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Identification of presynaptic neurotoxin complexes in the venoms of three Australian copperheads (*Austrelaps* spp.) and the efficacy of tiger snake antivenom to prevent or reverse neurotoxicity

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ABSTRACT

The venom of the Australian lowlands copperhead, *Austrelaps superbus*, produces significant and potentially lethal neurotoxic paralysis in cases of clinical envenomation. However, little is known about the neurotoxic components within this venom or venoms from the related alpine copperhead (*Austrelaps ramsayi*) or pygmy copperhead (*Austrelaps labialis*). Using the isolated chick biventer cervicis nerve-muscle preparation, all *Austrelaps* venoms were found to exhibit potent and rapid inhibition of nerve-evoked twitch contractions and block of contractures to nicotinic agonists, consistent with postsynaptic neurotoxic activity. Following separation by size-exclusion liquid chromatography under non-denaturing conditions, all *Austrelaps* venoms were found to also contain a high molecular mass fraction with only weak phospholipase A_2 (PLA_2) activity that caused a slow inhibition of twitch contractions, without inhibiting contractures to nicotinic agonists. These actions are consistent with the presence of additional snake presynaptic PLA_2 neurotoxin (SPAN) complexes in all three *Austrelaps* venoms. However, there was no evidence of direct muscle damage produced by any *Austrelaps* venom or SPAN complex. Monovalent tiger snake antivenom was effective in neutralising the neurotoxicity of both whole venom and the SPAN complex. However antivenom was unable to effectively reverse whole venom neurotoxicity, or prejunctional SPAN neurotoxicity, once established. Given the strong neurotoxicity of all *Austrelaps* venoms, particularly *A. ramsayi* and *A. labialis*, effective bites from these copperhead species should be considered potentially lethal. Furthermore, clinicians need to be aware of possible irreversible presynaptic neurotoxicity following envenomation from all copperhead species and that early antivenom intervention is important in preventing further development of toxicity.

1. Introduction

Despite Australia being home to some of the world's most venomous snakes, there are only around 2 deaths per year, despite 1000–3000 cases of snakebite (White, 1998). This is due to Australia possessing high quality species-specific antivenoms, well-trained emergency and intensive care medical services and effective first aid techniques that specifically assist in elapid snakebite (Currie, 2006). To maintain such a low rate of mortality, despite increasing population spread, adequate knowledge of the likely

Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; BCA, bicinchoninic acid; BSA, bovine serum albumin; CBCNM preparation, chick biventer cervicis nerve-muscle preparation; CCh, carbachol; FPLC, fast-perfusion liquid chromatography; LD_{50}, median lethal dose; MALDI-TOF, matrix-assisted laser desorption ionisation time-of-flight; SEM, standard error of the mean; SPAN, snake presynaptic $sPLA_2$ neurotoxin; $sPLA_2$, secretory phospholipase A_2; TSAV, monovalent tiger snake antivenom; t_{90}, time to 90% neuromuscular blockade; V_e, elution volume; V_o, void volume.

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clinical actions of all potentially dangerous snake venoms is critical.

An important complication of systemic envenomation by many Australian elapid snakes is skeletal muscle paralysis due to the presence of neurotoxins. If left untreated, systemic neurotoxicity can lead to paralysis of respiratory muscles, ultimately resulting in death by asphyxiation. Although these neurotoxic components have been pharmacologically characterised from the venoms of several Australian snakes, many others have been understudied. Members of the genus *Austrelaps*, the Australian copperheads (Worrell, 1963; Serpentes: Elapidae: Acanthophiinae), are among those that have been poorly characterised with limited biochemical or pharmacological studies. Importantly these snakes should not be confused with North American copperheads belonging to the genus *Agkistrodon*. It is generally accepted that there are three species within the *Austrelaps* genus (Rawlinson, 1991): *Austrelaps superbus* (common or lowland copperhead, Günther, 1858), *Austrelaps ramsayi* (alpine or highland copperhead, Krefft, 1864) and *Austrelaps labialis* (pygmy or Adelaide Hills copperhead, Jan, 1859). These snakes prefer cooler climates and are usually found in damp areas around streams, rivers, swamps or marshlands and are distributed in the southeastern states of Victoria and Tasmania, the highlands of New South Wales and southern parts of South Australia (Fig. 1).

Whilst there is a paucity of clinical data on copperhead envenoming, the toxicity of *A. superbus* venom has been established, with a median lethal dose (LD_{50}) of 0.50 mg/kg (s.c. in mice with 0.1% BSA), exactly the same lethality as that of the Indian cobra *Naja naja* (Broad et al., 1979). Furthermore, *A. superbus* has been attributed to at least one human fatality and profound paralysis in a dog following envenoming (Sutherland and Tibballs, 2001). Given the high venom potency and venom yield of 26–85 mg (Sutherland and Tibballs, 2001), *A. superbus* should be regarded as dangerous and capable of lethal envenomation. To date, a number of coagulopathic proteins, including anticoagulants and inhibitors of platelet aggregation have been characterised from this venom (Singh et al., 2000; Subburaju and Kini, 1997; Yuan et al., 1993) and one study has reported the presence of myotoxic fractions causing myoglobinuria (Mebs and Samejima, 1980). However, while an anticoagulation coagulopathy is observed, symptoms of major myolysis and defibrination coagulopathy are not commonly observed in a clinical setting as they are with tiger snake envenoming. Despite these studies, there is very limited knowledge about the neurotoxicity of this venom with a single study indicating the likely presence of postsynaptic neurotoxins (Hodgson et al., 2003).

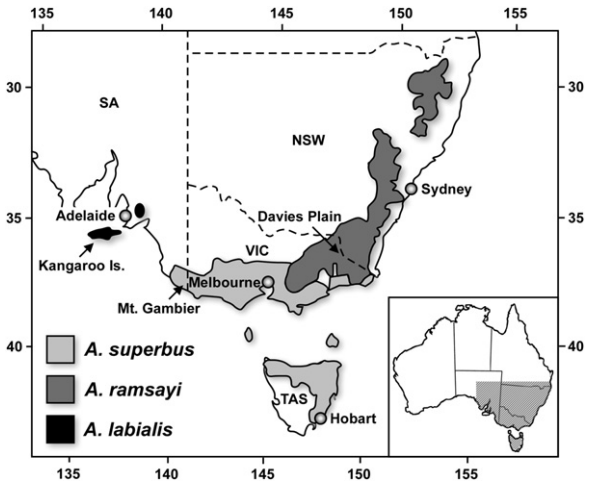

Fig. 1. Distribution of the three Australian copperhead species. Venom was sourced from specimens captured at Kangaroo Island, South Australia (*A. labialis*), Mount Gambier, South Australia (*A. superbus*) and Davies Plain, Victoria (*A. ramsayi*). Modified from Rawlinson, 1991 (Rawlinson, 1991).

While the yield of venom from *A. ramsayi* and *A. labialis* is very low, the LD_{50} values were found to be 0.6 mg/kg and 1.3 mg/kg, respectively (Sutherland and Tibballs, 2001). However there is little data available about the venom components of these two species compared to *A. superbus*. To date, there is only one study that describes the venom composition of *A. ramsayi* using a proteomics approach to identify proteins by mass and sequence homology (Birrell et al., 2007). Similarly, there is a single published study describing the venom composition of *A. labialis*, using transcriptomics to sequence cDNA from the venom gland of a specimen and identify proteins by sequence homology (Doley et al., 2008). A limitation with proteomics- and transcriptomics-based approaches is that proteins identified by sequence homology provide little if any information about their pharmacological action(s) or their potency, nor can they identify non-covalently bound protein complexes. These previous studies have shown that secretory phospholipase A_2 ($sPLA_2$) enzymes are present in the venoms of all three copperheads (Birrell et al., 2007; Doley et al., 2008). However, the pharmacological activities of $sPLA_2$ enzymes are quite diverse: neurotoxic ± myotoxic, cardiotoxic, convulsant or coagulopathic activity (Kini, 1997).

In addition to the common postsynaptic α-neurotoxins that block postjunctional nicotinic acetylcholine (ACh) receptors, monomeric SPANs (Harris et al., 1973) and multimeric SPAN complexes (Blacklow et al., 2010a; Chaisakul et al., 2010; Fohlman et al., 1976; Hodgson et al., 2007; Su et al., 1983) have been isolated from the venoms of several Australo-Papuan elapid snakes. SPANs bind irreversibly to the presynaptic nerve terminal at the skeletal neuromuscular junction to inhibit the release of ACh without affecting the sensitivity of the motor endplate to ACh. Given that these toxins block neurotransmitter release in a potent and irreversible fashion, in addition to damaging the nerve terminal, knowledge of their presence can have implications for the management of envenomed patients. Therefore, *in vitro* studies are critical to determine the pharmacological actions of the components within these venoms, with particular attention paid to neurotoxic components that are responsible for the most serious complications in systemic copperhead envenomation.

Despite a series of monovalent antivenoms being available to treat envenomation by a range of different Australo-Papuan snakes, there are no specific antivenoms to treat several potentially lethal snakes, including