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ORIGINAL ARTICLE

Widespread and Differential Neurotoxicity in Venoms from the *Bitis* Genus of Viperid Snakes

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Abstract

Research into the neurotoxic activity of venoms from species within the snake family Viperidae is relatively neglected compared with snakes in the Elapidae family. Previous studies into venoms from the *Bitis* genus of vipers have identified the presence of presynaptic phospholipase A_2 neurotoxins in *B. atropos* and *B. caudalis*, as well as a postsynaptic phospholipase A_2 in *B. arietans*. Yet, no studies have investigated how widespread neurotoxicity is across the *Bitis* genus or if they exhibit prey selectivity of their neurotoxins. Utilising a biolayer interferometry assay, we were able to assess the binding of crude venom from 14 species of *Bitis* to the neuromuscular α-1 nAChR orthosteric site across a wide range of vertebrate taxa mimotopes. Postsynaptic binding was seen for venoms from *B. arietans*, *B. armata*, *B. atropos*, *B. caudalis*, *B. cornuta*, *B. peringueyi* and *B. rubida*. To further explore the types of neurotoxins present, venoms from the representatives *B. armata*, *B. caudalis*, *B. cornuta* and *B. rubida* were additionally tested in the chick biventer cervicis nerve muscle preparation, which showed presynaptic and postsynaptic activity for *B. caudalis* and only presynaptic neurotoxicity for *B. cornuta* and *B. rubida,* with myotoxicity also evident for some species. These results, combined with the biolayer interferometry results, indicate complex neurotoxicity exerted by *Bitis* species, which varies dramatically by lineage tested upon. Our data also further support the importance of sampling across geographical localities, as significant intraspecific variation of postsynaptic neurotoxicity was reported across the different localities.

Keywords *Bitis* · Neurotoxicity · Nicotinic acetylcholine receptor · Viperidae · Postsynaptic · Presynaptic

Introduction

Snake venom toxins exhibit a variety of pathophysiological activities which act synergistically, targeting key physiological systems to immobilise prey (Fry et al. 2012). Species within the family Viperidae possess highly complex venoms, with coagulotoxic effects being the most prominent and investigated activity (Serrano et al. 2005). Although neurotoxicity is a main pathophysiology of venom from most species within the family Elapidae, it has been relatively understudied within Viperidae (Barber et al. 2013; Harris et al. 2020b; Nirthanan and Gwee 2004).

The most common function of snake venom neurotoxins is to cause flaccid paralysis via inhibition of the orthosteric site (acetylcholine binding region) of postsynaptic muscle-type α-1 nicotinic acetylcholine receptors (nAChRs). This postsynaptic neurotoxicity is primarily seen in venoms which possess three finger toxins (3FTxs) which are a non-enzymatic toxin class ubiquitous to the Elapidae family along with some species of Colubridae and Lamprophiidae (Barber et al. 2013; Fry et al. 2003, 2008; Nirthanan and Gwee 2004; Pawlak et al. 2009). Snake toxins that target postsynaptic nAChRs are known as α-neurotoxins (Barber et al. 2013; Nirthanan and Gwee 2004).

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Conversely, only some species of vipers have been shown to have neurotoxic activities. A toxin isolated from *Crotalus durissus terrificus*, known as crotoxin, is a snake venom phospholipase A_2 (PLA_2) (Chang and Lee 1977a). Crotoxin blocks neuromuscular transmission at the presynapse by binding to the motor nerve terminal to deplete synaptic acetylcholine (ACh) vesicles, thus impairing the release of ACh into the synaptic cleft. Toxins that target the presynapse to impair ACh vesicle release are known as β-neurotoxins. This toxin complex is widespread in *Crotalus*, and the relative presence may vary dramatically between populations, as has been shown for *Crotalus scutatus* (Dobson et al. 2018). Other neurotoxic peptides from vipers include the Azemiopsin peptides from *Azemiops species* and the waglerin peptides from *Tropidolaemus* species, which are two types of post-synaptic neurotoxins that are derived from the propeptide region of the C-type natriuretic gene (Brust et al. 2013; Debono et al. 2017; Harris et al. 2020b; Utkin et al. 2012).

Neurotoxic effects have also been identified in some African vipers within the genus *Bitis*. Neurotoxicity is a common pathophysiology from *Bitis atropos* envenomings (Van Der Walt and Muller 2018; Wium et al. 2017), with two neurotoxic PLA_2 toxins having been isolated from the venom and tested for their LD_{50} in vivo (Van Zyl et al. 2001). Caudoxin, a presynaptic PLA_2 neurotoxin, isolated from the venom of *B. caudalis* has been investigated using both chick biventer cervices nerve muscle (CBCNM) and mouse phrenic nerve diaphragm (MPND) (Lee et al. 1982). Further, the crude venom of *B. arietans* has shown postsynaptic neurotoxic activity (Fernandez et al. 2014). Additionally, a PLA_2 toxin named Bitanarin has been isolated that competed with α-bungarotoxin for binding to α-7 nAChRs suggesting postsynaptic activity (Vulfius et al. 2011). However, unlike α-1, α-7 is not a physiologically relevant target, and the evolutionary implications of this activity remain to be elucidated. Regardless, these studies show that both post- and presynaptic acting PLA_2 neurotoxins are prevalent within *Bitis* venoms.

Thus, neurotoxicity in African vipers seems to be a neglected area, especially considering the overwhelming amount of neurotoxic research conducted on elapids (Barber et al. 2013; Nirthanan and Gwee 2004). This is potentially due to the lack of neurotoxic symptoms observed in human envenoming reports from African viper species. However, since humans are not natural prey items of viperid snakes, and given the potential for both α- and β-neurotoxins to exhibit prey-specific targeting (Chang and Lee 1977b; Chang et al. 1977; Harris et al. 2020c; Heyborne and Mackessy 2013; Pawlak et al. 2006, 2009; Su and Chang 1984), the absence of neurotoxic effects in human bite victims is not evidence of the absence of neurotoxins. Therefore, the exploration of prey-selective neurotoxins in viper venoms is a rich but neglected area of research.

Bitis continues to be one of the most medically significant and widespread genera across Africa (Kasturiratne et al. 2008). Despite some evidence for neurotoxicity within the genus (Fernandez et al. 2014; Van Zyl et al. 2001; Viljoen et al. 1982), research has primarily focussed on the coagulotoxic effects of their venom (Mackay et al. 1970; Marsh and Whaler 1974; Morné et al. 2016; Pirkle et al. 1986; Sánchez et al. 2011; Viljoen et al. 1979; Youngman et al. 2019, 2020). This lack of research leaves a major gap in our understanding of the evolutionary history and presence of neurotoxicity throughout the genus. Thus, we aimed to assess the neurotoxicity of *Bitis* venoms utilising a well-established biolayer interferometry (BLI) assay designed to test the binding to orthosteric site (ACh binding region) of postsynaptic α-1 nAChRs to a plethora of taxon-specific mimotopes (Harris et al. 2020a, 2020b, 2020c; Zdenek et al. 2019a). We set out to determine what other members of the *Bitis* genus utilised postsynaptic nAChR targeting toxins and then further assess if they exhibit prey-specific targeting which is known to occur with some α-neurotoxins (Heyborne and Mackessy 2013; Pawlak et al. 2006, 2009). *Bitis* are also the most geographically widespread viper genus across Africa, with some species having extremely wide distributions (e.g. *B. arietans*) and others having multiple allopatric populations (e.g. *B. atropos*, *B. caudalis* and *B. cornuta*) (Barlow et al. 2019; Spawls and Branch 2020). Sampling across multiple populations is critically important to determine evolutionary facets about the venom activity. Thus, different locations of some *Bitis* species were also tested to determine if geographical variation of neurotoxicity occurs which has been revealed in other venomous snakes (Dobson et al. 2018; Forstner et al. 1997; Glenn and Straight 1978; Glenn et al. 1983). To further support our BLI results, representative *Bitis* species which exhibited postsynaptic binding were additionally tested on a CBCNM assay to further confirm the presence of neurotoxic activity.

Methods and Materials

Venoms and Reagents

All venom work was undertaken under University of Queensland IBSC Approval #IBC134BSBS2015. All venoms were sourced from captive snakes: *Bitis arietans* (Kenya, Mali, Saudi Arabia, Tanzania and West Africa locales) and *B. nasicornis* (Burundi) venoms were purchased from Latoxan (Portes-les-Valence, France); *B. atropos unicolor* (Limpopo province), *B. cornuta* (Kleinzee and Springbok) and *B. parviocula* (Ethiopia) were supplied by the Universeum, Gothenburg; *B. armata* (Cape Arguilas), *B. peringueyi* (UL) and *B. schneideri* (UL) were supplied by the Serpentarium Calden, Germany. All other venom

samples were sourced from the Toxin Evolution Lab long-term cryogenic research collection. For samples from species where the geographical locality of the founding stock was unknown, the abbreviation UL (unknown locality) was used. Venoms were lyophilised and stored at − 80 °C. Working stock solutions of venom (50% glycerol and 50% double-deionised water (ddH_2O) to preserve enzymatic activity) were made at a concentration of 1 mg/ml and stored at − 20 °C until required. Working stock concentrations were determined using a NanoDrop 2000 UV–Vis Spectrophotometer in triplicate.

Mimotope Production and Preparation

Extending previous research (Harris et al. 2020b, 2020c; Zdenek et al. 2019a), a 13–14 amino acid mimotope of the nAChR orthosteric site of vertebrate α-1 nAChR subunit was developed by GenicBio Ltd. (Shanghai, China) designed upon specification and adapted from publicly available sequences of cholinergic receptors (Chrna1) from Genbank and UniProt. For each taxon, the α-1 orthosteric site amino acid sequences were obtained using the following accession codes: amphibian α1 (uniprot F6RLA9), lizard α1 (Genbank XM_015426640), avian α1 (uniprot E1BT92), rodent α-1 (uniprot P25108) and human α-1 (uniprot G5E9G9). The only exception was the α-1 sequence for the snake α-1 (*Coelognathus radiatus*), which was Sanger sequenced in a previous study (Zdenek et al. 2019a). During peptide synthesis, the Cys-Cys of the native mimotope is replaced with Ser-Ser to avoid uncontrolled postsynthetic thiol oxidation. Replacement with Ser-Ser is not expected to have any effect upon the analyte-ligand complex formation as the Cys-Cys bond in the nAChR binding region does not participate directly in analyte-ligand binding (McLane et al. 1994, 1991; Tzartos and Remoundos 1990). However, the Cys-Cys bond is important in the conformation of the interaction site of whole receptors. Thus, direct comparisons between nAChR mimotopes and whole receptor testing using kinetics data should be avoided or approached with caution.

Mimotopes were further synthesised to a biotin linker bound to two aminohexanoic acid (Ahx) spacers, forming a 30 Å linker. Dimethyl sulfoxide (DMSO) was used to solubilise mimotope dry stocks, then diluted in 1:10 dilution of double deionised water to create a working stock of 50 µg/ml and stored at − 80 °C until use.

Biolayer Interferometry

The biolayer interferometry (BLI) assay was performed on an Octet HTX system (ForteBio™, Fremont, CA, USA). All methodology followed previous research that developed this nAChR binding assay (Harris et al. 2020c; Zdenek et al. 2019a). In brief, Streptavidin biosensors were hydrated in assay running buffer for 30–60 min and agitated at 2.0 revolutions per minute (RPM) on a shaker, prior to experimentation. Venom (analyte) samples were diluted to make a final experimental concentration of 50 µg/ml per well and mimotope aliquots were diluted to an experimental concentration of 1 µg/ml per well. 1× DPBS with 0.1% BSA and 0.05% Tween-20 was used for the assay running buffer. Analyte dissociation occurred using a standard acidic solution (glycine buffer), made up of 10 mM glycine (pH 1.5–1.7) in ddH_2O. Raw data is provided in supplementary file 1. All experiments were conducted in triplicate across the mimotopes. Due to limitations in venom supply, *B. worthingtoni* was unable to be tested upon the human mimotope.

All data obtained from the BLI assay was processed in exact accordance to the validation of this assay (Zdenek et al. 2019a). The association step data was obtained in an excel.csv file extracted from raw outputs of the Octet HTX system and then imported into Prism 8.0 software (GraphPad Software Inc., La Jolla, CA, USA) for analysis and graphing. Raw data is provided in supplementary file 1.

Chick Biventer Cervicis Nerve Muscle Preparation

The chick biventer work was undertaken under Monash University Animal Ethics Committee (MARP2 committee) approval # 22575. Chicks aged 4 to 10 days were euthanized with CO_2. After dissection, the chick biventer cervicis nerve muscle (CBCNM) preparations were mounted under 1 g tension in 5-ml organ baths containing physiological salt solution (NaCl, 118.4 mM; KCl, 4.5 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25 mM; and glucose, 11.1 mM). Organ baths were maintained at 34 °C and bubbled with carbogen (95% O_2; 5% CO_2). Electrodes were placed around the tendon of the biventer muscle and the motor nerve stimulated (0.2 ms duration, 0.1 Hz, supramaximal V), using a Grass S88 stimulator (Grass Instruments, Quincy, MA, USA), to evoke indirect twitches. Selective stimulation of the nerve was confirmed by the abolition of twitches with d-tubocurarine (10 µM), a nAChR competitive antagonist. Tissues were then washed repeatedly with physiological salt solution to restore twitch responses to nerve stimulation. The stimulation was ceased, and the contractile responses to acetylcholine (ACh, 1 mM for 30 s), carbachol (CCh, 20 µM for 60 s) and potassium chloride (KCl, 40 mM for 30 s) were obtained and recorded. The organ bath was then washed, and electrical stimulation was resumed and maintained for 30 min to allow the preparation to equilibrate. Venom (10 µg/ml) was added to the organ bath and the twitch height was recorded until the abolition of twitch responses or after a 1 h period. The stimulator was turned off again and the bath was washed. Contractile responses to ACh, CCh and KCl were obtained again to compare with responses prior to venom

addition. The twitch responses to electrical stimulations and contractile responses to agonists (ACh, CCh and KCl) were measured using a Grass FT03 force displacement transducer (Grass Instruments, Quincy, MA, USA) and recorded on a PowerLab system (ADInstruments Pty Ltd., Bella Vista, NSW, Australia). To compare the responses to exogenous agonists, one-way ANOVAs were used followed by Tukey's multiple comparison post hoc tests. Graphs were prepared using Prism8.0 software (GraphPad Software Inc., La Jolla, CA, USA). Raw data is provided in supplementary file 2.

Results and Discussion

Previous research investigating the crude venom of *B. arietans* and isolated PLA_2 toxins has been the only indication of postsynaptic neurotoxicity within *Bitis* venom to date (Fernandez et al. 2014; Vulfius et al. 2011). Our investigation into the postsynaptic binding activity of the crude venom from 14 species across the *Bitis* genus revealed that postsynaptic neurotoxicity is more common throughout the genus than previously realised, and in particular is a trait of many dwarf *Bitis* species. However, no significant postsynaptic binding was observed for the dwarf species *B. worthingtoni* which is the most basal of the genus, or in any of the giant species tested which make up the *Macrocerastes* clade (i.e. *B. gabonica*, *B. nasicornis*, *B. parviocula*, *B. rhinoceros*) (Fig. 1). Intraspecific venom variation was also seen for multiple *Bitis* sp., further supporting the occurrence of geographical variation of venom (Fig. 1).

Significant geographical variation was found within *B. arietans*, with only the venoms from the Saudi Arabia and Eastern Cape localities possessing notable neurotoxic activity out of seven localities tested (Fig. 1). Geographical variation in venom composition and function has been documented within many snake species (Forstner et al. 1997; Glenn et al. 1983; Jayanthi and Veerabasappa Gowda 1988; Zdenek et al. 2019b), including previously for *B. arietans* venom (Currier et al. 2010; Youngman et al. 2019). Interestingly, the two *B. arietans* populations which showed postsynaptic binding are not located close to each other, being highly separated at the north-eastern and south-eastern extents of *B. arietans'* range (Barlow et al. 2019; Spawls and Branch 2020). Analysis of mitochondrial data across *B. arietans* has also shown that the Arabian population is allopatric and has diverged into its own clade, separate from other *B. arietans* populations including that of the Eastern Cape (Barlow et al. 2013). Since nothing is known that distinctively links these two populations over other populations of *B. arietans,* either ecologically or geographically, it is possible that this postsynaptic neurotoxic activity was amplified independently twice within *B. arietans* or that these are the only two populations to retain this activity while all other sampled populations have secondarily lost this activity.

Although the basal dwarf species *B. worthingtoni* did not show binding to the nAChR mimotopes, several species of dwarf *Bitis* from the subgenus *Calechidna* displayed binding to the nAChR mimotopes (Fig. 1). These results therefore indicate that postsynaptic neurotoxicity has been amplified at the base of the *Calechidna* group of dwarf *Bitis*. *Bitis atropos*, *B. caudalis*, *B. cornuta*, *B. peringueyi* and *B. rubida* all showed postsynaptic binding activity to the orthosteric site of the neuromuscular α-1 nAChR mimotopes (Fig. 1). This activity was also subject to intense geographical variation among different populations of *B. atropos*, *B. caudalis* and *B. cornuta*, all of which have multiple allopatric populations (Fig. 1). Three localities of *B. caudalis* showed the highest binding affinity of all *Bitis* species. However, the Messina locality did not show evidence of neurotoxicity, indicating that this locality has had a secondary loss of this trait (Fig. 1). One locality of *B. atropos* showed binding while three localities did not. *B. cornuta* also showed significant variation, with two out of three localities showing evidence of neurotoxicity but the third secondarily lacking this trait. Therefore, our results indicate that within the dwarf *Bitis*, there has been multiple secondary losses of this postsynaptic binding activity, underscoring how extremely dynamic this trait is.

For most species that showed postsynaptic nAChR activity, there was no prey-specific targeting (Fig. 1). However, some species such as *B. arietans* (Eastern cape), *B. armata* and all locales of *B. caudalis* seemed to show some degree of preferential prey binding (Fig. 1). *Bitis arietans* (Eastern cape) and *B. armata* had their highest binding toward the amphibian mimotope. *Bitis caudalis* showed preferential targeting towards the bird mimotope with amphibian close behind (Fig. 1) across all localities to varying degrees of binding intensity. Although these results are not indicative of true prey selectivity, they do suggest that certain proportions of postsynaptic nAChR toxins within *Bitis* have the propensity to target certain prey orthosteric sites than others. This is also evident in that all venoms were either very low binding or did not bind to the human and rodent mimotopes.

Previous research utilising the BLI method has shown that the orthosteric site of the human α-1 nAChR is susceptible (albeit weak compared with taxa types tested) to other classes of α-neurotoxins (Harris et al. 2020b). This suggests that the α-neurotoxin susceptibility of *Bitis* venoms toward the human α-1 orthosteric site is low. This also further supports the lack of neurotoxic symptoms in *Bitis* envenomations, except *B. atropos* which is likely to be caused by presynaptic neurotoxins. These results further support the importance of using taxa which are representative of potential prey to capture the whole function of the venom when investigating activity. Numerous studies only aim to understand venom activity regarding medical significance and thus are focussed on the pathophysiology

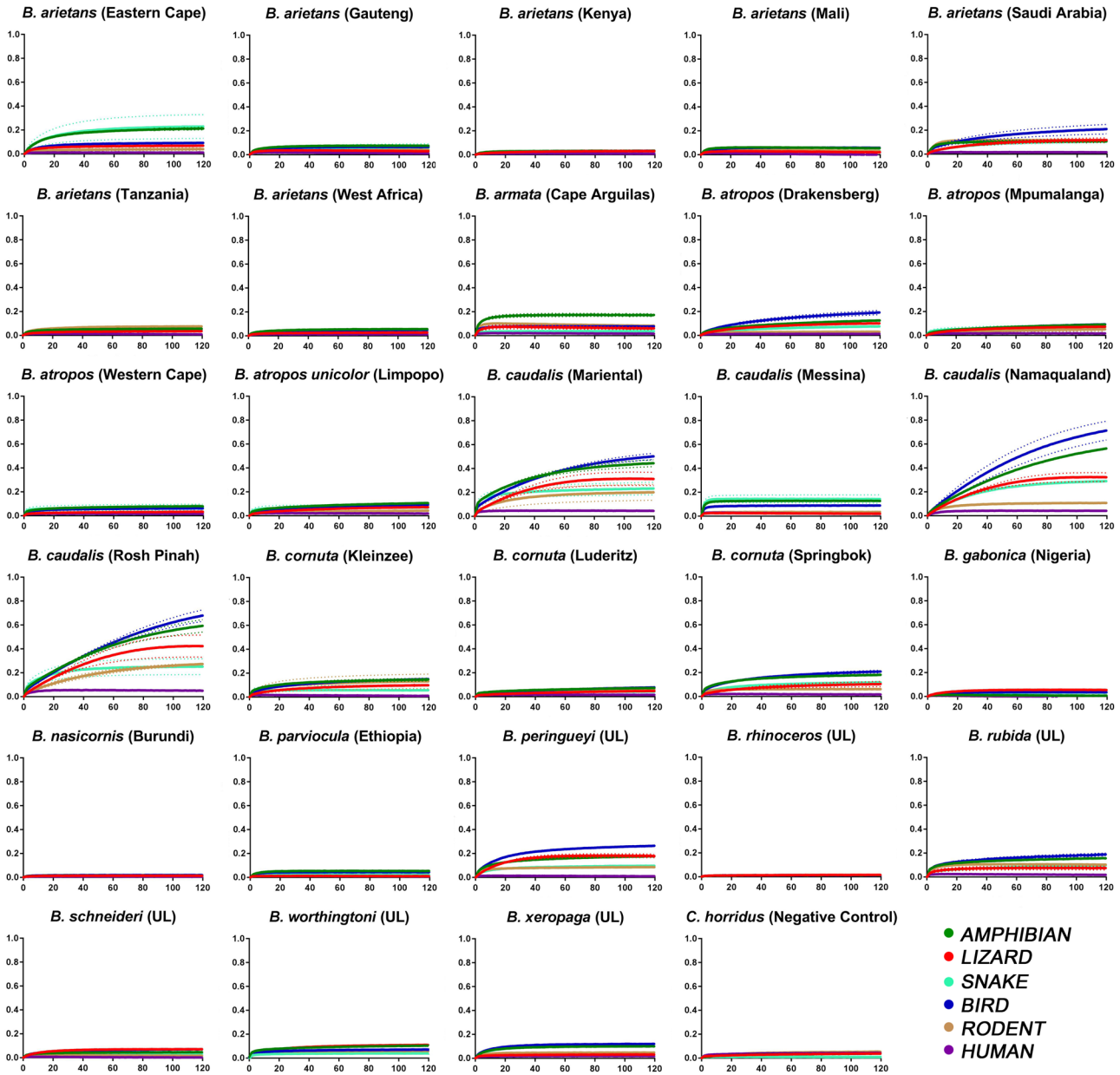

Fig. 1 Comparison of wavelength (nm) curves of the association step (k_a binding step) for *Bitis*, conducted over a 120-s assay period. Venoms were tested against amphibian (green), lizard (red), snake (aqua), bird (blue), rodent (brown) and human (purple) mimotopes in triplicate ($n=3$). *Crotalus horridus* was included as a negative control to represent a venom rich in non-binding toxin types. The dots surrounding the curve lines represent the standard error of the mean (SEM). Due to the high precision of the BLI assay, the SEMs are too small to be viewed for some curves

of human envenomations, which is not always an accurate reflection of the physiological system specific toxins have evolved toward natural prey. Future work should endeavour to determine the biochemical interactions associated with the human α-1 orthosteric site which prevent the binding of *Bitis* α-neurotoxins.

To further investigate the neurotoxic activity of *Bitis* venoms, we tested representative species using the in vitro CBCNM preparation. The selected species were *B. armata* (Cape Arguilas), *B. caudalis* (Namaqualand), *B. cornuta* (Springbok) and *B. rubida*. Interestingly, although *B. caudalis* had the strongest response on the BLI, it displayed a relatively weak neurotoxic response on the CBCNM, only reducing twitch height by approximately 50% over 60 min (Fig. 2a). However, *B. caudalis* venom showed the largest inhibitory effect on contractile responses to ACh and CCh, potentially indicating the highest postsynaptic activity (Fig. 2b). However, only the decrease in ACh response ($F(4,14)=6.156$,

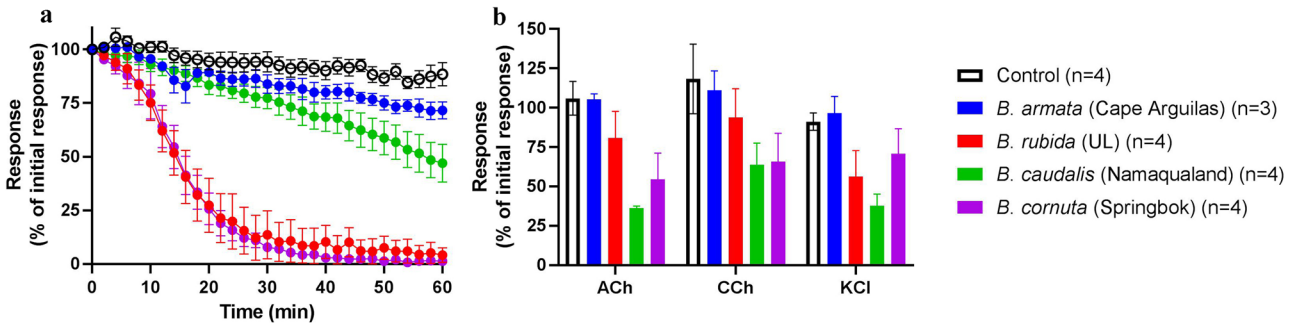

Fig. 2 Chick biventer cervicis nerve muscle preparation showing the neurotoxic activity of venoms at 10 µg/ml from *B. armata*, *B. rubida*, *B. caudalis* and *B. cornuta* and relative agonist blockage. **a** Inhibition of indirect twitches by the crude venom of *B. armata* (blue), *B. rubida* (red), *B. caudalis* (green) and *B. cornuta* (purple). Vehicle (white) represents the time control. **b** Effect of the venoms on contractile response to exogenous acetylcholine (ACh), carbachol (CCh) and potassium chloride (KCl). All venoms were tested in quadruplicate ($n=4$), except *B. armata* which could only be tested in triplicate ($n=3$) due to a limited amount of venom. Error bars represent the SEM

$p=0.0084$) was statistically significant when compared with the time control and this was accompanied by a significant drop in the contractile response to KCl ($F(4,14)=3.84$, $p=0.0466$) (Fig. 2b). The drop in response to KCl is suggestive that there may be myotoxic effects in addition to postsynaptic neurotoxic binding, causing the reduction in response to the exogenous agonists ACh and CCh in this tissue preparation.

The neurotoxic activity of *B. armata*, *B. cornuta* (Springbok) and *B. rubida* venom all showed varying levels of binding to the nAChR mimotopes. *Bitis armata* venom displayed almost no neurotoxicity on the CBCNM assay (Fig. 2a) which is also consistent with the lack of binding toward the bird mimotope (Fig. 1). However, the level of binding which occurred to the amphibian mimotope by *B. armata* venom in the BLI assay is suggestive that there may be a small proportion of postsynaptic neurotoxins in the venom which specifically target amphibian postsynaptically (Fig. 1). However, more research is needed to confirm the level of specificity which these kinds of toxins might exhibit, such as using amphibian neuromuscular tissue preparations. Venoms from *B. cornuta* (Springbok) and *B. rubida* showed binding in the BLI assay to various nAChR mimotopes tested including the bird mimotope. Testing upon the CBCNM assay showed potent neurotoxic activity from *B. cornuta* (Springbok) and *B. rubida* venom, both venoms causing 100% inhibition of twitch height (Fig. 2a). These venoms also partially reduced contractile responses to ACh and CCh indicating that this neurotoxic effect may be due to the activity of both presynaptic and postsynaptic neurotoxins, although this was also accompanied by a decrease in response to KCl which likely indicates a myotoxic in addition to postsynaptic neurotoxic effect similar to the results for the venom of *B. caudalis* (Fig. 2b).

A potential scenario for the neurotoxic activity of *B. caudalis*, *B. cornuta* and *B. rubida* is that the binding seen towards the nAChR mimotopes in the BLI assays is due to the crude venoms possessing a low proportion of neurotoxins which target the postsynaptic nAChR orthosteric site. However, this activity is not discernible in the CBCNM due to the venom being proportionally higher in presynaptic acting neurotoxins (Lee et al. 1982; Van Zyl et al. 2001; Viljoen et al. 1982) as well as, potentially, myotoxins which obscures any potential postsynaptic activity. PLA_2 toxins possessing both presynaptic neurotoxic activity as well as myotoxic activity have indeed been described, such as notexin from the venom of the elapid snake *Notechis scutatus* (Dixon and Harris 1996; Harris and Johnson 1978; Harris et al. 1973). Due to the propensity for PLA_2 toxins to have multiple functions, it is possible that some of the α-neurotoxins present in *B. caudalis*, *B. cornuta*, *B. rubida* which bind to the postsynaptic mimotopes in the BLI assay are also displaying presynaptic or myotoxic activity on the CBCNM causing our observed results. This is thus a rich area for future research.

Conclusions

Our study identifies for the first time the wide prevalence of postsynaptic α-1 nAChR orthosteric targeting venoms across the genus *Bitis*. Postsynaptic neurotoxicity was present in the venom of *B. arietans* as well as numerous dwarf *Bitis* species within the *Calechidna* clade. This suggests that this form of neurotoxicity may be a basal trait that has been independently amplified on multiple occasions within *Bitis*. *Bitis caudalis*, *B. cornuta* and *B. rubida* all showed evidence for possessing both presynaptic and postsynaptic neurotoxicity, in addition to myotoxicity. These results therefore suggest that neurotoxic venom activity is more widespread throughout the *Bitis* genus than previously known. Significant intraspecific geographical variation was also revealed for the postsynaptic neurotoxic activity of *B. arietans*, *B. atropos*, *B. caudalis* and *B. cornuta*. Thus, these results further support the growing body of literature which

establishes the importance in assessing geographical variation in venom activity, particularly for species with extensive or isolated ranges. Future work should investigate additional species across a wider range of concentrations and with more replicates in order to more fully investigate this neglected area of research. The isolation and characterisation of neurotoxins from the venoms would also be beneficial to elucidate their site of action. This is particularly important for *B. atropos* which is well-characterised as producing potent human effects yet was not very strong in this assay. Thus, the human medicine site of action appears to lie outside the orthosteric site, and thus, follow-up studies should investigate using the chick biventer assay and also ascertain the efficacy of the available South African antivenom. This and other neglected aspects of *Bitis* venom neurotoxicity is a rich area of future research.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12640-021-00330-4.

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