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Research Article

Distinct activities of novel neurotoxins from Australian venomous snakes for nicotinic acetylcholine receptors

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Abstract. Envenomation from Australian elapid snakes results in a myriad of neurological effects due to post-synaptic neurotoxins that bind and inhibit nicotinic acetylcholine receptors (nAChRs) of neurons and muscle fibres. However, despite the significant physiological effects of these toxins, they have remained largely undercharacterised at the molecular level. This study describes the identification and comparative analysis of multiple neurotoxin isoforms from ten Australian snakes, including functional characterisation of two of these isoforms, Os SNTX-1 from *Oxyuranus scutellatus* and the more potent Pt LNTX-1 from *Pseudonaja textilis*. Electrophysiological recordings from adrenal chromaffin cells demonstrate that both neurotoxins act as competitive antagonists of nAChRs in a concentration-dependent manner. Their effects upon spontaneous and nerve-evoked membrane responses at the amphibian neuromuscular junction provide further evidence that both toxins bind muscle nAChRs in an irreversible manner. This study represents one of the most comprehensive descriptions to date of the sequences and activity of individual Australian elapid neurotoxins.

Keywords. Australian elapid snake, neurotoxin, cDNA cloning, recombinant protein expression, nicotinic acetylcholine receptor, venom.

Neurotoxins present in Australian elapid snake venoms serve a primary role in the immobilisation of prey by rapid and complete systemic paralysis which may result in death via asphyxiation. The toxins responsible for these effects are typically small and disulphide-rich and specifically target and block neuromuscular transmission via the post-synaptic skeletal muscle acetylcholine receptor [1]. The nicotinic acetylcholine receptor (nAChR) family are ligand-gated, cation-selective ion channels that span the cell membrane of nerves and muscle fibres. They contain various pentameric assemblies of structurally related subunits that consist of the agonist-binding α subunits (α1 – α10) as well as β1 – β4, γ, δ and ε subunits [2]. Binding of the elapid post-synaptic neurotoxins does not result in ultrastructural changes to the cell, and their paralytic effects are more easily reversed by antivenom than those of the pre-synaptic neurotoxins [3].

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Snake venom α-neurotoxins may be classified as either short or long based on the number of amino acids in the mature protein. Mature short-chain α-neurotoxins are typically 60–62 amino acids in length with four disulphide bonds, whereas long-chain α-neurotoxins have five internal disulphide bonds and are 66–79 (average 73) residues long [4]. Both groups share a basic three-finger loop structure, with the majority of variation at the C terminus, and exist in a monomeric form [5]. Hundreds of neurotoxin peptide sequences have been identified worldwide from various snakes and have been shown to bind to the nAChR in a competitive manner with endogenous cholinergic agonists and antagonists, thereby blocking the receptor without induction of ion channel opening [6]. Only the α subunit of the nAChR is capable of binding neurotoxins; however, other than variation in the kinetics of association and dissociation with nAChRs, the major functional difference between these toxins is that while long-chain neurotoxins can bind with high affinity to muscular and α7 homo-oligomeric neuronal nAChRs, short-chain neurotoxins bind only to muscular nAChRs [7]. It is postulated that the presence of the fifth disulphide bond in long-chain neurotoxins facilitates their binding to neuronal nAChRs [8]. Neurotoxin amino acid residues involved in the binding to distinct nAChRs have also been characterised by both sited-directed mutagenic and biochemical means [9, 10].

Although much research has focused on the structure of snake neurotoxins, and in particular on their relationship with the mammalian nAChR, the post-synaptic neurotoxins of Australian elapids have remained largely understudied, particularly at the molecular level, despite the significant neuropathology and toxicity of the venom of these snakes. The single exception is the common brown snake, *Pseudonaja textilis*, from which seven short-chain and one long-chain- (pseudonajatoxin b) coding cDNA sequences have been identified [11–13]. These short-chain neurotoxin isoforms are highly conserved, 57- to 58-amino-acid mature proteins that demonstrate lethality similar to that of the native toxin when expressed in recombinant form. Pseudonajatoxin b has also been cloned and both the recombinant and native forms are highly toxic in mammalian systems, particularly when compared to the short-chain neurotoxins.

This study describes the cloning and comparative analysis of families of both long- and short-chain neurotoxins from the venom glands of a total of ten Australian venomous snakes, including functional studies of two of these peptides. It represents the most comprehensive study to date of the genes responsible for the neurotoxic properties of Australian elapid venoms and has important implications not only for our understanding of the envenomation process, but also the clinical and diagnostic potential for these biological molecules.

Materials and methods

RNA isolation and cDNA synthesis. Venom glands were excised from Australian elapids including the coastal taipan (*Oxyuranus scutellatus*), inland taipan (*O. microlepidotus*), common brown snake (*P. textilis*), tiger snake (*Notechis scutatus*), rough-scaled snake (*Tropidechis carinatus*), Stephen's banded snake (*Hoplocephalus stephensii*), copperhead snake (*Austrelaps superbus*), red-bellied black snake (*Pseudechis porphyriacus*), mulga (*Pseudechis australis*) and small-eyed snake (*Rhinoplocephalus nigrescens*) collected under National Parks and Wildlife permit number W4\00261\01\SAA. Snap-frozen glands were homogenised with a polytron and RNA isolated using the Tri Reagent method (Sigma, St. Louis, Mo.). First-strand cDNA was synthesised from 1 μg of total RNA with an oligo$(dT)_{12-18}$ primer via reverse transcription with 200 units of Superscript III RNAse H^- Reverse Transcriptase (Invitrogen, Mt Waverly, Australia). The final reaction was ethanol precipitated and cDNA samples were resuspended in sterile water and stored at $-20\,^{\circ}C$.

Identification of short-chain neurotoxins. A partial, short-chain α-neurotoxin transcript was previously identified from screens of a cDNA library produced from the venom gland of *O. scutellatus* [14]. 5′RACE was performed with a SMART RACE cDNA Amplification kit (Clontech, Palo Alto, Calif.), from the *O. scutellatus* cDNA template with a single-gene-specific primer (5′-GGT CGT CGA TGG ATG AGA GCA AAA CTC-3′) to identify the full-length coding sequence. The 5′RACE product was thermocycled according to the manufacturer's protocol, separated on a 1% TAE agarose gel, excised using a QIAex II gel extraction kit (Qiagen, Hilden, Germany) and cloned via the pGEM-T vector system (Promega, Madison, Wis.). The ligation mix was transformed into competent dH5α, *Escherichia coli*, with recombinant clones selected on LB-Ampicillin plates (50 μg/ml) supplemented with IPTG and x-Gal. Multiple clones were then sequenced from each snake with a Big Dye version 3.1 Terminator sequence ready reaction kit (Perkin-Elmer, Norwalk, Conn.) and alignments performed with BioEdit software (Isis Pharmaceuticals Inc., Carlsbad, Calif.). Upon cloning of the entire neurotoxin-coding sequence, similar neurotoxin transcripts were identified in related snakes by PCR. The short-chain α-neurotoxin coding region was amplified

from all ten Australian snake cDNAs with 1 unit of AmpliTaq gold (Applied Biosystems, Foster City, Calif.) buffered in 1× buffer, 2.25 mM $MgCl_2$ and 200 μM dNTPs with 25 pmol of the forward (5′-CGC AAG ATG AAA ACT CTG CTG C-3′) and reverse (5′-GCC ACT CGT AGA GCT AAT TGT TG-3′) primers. The reaction was thermocycled at 95 °C for 10 min followed by 30 cycles of 95 °C for 30 s, 61 °C for 40 s and 72 °C for 40 s with a final extension of 72 °C for 7 min. All PCR products were visualised on a 1% TAE agarose gel, purified, cloned, sequenced and aligned.

Identification of long-chain neurotoxins. Multiple, putative long-chain α-neurotoxins were identified from the venom gland cDNA of Australian snakes by PCR amplification. Primers were designed against the previously published, *P. textilis* long-chain neurotoxin pseudonajatoxin b (AF082982). PCR was performed as previously described, with forward (5′-ATG AAA ACT CTG CTG CTG ACC-3′) and reverse (5′-GTC GAG ATG TCA AAG ACG CA-3′) primers. The reaction mixture was then thermocycled at 95 °C for 8 min, followed by 30 cycles of 95 °C for 20 s, 59 °C for 20 s and 72 °C for 45 s with a final extension of 72 °C for 7 min. A single PCR product from each snake was cloned and sequenced and alignments performed as previously described.

Recombinant protein expression of selected neurotoxins. Two neurotoxins, the putative short-chain α-neurotoxin Os SNTX-1 from *O. scutellatus* and the putative long-chain α-neurotoxin Pt LNTX-1 from *P. textilis*, were selected for further characterisation via recombinant protein production using the pGEX-6P-1, *E. coli* expression system (GE Healthcare, Rydalmere, Australia). A PCR product corresponding to the mature protein sequence, without propeptide, of Os SNTX-1 was amplified with forward (5′-CGG GAT CCA TGA CAT GTT ACA ACC AAC AGT C-3′) and reverse (5′-GGA ATT CCT AAT TGT TGC ATT TGT CTG TTC-3′) primers, while Pt LNTX-1 was amplified with forward (5′-CGG GAT CCT TGA TAT GCT ACC TGG ATT TTA GTG-3′) and reverse (5′-GGA ATT CTC AAT GAG GTT TCT GTT TCG-3′) primers as described above. The PCR products and pGEX-6P-1 vector were subsequently double digested with *Bam*H1 and *Eco*R1 (New England Biolabs, Beverly, Mass.), purified from a 1% TAE agarose gel and ligated into pGEX-6P-1 with T4 DNA Ligase (Promega) prior to transformation into BL21, *E. coli* cells. Recombinant protein was purified according to the protocols described by Frangioni and Neel [15]. Cleavage of the fusion protein was induced with 20 units/ml PreScission protease (GE Healthcare) at 4 °C overnight in cleavage buffer (50 mM Tris-HCl pH 7.0, 150 mM NaCl and 1 mM EDTA) and the protein eluted with multiple washes. Both recombinant proteins were then further purified on an HPLC column before analysis on 15% SDS-PAGE stained with Coomassie and quantitated via a Lowry assay prior to functional analysis. Sizes of the recombinant proteins were also confirmed by MALDI-TOF analysis as previously described [16].

Electrophysiological recordings from bovine adrenal chromaffin cells. Chromaffin cells were prepared from bovine adrenal glands as described previously [17]. Glass electrodes (GF150F-7.5, Harvard Apparatus, Edenbridge, UK) had resistances of 2–3 MΩ when filled with intracellular solution (140 mM CsCl, 2 mM $CaCl_2$, 11 mM EDTA, 2 mM MgATP and 10 mM HEPES-KOH, pH 7.2). Agonists were diluted in bath solution (140 mM NaCl, 3 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 7.7 mM glucose and 10 mM HEPES-NaOH, pH 7.35) and applied to cells by brief (10 ms) 15-psi pressure ejection from an extracellular patch pipette positioned 50–100 μm from the cell soma to evoke maximal responses to agonists [18]. Recombinant neurotoxins were then bath applied at varying concentrations (0.3–250 μg/ml) at room temperature. Membrane currents evoked by agonist application were amplified and low-pass filtered (10 kHz) using a MultiClamp 700B patch-clamp amplifier and voltage steps were generated using pCLAMP version 9.2 software and a Digidata 1322A interface (Axon Instruments, Union City, Calif.). Statistical analysis was performed with SigmaPlot version 8.0. Full concentration-response curves for agonists were carried out for estimation of EC_{50} values for agonists and for curve shifts caused by competitive antagonists. IC_{50} values were defined as the concentration of an antagonist that caused 50% inhibition of the response to a fixed concentration of an agonist.

Ex vivo *amphibian electrophysiological assays.* The iliofibularis muscles together with their innervating motor axons were removed from toads (*Bufo marinus*), pinned out in an organ bath and perfused with Krebs solution (118.4 mM NaCl, 25 mM $NaHCO_3$, 1.13 mM NaH_2PO_4, 4.7 mM KCl, 1 mM $MgCl_2$, 11.1 mM glucose, 0.2–0.3 mM $CaCl_2$) at a rate of 2–3 ml per minute at room temperature. The reservoir supplying the bath was gassed continuously with 95% O_2 and 5% CO_2 and pH maintained at 7.3. Motor axons were secured into a glass pipette filled with Krebs solution and containing two Ag/AgCl wires, one inside and one outside the electrode, which supplied a square-wave pulse of 0.08 ms in duration and 20 V in strength, at a frequency of 0.2 Hz. End-plate potentials (EPPs) and miniature EPPs (MEPPs) were recorded by intracellular impalement of the end-plate region of muscle fibres with microelectrodes filled with 2 M KCl (30–40 MΩ resistance). Record-

ing sites were accepted if the rise times of MEPPs were less than 1.5 ms and the resting membrane potential (RMP) stable and greater than – 50 mV. After control signals had been recorded, recombinant Os SNTX-1 and Pt LNTX-1 were either perfused into, or directly applied to the bath. For recovery conditions, the preparation was washed with toxin-free Krebs solution for at least 30 min. Analog signals were digitised using MacLab 4 and Scope 3.5.5 software. EPP and MEPP signals were analysed with the Scope and Microsoft Excel programs.

Results

Identification of Australian snake short-chain neurotoxin cDNAs. A full-length, 532-bp cDNA transcript, identified by 5′RACE from the venom gland of the coastal taipan (*O. scutellatus*), was predicted by BLAST homology searches to code for a short-chain α-neurotoxin, 83 amino acids in length, designated Os SNTX-1 (GenBank DQ085855). This transcript shared significant identity (78%) with a short-chain α-neurotoxin from the venom of Hardwick's sea snake, *Lapemis hardwickii* (AAL54895) (Fig. 1a). The mature protein sequence of Os SNTX-1, excluding propeptide, had previously been determined by Zamudio et al. [19], who described the isolation and characterisation of two toxins, toxins 1 and 2, from the venom of the coastal taipan which differed by only a single residue. Primers were designed from Os SNTX-1 to identify related neurotoxins in the venom gland of other Australian elapid snakes by PCR. This approach led to the identification of an additional putative short-chain α-neurotoxin, designated Os SNTX-3 (DQ917497) from *O. scutellatus* (Fig. 1a). Os SNTX-3 demonstrates significant identity with a precursor protein scutelatoxin (AY691665), also identified from the venom gland of the same snake, differing by only three amino acids. However, despite relative conservation of the propeptide leader sequence and cysteine residues, only 33% identity existed between the mature protein sequences of Os SNTX-1 and Os SNTX-3.

Full-length coding sequences of related neurotoxins were identified in nine other Australian elapids (DQ917498–DQ917507, EF599317). Figure 1a represents an alignment of the deduced amino acid sequences of these toxins compared to five of the most closely related proteins previously characterised from other snakes. Note the complete conservation of cysteine residues involved in disulphide bond formation, as well as a high degree of identity within the 21-amino-acid, hydrophobic propeptide leader sequence in all cases. Novel clones identified from *O. microlepidotus*, *P. porphyriacus*, *T. carinatus*, *H. stephensii*, *A. superbus*, *R. nigrescens* and *N. scutatus* represent the first description of a short-chain α-neurotoxin at either the nucleotide or protein level from the venom gland of these snakes. The deduced protein sequence from the *P. australis* clone Pa-a is 100% identical to that reported at the mature protein level from the venom from that snake (P25497), which has been shown to produce peripheral paralysis by post-synaptic blockage of neuromuscular transmission [20]. This related family of short-chain α-neurotoxins share 76–93% identity with Os SNTX-1. A single clone sharing little identity to the short-chain α-neurotoxins from the other elapids was amplified from the venom gland cDNA of *P. textilis* (Pt SNTX-8) (Fig. 1a). A total of seven short-chain α-neurotoxin isoforms have previously been isolated from *P. textilis*, with Pt SNTX-8 demonstrating closest homology to that of Pt SNTX-1 [11, 12].

Identification of Australian snake long chain neurotoxin cDNAs. A long-chain α-neurotoxin, pseudonajatoxin b, has previously been cloned from the venom gland of *P. textilis* [13]. To detect the presence of other long-chain neurotoxins from related Australian elapids, primers were designed from the mRNA sequence of pseudonajatoxin b and a single PCR product subsequently cloned from all ten snakes. An alignment of the deduced amino acid sequence of all unique long-chain α-neurotoxins identified, compared to pseudonajatoxin b, is shown in Figure 1b (DQ917508–DQ917514, EF599318). These transcripts have been defined as long-chain neurotoxins on the basis of their mature protein length (68–73 amino acids) and homology to other long-chain neurotoxins as a result of BLAST searches. Although primers were designed specifically for the pseudonajatoxin b sequence, this transcript was not identified by PCR from *P. textilis* cDNA. A second toxin was identified, however, designated Pt LNTX-1, which exhibited little sequence identity with pseudonajatoxin b. Indeed the closest known match to the mature protein sequence is a long-chain neurotoxin from the yellow-lipped sea krait (*Laticauda colubrina*), neurotoxin b (0901189B), which shares only 51.4% identity. Although mature Pt LNTX-1 is 72 amino acids in length, it does not contain the conserved cysteine residues that form the fifth disulphide bond characteristic of long-chain neurotoxins, and has an alternate cleavage site between the signal peptide and mature protein (Fig. 1b).

A significant degree of variation was observed in the putative long-chain neurotoxins identified from the other Australian elapids. Os LNTX-1 from *O. scutellatus* and Om LNTX-1 and LNTX-2 from *O. microlepidotus* are highly related to each other (90.2%) and

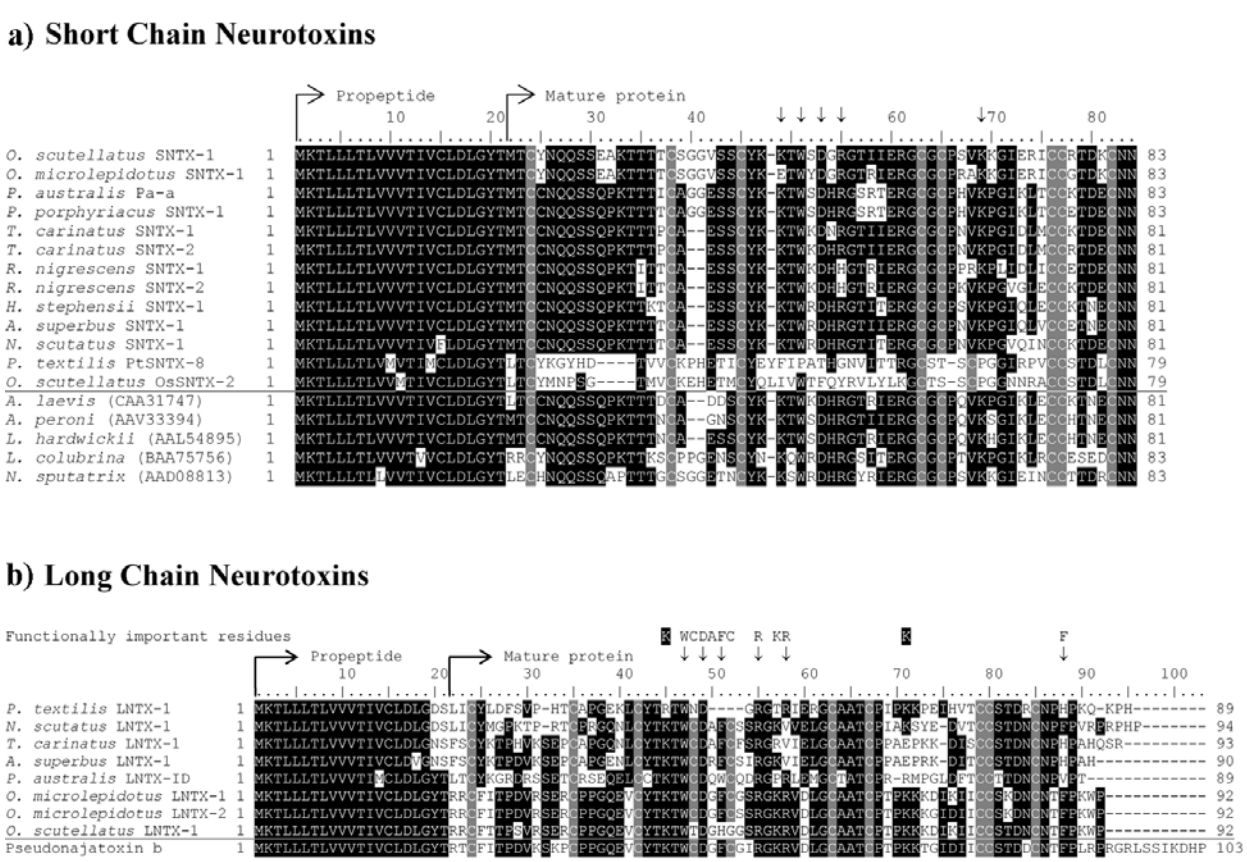

Figure 1. Neurotoxin sequence alignments. (*a*) Protein alignment of precursor short-chain neurotoxins. Comparisons are made between sequences identified in ten Australian elapid snakes (above the line) compared to the previously characterised toxins from the olive sea snake (*Aipysurus laevis*), horned sea snake (*Acalytophis peroni*), Hardwick's sea snake (*Lapemis hardwickii*), yellow-lipped sea snake (*Lacticauda colubrina*) and spitting cobra (*Naja sputatrix*). Conserved cysteine residues involved in putative disulphide bond formation are shaded grey, and propeptide and mature protein sequences denoted by arrows. Conserved residues implicated in receptor binding are denoted by ↓. (*b*) Protein alignment of precursor long-chain neurotoxins. Comparisons are made to pseudonajatoxin b (AAK15774) previously identified from the venom of *Pseudonaja textilis* [13]. Residues implicated in the binding of both muscular and neuronal nAChRs by α-cobratoxin are denoted by ↓ [29]. Other residues which are required for either specifically binding just muscular nAChRs (lysine residues shaded black) or neuronal nAChRs by α-cobratoxin are also shown [7].

to pseudonajatoxin b; however, they lack the 11-amino-acid C-terminal extension present in pseudonajatoxin b. Another notable difference is the absence of the cysteine residues involved in the formation of the fifth disulphide bond in Os LNTX-1. Other unique long-chain neurotoxins were also identified from the venom gland of *N. scutatus* and *P. australis*, which have previously been described at the mature protein level, but not at the level of cDNA. PA-ID (P14612) is a long-chain neurotoxin from the venom of *P. australis* previously identified by Takasaki [21] and has been postulated to be non-lethal as it is unable to bind to the acetylcholine receptor of an electric ray eel. Only a single neurotoxin protein sequence (P01384) has been previously identified from the venom of *N. scutatus*, with an intravenous LD_{50} value of 0.125 mg/kg in mice [22]. The mRNA transcript of Ns LNTX-1 identified by PCR in this study codes for a 73-amino-acid protein similar to that described by Halpert et al. [22], differing by a reversal of the second-last and third-last amino acids compared to the published protein. In all instances, the long-chain neurotoxins identified in this study represent the first description of a cDNA transcript for this toxin family in these snake species. Finally, unique long-chain neurotoxins were also identified in the venom gland of, *T. carinatus* (Tc LNTX-1) and, *A. superbus* (As LNTX-1) which shared little identity with the other long-chain neurotoxins identified in this study (Fig. 1b), but which demonstrated significant identity with a related long-chain neurotoxin from the Central Asian cobra, *Naja oxiana* (P01382) [23]. It should be noted that a PCR product was also obtained from *P. porphyriacus*, *H. stephensii* and, *R. nigrescens*; however, subsequent cloning revealed that the only transcripts amplified were the short-chain neurotoxins identified previously

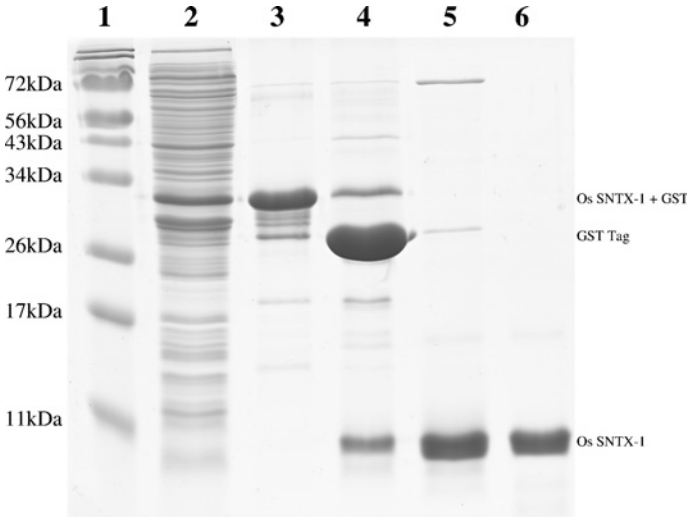

Figure 2. Purification of recombinant Os SNTX-1. Fractions were loaded on 15% SDS-PAGE and stained with Coomassie as follow: 1, molecular-weight marker; 2, induced cell lysate; 3, GST-fusion prior to PreScission cleavage; 4, GST-fusion after PreScission cleavage; 5, eluted Os SNTX-1; 6, HPLC-purified Os SNTX-1.

in Figure 1a. The signal peptide had a high degree of identity for all long-chain neurotoxins, and also between long- and short-chain neurotoxins, suggesting a similar mechanism of secretion and activation in Australian elapids. The mature neurotoxins identified in this study would be predicted to have molecular weights within the range of 6.34–8.98 kDa.

Recombinant protein expression and characterisation. Two neurotoxins identified above were selected for further characterisation by recombinant protein expression and functional analysis. Constructs for both Os SNTX-1 from *O. scutellatus* and Pt LNTX-1 from *P. textilis* were cloned into pGEX-6P-1 and subsequently expressed and purified (see Fig. 2). Protein yields of 900 μg for Os SNTX-1 and 200 μg for Pt LNTX-1 per litre of bacterial culture were obtained. Subsequent to HPLC purification, the molecular masses of Os SNTX-1 and Pt LNTX-1 were determined by MALDI-TOF analysis to be 7145 Da and 8078 Da, respectively.

In vitro *electrophysiological assays.* Functional assays were performed to determine the effects of both recombinant neurotoxins upon muscle and neuronal nAChRs. To address this, Os SNTX-1 and Pt LNTX-1 recombinant proteins were investigated for their effects on nicotine-evoked membrane currents in dissociated bovine adrenal chromaffin cells. Bath application of increasing concentrations of both neurotoxins reversibly inhibited nicotine-evoked membrane currents in cells voltage-clamped at −100 mV (Fig. 3). The initial potentiation observed at low toxin concentrations has been observed for other competitive antagonists of neuronal nAChRs [24]. High concentrations of toxin, however, almost completely inhibited the nAChR-mediated currents in chromaffin cells. This inhibition was significantly greater for Pt LNTX-1 than Os SNTX-1, with a half-maximal inhibitory concentration (IC_{50}) of 2 μM for Pt LNTX-1 compared to 25 μM for Os SNTX-1. The slope factors (Hill coefficient) of 1.3 and 1.5 for Os SNTX-1 and Pt LNTX-1, respectively, indicate that binding of both toxins to the nAChR occurs in an approximately 1:1 stoichiometry. Both neurotoxins depressed nicotine-evoked current amplitude at all membrane potentials examined, although again, Pt LNTX-1 was more potent (Fig. 3). This inhibition was voltage insensitive, with nicotine-evoked current amplitude reduced by ~26% and ~23% at all membrane potentials in the presence of 12 μM Os SNTX-1 or 1 μM Pt LNTX-1, respectively (data not shown). These data suggest that both neurotoxins are competitive antagonists of neuronal nAChRs, thereby inhibiting the binding of nicotine to the receptor rather than blocking the open-channel pore.

Ex vivo *electrophysiological assays.* The effects of the recombinant neurotoxins upon spontaneous and evoked neurotransmitter release was further examined in an *ex vivo* amphibian electrophysiological assay. Direct application of the neurotoxins (1 μM Pt LNTX-1 or 5 μM Os SNTX-1) produced a rapid decrease in, and an eventual elimination of both EPP and MEPP amplitudes (Fig. 4a,b). Both neurotoxins completely and irreversibly abolished the mean amplitude of EPPs, with no stimulus recovery in high extracellular Ca^{2+} (0.5 mM) conditions post-toxin exposure (data not shown). The results are consistent with both recombinant toxins inhibiting the nAChR post-synaptically, as both evoked and spontaneous EPP amplitudes were decreased to the same degree simultaneously. Furthermore, both Pt LNTX-1 and Os SNTX-1 inhibited evoked EPPs and MEPPs in a concentration-dependent manner (Fig. 4a,b). A comparison of the efficacy of the two neurotoxins by measuring the mean decline in EPP amplitude again

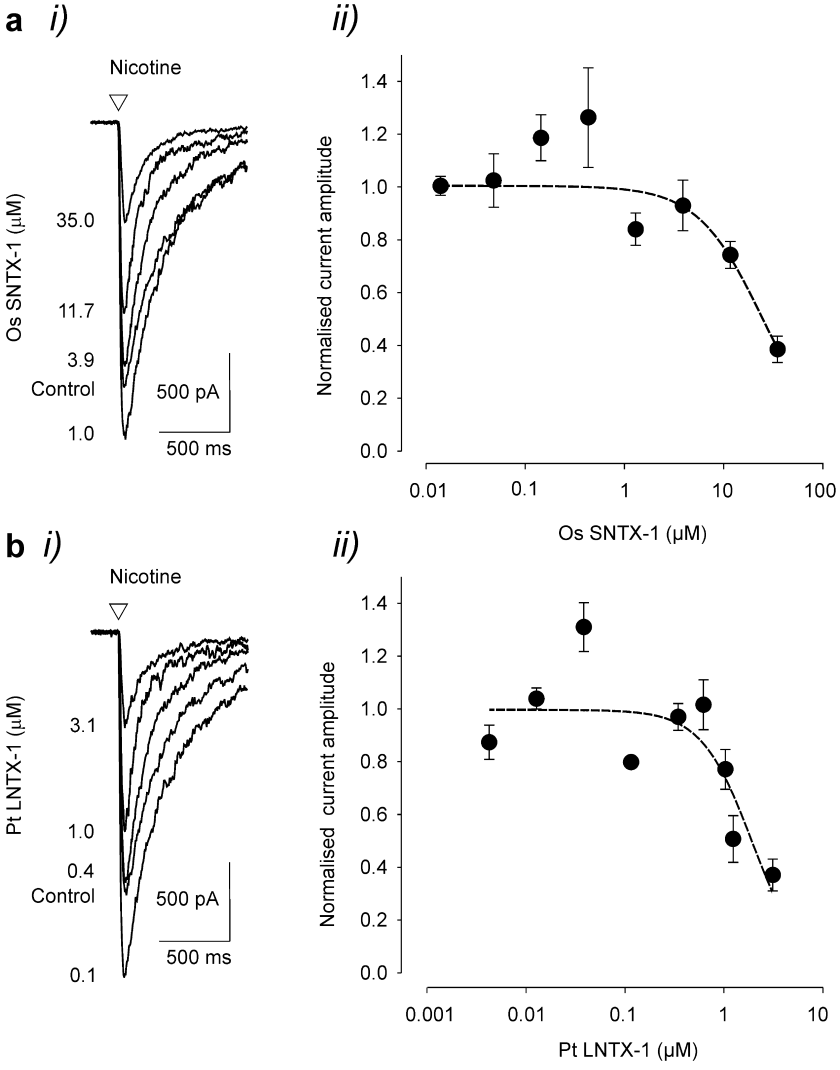

Figure 3. *In vitro* electrophysiological assays. Concentration dependence of the inhibition of nAChRs in bovine adrenal chromaffin cells by the recombinant neurotoxins Os SNTX-1 and Pt LNTX-1. On the left are superimposed nicotine (100 μM)-evoked currents recorded from isolated chromaffin cells voltage-clamped at −100 mV in the absence (control) and presence of different concentrations of Os SNTX-1 (*a*) and Pt LNTX-1 (*b*). The righthand diagrams show concentration-response relationships obtained for the inhibition of nicotine-evoked currents in isolated chromaffin cells by the neurotoxins, giving IC_{50}s of 25 μM and 2 μM for Os SNTX-1 (*a*) and Pt LNTX-1 (*b*), respectively. Data represented as mean ± SE (n = 3–6).

confirmed the potency of Pt LNTX-1 nAChR inhibition, indicating that Pt LNTX-1 (IC_{50} = ~0.1 μM) is approximately five times more potent in its ability to block neuromuscular transmission compared to Os STNX-1 (IC_{50} = ~0.5 μM) (Fig. 4c).

Discussion

Australian elapid snake envenomation results in a myriad of neurological effects within its prey that are, in part, a result of the presence of post-synaptic neurotoxins that bind the nAChRs of neurons and muscle fibres and inhibit synaptic transmission. We described here the identification of a neurotoxin from the venom gland cDNA library of *O. scutellatus*, and its further characterisation by 5'RACE and cloning. The transcript was identified as a short-chain neurotoxin which was previously described at the mature protein level from the venom of the coastal taipan [19]. Subsequent PCR amplification identified a related family of toxins within the venom glands of eight other Australian elapids, including the short neurotoxin Pa-a from *P. australis*, which shares 72.6% identity at the protein level (Fig. 1a) [20]. Note that these short-chain neurotoxins fall into the type I α-neurotoxin sub-family as described by Fry et al. [25] which includes toxins from numerous other elapid snakes, including *L. colubrina*, *Naja atra* and *Aipysurus laevis*, that are known to be antagonists of the α1 site of nAChRs. Os SNTX-1 and Pa-a have previously been shown to be toxic to rats and mice with similar intravenous LD_{50} values of 63 and 76 μg/kg, respectively. Zamudio et al. [19] reported an inability of native Os SNTX-1 to bind neuronal nAChRs (typical of other short-chain neurotoxins) and a reduced affinity for muscle nAChRs, attributing this decrease in binding efficiency potentially to the substitution of either a phenylalanine or a histidine residue with a glycine residue at position 53 of the precursor

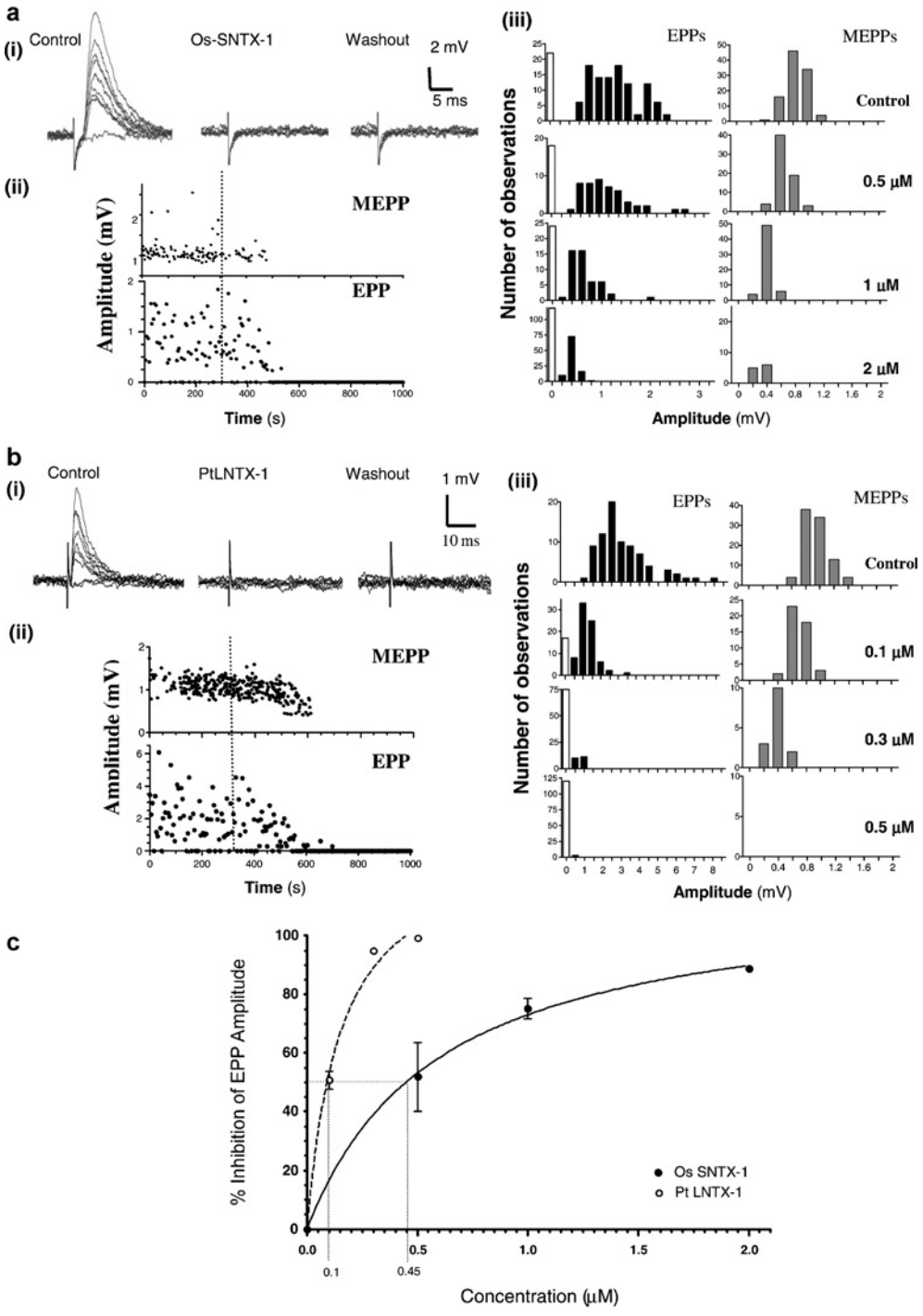

Figure 4. *Ex vivo* electrophysiological assays. Effect of Os SNTX-1 (*a*) and Pt LNTX-1 (*b*) on spontaneous and evoked neurotransmitter release at the amphibian neuromuscular junction. (*i*) Representative superimposed traces of EPPs recorded before the neurotoxin addition, in the presence of either Os SNTX-1 (5 mM) or Pt LNTX-1 (1 mM) and washout. (*ii*) Scatter plot of EPP and MEPP amplitudes before and during the application of either Os SNTX-1 (5 mM) or Pt LNTX-1 (0.5 mM). Dashed line indicates addition of toxin. (*iii*) Number of observations versus amplitude histograms of EPPs and MEPPs for control, 0.5, 1 and 2 mM Os SNTX-1 and 0.1, 0.3 and 0.5 mM Pt LNTX-1. Note that both neurotoxins completely and rapidly abolish EPPs in (*i*) and (*ii*), with a concurrent decrease in MEPP amplitudes shown in (*ii*) and (*iii*). There is a progressive decrease in both EPP and MEPP amplitudes with increasing concentrations of either neurotoxin, shown in (*iii*). The elimination of EPPs was not recovered by the washout phase, even at higher concentrations of Ca^{2+}, indicating that both neurotoxins irreversibly block neurotransmitter release. (*c*) Concentration-response relations obtained for the inhibition of neurotransmitter release at the amphibian neuromuscular junction by Os SNTX-1 (solid line) and Pt LNTX-1 (dashed line). Note that Pt LNTX-1 produced an ~50% inhibition at 0.1 mM, compared to 0.45 mM for Os SNTX-1. Pt LNTX-1 is approximately five times more potent in blocking neurotransmitter release than Os SNTX-1. Data represent mean ± SE for Pt LNTX-1 (n = 5) and Os SNTX-1 (n = 3).

molecule (Fig. 1a). This substitution is also observed in the closely related, *O. microlepidotus* molecule, Om SNTX-1, and would be predicted to have a similar effect. Interestingly, while the conserved histidine residue is observed in all other snake species for this particular family of neurotoxins, a second clone was also identified in the rough-scaled snake (*T. carinatus*) with a non-conservative amino acid substitution (H51N in Tc SNTX-1). A non-conservative substitution at this position may decrease the efficiency of neurotoxin binding to the nAChR as it occurs immediately adjacent to an arginine residue at position 52 of the precursor molecule that is conserved in most members of this family of proteins and which has previously been shown to be crucial for optimal neurotoxin activity [9].

Two additional, unique short-chain neurotoxin sequences distinct from those described above were identified in this study: Pt SNTX-8 from *P. textilis* and Os SNTX-3 from *O. scutellatus* (Fig. 1a). Pt SNTX-8 represents the eighth member of a unique family of short-chain neurotoxins previously isolated from the venom gland of *P. textilis*, sharing 75% overall identity and classified as type III α-neurotoxins by Fry et al. [25]. Functional studies revealed that these proteins exert the typical effects of short-chain neurotoxins including high binding affinity with skeletal muscle nAChRs resulting in muscle paralysis, spasms and increased respiration; however, their intravenous LD_{50} values in mice are much lower than those of other neurotoxins [11]. Variation in activity between these sequences probably arises from point mutations, and the significant differences observed in primary structure (for example the loss of a proline from loop 1) between Pt SNTX-8 and the other neurotoxins may contribute to this decrease in lethality in a mammalian system. Conversely, Os SNTX-3 is a novel neurotoxin sequence whose activity remains undefined. Zamudio et al. [19] briefly alluded to the presence of a third neurotoxin within the venom of the coastal taipan with little sequence identity to toxins 1 and 2, which was completely ineffective in all nAChR assays performed and its precise pharmacological role within the venom unknown. The great variation observed in the primary sequence of neurotoxins within a single species has been attributed to the diversity, availability and susceptibility of prey, that is, a direct correlation between the deviation in venom composition with the target prey population and the snake's diet [26]. The variation in neurotoxin sequences observed within a single species of Australian snake has probably arisen as a response to the variation in nAChR targets presented by a wide range of target prey organisms, including mammals, amphibians and other reptiles.

The secondary fold adopted by all post-synaptic neurotoxins is characterised by three adjacent loops rich in β-pleated sheets, held in a tight conformation by four disulphide bonds [27, 28]. Typically occurring as homodimers, single-amino-acid mutations within this framework are responsible for the variation in activity and binding efficiency observed between toxins, with a number of key conserved residues that are important for neurotoxin recognition and binding to nAChRs [29]. These include residues K27, W29, D31, R33 and K47 of the mature protein sequence of the short-chain neurotoxin, erabutoxin, from the broad-banded blue sea krait, *Laticauda semifasciata* [9, 30]. The corresponding amino acids, K48, W50, D52, R54 and K68, of the precursor protein Os SNTX-1 are completely conserved, along with the eight cysteine residues that form disulphide linkages (Fig. 1a, denoted by arrows). Indeed, these residues are highly conserved in all short-chain neurotoxins identified in this study except for the two unusual isoforms Os SNTX-3 and Pt SNTX-8, suggesting a decreased ability to bind nAChR or an alternate target site in these two proteins. By maintaining the robust three-finger protein structure, it is possible for the snake to accumulate an arsenal of toxins with a wide variation in function and activity by just a few subtle changes to residues within functional sites [27, 31]. It has also been suggested that post-synaptic neurotoxins and other venom proteins have undergone a process of accelerated evolution via directional mutation to allow for this adaptation [32, 33]. Hence, selection pressure may have favoured the multiplicity of isoforms observed in short- and long-chain neurotoxins observed in Australian snake venoms.

In addition to the short-chain neurotoxins, a number of unique long-chain neurotoxin sequences were also identified, representing their first description at the nucleotide level within an Australian elapid (Fig. 1b). These toxins, classified as type II α-neurotoxins, are significantly more heterogenous, with a higher degree of sequence diversity, as has previously been observed for this sub-family of proteins [25]. Besides the increase in length, and often the presence of a fifth disulphide bond located at the tip of loop 2, long-chain neurotoxins are distinguished from short-chain neurotoxins by their ability to bind and inhibit both muscle and neuronal nAChRs [34]. In mutagenic studies of α-cobratoxin from the venom of the monocled cobra, *Naja kaouthia*, both common and specific residues determined binding to the two receptor types. It was observed that the amino acids W25, D27, F29, R33, R36 and F65 in the mature protein were involved in binding to both muscle and neuronal nAChRs [29]. However, while A28, K35 and the C26-C30 disulphide bond were specifically involved in the association with

α7 neuronal nAChRs, residues K23 and K49 solely bound to muscle nAChRs [7]. The corresponding residues in the long-chain neurotoxins identified in Australian elapids in this study are shown in Figure 1b, denoted by arrows. Two of these sequences, PA-ID from *P. australis* and Ns LNTX-1 from *N. scutatus*, have previously been characterised at the protein level from the venom of these snakes [21, 22]. While Ns LNTX-1 (with complete conservation of these key residues) has been shown to be highly toxic in a mammalian system, PA-ID (with a number of substitutions within key residues) demonstrated an inability to bind nAChRs. Long-chain neurotoxins were also identified from *O. microlepidotus* and *O. scutellatus* which demonstrated 74–78% identity with pseudonajatoxin b, a potently toxic long-chain neurotoxin from the venom of *P. textilis*. These sequences, however, lack the C-terminal extension present in pseudonajatoxin b. Some of the variable toxicity observed in other long-chain neurotoxins arises from the diverse C-terminal extensions that distinguish them from short-chain neurotoxins, particularly the presence of basic amino acid residues [35]. In addition, Os LNTX-1 is missing the fifth disulphide bond, which is still present in Om LNTX-1 and -2, suggesting an inability to bind and inhibit α7 neuronal nAChRs. The presence of only four disulphide bonds in a long-chain neurotoxin is unusual, but not unheard of, and does not necessarily result in loss of lethality [36].

A 21-amino-acid signal peptide was identified for all clones, representing the first description of this sequence for the majority of snake species examined. This propeptide was observed to be highly conserved both within and between the short-chain and long-chain neurotoxin families, indicating a common pathway for the folding and secretion of a functional protein. The high degree of conservation of propeptide sequences is a feature shared by all Australian snake venom toxin families [14]. This leader sequence contains a high proportion (approximately 60%) of hydrophobic residues, consistent with its role as a secretion signal. Previous studies have indicated that the presence of a cysteine residue at position 15 of the precursor neurotoxin is known to influence the rate of folding of the mature protein [37, 38]. This cysteine residue is completely conserved in all neurotoxin sequences reported in this study with the exception of the clone Ns SNTX-1 from *N. scutatus*, which has possible implications for the correct folding and processing of this molecule.

The single long-chain neurotoxin identified in this study in *P. textilis*, Pt LNTX-1, while containing the four conserved disulphides common to three-finger toxins and a highly similar propeptide, possessed a number of unique features. These include an alternative cleavage site for the propeptide, the absence of the fifth disulphide bond as well as other deletions within the tip of loop 2 (amino acids 54 and 55 of the precursor) along with further non-conserved substitutions within other functionally important areas of the molecule. Hence, to determine the activity of Pt LNTX-1, a recombinant protein was produced in an, *E. coli* expression system along with Os STNX-1 for comparative analysis. The reduced affinity of Os SNTX-1 for nAChRs noted in all assays in this study is consistent with the observations by Zamudio et al. [19] for the native venom protein. The patch-clamp assays in bovine adrenal chromaffin cells identified both toxins as competitive antagonists of neuronal nAChR, directly competing with the binding of nicotine for activation of the receptor, and hence they do not directly block cation influx through the open-channel pore. Both neurotoxins bind to the nAChR in a 1:1 stoichiometry (Fig. 3), compared to the 2:1 stoichiometry observed for the binding of acetylcholine [39].

In *ex vivo* electrophysiological assays examining neurotransmitter release at the amphibian neuromuscular junction, both toxins completely abolished EPPs and MEPPs in a concentration-dependent manner, confirming the findings of the functional studies in adrenal chromaffin cells. Given the progressive reduction in EPP and MEPP amplitudes, both neurotoxins are postulated to inhibit neurotransmission by the irreversible blockade of post-synaptic muscle nAChRs, with Pt LNTX-1 being approximately five times more potent than Os SNTX-1. The inhibition of EPPs and MEPPS by the short-chain neurotoxin from *O. scutellatus* is consistent with the inhibition of muscle twitch reported recently from the venom of the closely related inland taipan (*O. microlepidotus*) [40]. Interestingly, the novel long-chain neurotoxin from *P. textilis* had a significantly enhanced effect when compared to that of the short-chain neurotoxin from *O. scutellatus* in both functional assays. This increased inhibitory effect likely arises from differences in the primary structure between the two molecules as previously highlighted, since both toxins contain only four internal disulphide bonds. This difference in the inhibition of neuromuscular transmission between the two toxins is consistent across both mammalian and amphibian muscle nAChRs.

In conclusion, this study describes the cloning and partial characterisation of a number of novel long- and short-chain neurotoxins from the venom glands of ten venomous Australian snakes. It represents the most detailed study to date of the post-synaptic neurotoxic components from the venom of these elapids at the molecular level. The results of the present study have

important implications for the treatment of Australian snake envenomation, the phylogenetic relationships of Australian elapids, as well as for our further understanding of the mechanisms of action of the neurotoxic components of Australian snake venoms.

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