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A novel mechanism of inhibition of high-voltage activated calcium channels by \( \alpha \)-conotoxins contributes to relief of nerve injury-induced neuropathic pain

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Abstract

α-Conotoxins that are thought to act as antagonists of nicotinic acetylcholine receptors (nAChRs) containing α3-subunits are efficacious in several preclinical models of chronic pain. Potent interactions of Vc1.1 with other targets have suggested that the pain relieving actions of α-conotoxins might be mediated by either α9α10 nAChRs or a novel GABA_B receptor-mediated inhibition of N-type calcium channels. Here we establish that three α-conotoxins, Vc1.1, AuIB and MII, have distinct selectivity profiles for these three potential targets. Their potencies after intramuscular administration were then determined for reversal of allodynia produced by partial nerve ligation in rats. Vc1.1, which potently inhibits α9α10 nAChRs and GABA_B/Ca^{2+} channels but weakly blocks α3β2 and α3β4 nAChRs, produced potent, long-lasting reversal of allodynia that were prevented by pretreatment with the GABA_B receptor antagonist, SCH50911. α-Conotoxin AuIB, a weak α3β4 nAChR antagonist, inhibited GABA_B/Ca^{2+} channels but did not act on α9α10 nAChRs. AuIB also produced reversal of allodynia. These findings suggest that GABA_B receptor-dependent inhibition of N-type Ca^{2+} channels can mediate the sustained anti-allodynic actions of some α-conotoxins. However, MII, a potent α3β2 nAChR antagonist but inactive on α9α10 and α3β4 nAChRs and GABA_B/Ca^{2+} channels, was demonstrated to have short-acting anti-allodynic action. This suggests that α3β2 nAChRs may also contribute to reversal of allodynia. Together, these findings suggest that inhibition of α9α10 nAChR is neither necessary nor sufficient for relief of allodynia and establish that α-conotoxins selective for GABA_B receptor dependent inhibition of N-type Ca^{2+} channels relieve allodynia, and could therefore be developed to manage chronic pain.
1. Introduction

Chronic pain is a significant worldwide health problem and there is a need for new drug classes for treatment [5, 13, 16]. Conotoxins are peptides from the venom of the Conus genus of predatory marine snails [441]. Many conotoxins are selective antagonists of a range of ion channels, transporters and membrane receptors associated with pain. Previous studies have demonstrated the analgesic potential of several different α-conotoxins that competitively inhibit neuronal nicotinic acetylcholine receptors (nAChRs) with varying degrees of subtype selectivity [2,15,24,29,36,37]. One α-conotoxin, Vc1.1 from Conus victoriae, potently suppresses signs of neuropathic pain following intramuscular (i.m.) administration in rats [37] and recently progressed to Phase II clinical trials [2,18,26].

How Vc1.1 and related α-conotoxins relieve neuropathic pain remains controversial. Vc1.1 more selectively, but weakly, antagonises peripherally expressed nAChR subtypes (α3β2, α3β4 and α3α5β2) than those more abundantly expressed in the CNS (α4β2, α4β4, and α7) or skeletal muscle (α1β1γδ) [12]. More recently, Vc1.1 was shown to have high potency as an antagonist at α9α10 nAChRs and it was proposed that this nAChR is the analgesic target of Vc1.1 [43]. However, the loss of anti-allodynic activity of several analogs of Vc1.1 that retain activity at α9α10 but not other nAChR subtypes [31] suggests that targets other than α9α10 contribute to the pain-relieving activity of α-conotoxins.

We have recently reported that Vc1.1 inhibits N-type calcium channels in rat sensory neurons via a novel GABA_B receptor-dependent signalling mechanism [8], which may also contribute to its pain-relieving actions [4,39]. The mechanism of inhibition of N-type calcium channel currents by Vc1.1 remains to be elucidated but does not involve a conventional GPCR-like modulatory mechanism.
because it is voltage-independent \[8\], requires src-kinase activity \[8\], does not modulate GABA_B receptor-activated GIRK channels expressed in *Xenopus* oocytes \[29\] and does not directly displace binding of GABA_B receptor ligands \[29\]. N-type calcium channel inhibition also persists in sensory neurons from \(\alpha9\) nicotinic receptor knockout mice \[7\]. To better understand the potential analgesic mechanisms of \(\alpha\)-conotoxins, the present study employed \(\alpha\)-conotoxins with vastly differing selectivities for \(\alpha3\)-containing nAChR, \(\alpha9\alpha10\) nAChRs and GABA_B/Ca^{2+} channels to determine whether one or several of these targets contributes to relief of neuropathic pain. Intramuscular Vc1.1, which interacts potently with both \(\alpha9\alpha10\) nAChRs \[31,42,43\] and GABA_B/Ca^{2+} channels \[8\] but quite weakly with \(\alpha3\beta2\) and \(\alpha3\beta4\) nAChRs \[11\], produced potent, long-lasting relief from allodynia after partial nerve ligation. Another \(\alpha\)-conotoxin, AuIB also produced potent, long-lasting relief from allodynia. AuIB, which is a relatively weak but selective \(\alpha3\beta4\) nAChR antagonist \[14,27\], potently inhibited N-type Ca^{2+} channels but did not interact with \(\alpha9\alpha10\) nAChRs. We found that a third \(\alpha\)-conotoxin, MII, produced weak, short-lived reversal of allodynia. MII did not interact with \(\alpha9\alpha10\) nAChRs or GABA_B/Ca^{2+} channels but is a potent inhibitor of \(\alpha3\beta2\) nAChRs \[7\]. Significantly, these results suggest that GABA_B receptor-dependent inhibition of N-type Ca^{2+} channels may be the major mechanism of sustained anti-allodynic actions of \(\alpha\)-conotoxins. As reported previously, we confirm inhibition of \(\alpha9\alpha10\) nAChR is not required for the anti-allodynic actions of \(\alpha\)-conotoxins \[31\].

### 2. Materials and Methods

#### 2.1. Electrophysiological recordings in oocytes

All experiments adhere to the guidelines of the Committee for Research and Ethical Issues of IASP published in *Pain*, 1983;16:109–110. Procedures for harvesting *Xenopus laevis* oocytes were approved by the University of Queensland Animal Ethics Committee, which complies with National
Health & Medical Research Council of Australia (NHMRC) guidelines. RNA preparation, oocyte preparation and expression of nAChR subunits in *Xenopus* oocytes were performed as described previously [31]. Briefly, plasmids with cDNA encoding the rat α9 and α10 nAChR subunits were provided by Dr. A.B. Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina). 5 ng of cRNA was injected into each oocyte which was then kept at 18°C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES at pH 7.4) supplemented with 50 mg/L gentamycin and 5 mM pyruvic acid for 2-5 days before recording.

Membrane currents were recorded from the *Xenopus* oocytes using an OpusXpress™ 6000A workstation (Molecular Devices, Sunnyvale, CA). Electrodes had resistances of 0.3-1.5 MΩ when filled with 3 M KCl. All recordings were conducted at room temperature (20-23°C) using a bath solution of ND96 as described above. During recordings, the oocytes were perfused continuously at a rate of 1.5 ml/min, with 5 min incubation times for the conotoxins. Acetylcholine (ACh; 30 μM) was applied for 2 s at 5 ml/min, with 10 min washout periods between applications. Conotoxins were bath applied and co-applied with the agonist. Oocytes were voltage clamped at a holding potential of –80 mV. Data were sampled at 500 Hz and filtered at 200 Hz. Peak current amplitude was measured before and following incubation of the peptide.

2.2. Electrophysiological recordings from DRG neurons

Dorsal root ganglion (DRG) neurons were enzymatically dissociated from ganglia of 7-14 day old Wistar rats. All procedures were approved by the University of Queensland Animal Ethics Committee, which complies with NHMRC guidelines. Briefly, rats were killed by cervical dislocation, the spinal column was hemi-segmented and the spinal cord removed. Ganglia were
removed and rinsed in cold Hanks’ balanced salt solution (HBSS; MultiCel), minced and incubated in 1 mg/ml collagenase (Type 2; 405U/mg, Worthington Biochemical Corp., Lakewood NJ) in HBSS at 37°C for ~30 min. Following incubation, ganglia were rinsed three times with warm (37°C) Dulbecco’s modified Eagle’s medium (DMEM; GIBCO) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin and gently tritutated with a fire-polished Pasteur pipette. Cells were plated on glass cover slips, incubated at 37°C in 95% O₂:5% CO₂ and used within 4-24 hrs.

The external recording solution for rat DRG neurons contained (in mM): 150 TEACl, 2 BaCl₂, 10 D-glucose, 10 HEPES, pH 7.4. Recording electrodes were filled with an internal solution containing (in mM): 140 CsCl, 1 MgCl₂, 5 MgATP, 0.1 NaGTP, 5 BAPTA-Cs₄, 10 HEPES, pH 7.3 with CsOH and had resistances of 1.0-2.5 MΩ. Membrane currents were recorded using the whole-cell configuration of the patch clamp technique with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). A voltage protocol using step depolarizations from −80 mV to 0 mV was used when examining high voltage-activated (HVA) Ca²⁺ channel currents. Test potentials 150 ms in duration were applied every 20 s. Leak and capacitative currents were subtracted using a −P/4 pulse protocol. Currents were generated by a computer using pClamp 9.2 software (Molecular Devices), filtered at 2 kHz and sampled at 8 kHz by the Digidata 1322A (Molecular Devices). Sampled data were stored digitally on a computer for further analysis.

2.3. Surgery: partial nerve ligation model

Male Sprague-Dawley rats weighing 200-250g were housed in groups of 3 in a constant temperature room (22 ± 1°C), under a 12/12 h light/dark cycle (lights were on between 05:30-17:30 h) with access to food and water ad libitum. Animals were anaesthetised for all surgical procedures
using isoflurane and were killed by anaesthetic overdose at the end of the experiment. All experiments were approved by the Royal North Shore Hospital and University of Technology Animal Ethics Committee, which complies with NHMRC guidelines.

Signs of neuropathic pain were produced using partial ligation of the left sciatic nerve (PNL) under isoflurane anaesthesia [40]. Briefly, the left sciatic nerve in the mid-thigh region of the rat was exposed by blunt dissection through the biceps femoris at a site near the trochanter just distal to the posterior biceps semitendinosus nerve branches off the common sciatic nerve. A 4-0 silk suture was inserted into the left sciatic nerve trunk approximately 3mm proximal to the trifurcation of the nerve at the popliteal fossa and was tightly ligated so that the dorsal 30-50% of the nerve thickness was trapped in the ligature.

2.4. Mechanical allodynia testing

Mechanical paw withdrawal threshold (PWT) was measured using a series of von Frey hairs with bending pressures ranging from 0.41-15.1 g. Rats were placed in elevated plastic cages with wire mesh bases suspended above a table. All rats were given 30 min to acclimatise to the testing environment. Beginning with the 2g filament, von Frey hairs were pressed perpendicularly against the plantar surface of the left hind paw and held for 2 s. Each von Frey filament was applied 7 times at random locations. A positive response was regarded as the sharp withdrawal of the paw, paw licking, or flinching upon removal of the von Frey filament. The mechanical PWT was calculated using the up-down paradigm [10]. If an animal did not respond to any hairs then the mechanical PWT was assigned as 15 g. Mechanical PWT to non-noxious mechanical stimuli were tested prior to surgery on day 0 (pre-PNL) and 1, 4-5, 7-8, 9-11 and 12-14 days following injury.
2.5. Motor performance

As nAChRs are expressed on skeletal muscle, an accelerating rotarod device (Ugo Basile, Italy), with a maximum cut-off time of 300 s, was used to examine motor side effects. Animals were placed on the rotating drum and were required to walk against the accelerating motion. Each latency recording consisted of the average of three measurements on the day of testing.

2.6. Drugs and drug treatment

Vc1.1 (1,810 Da), MII (1,711 Da) and AuIB (1,572 Da) were synthesised as previously described [9,12,27,38]. Briefly, all α-conotoxins were manually synthesized by Boc solid phase chemistry, deprotected and cleaved from the resin as described previously [38]. The HPLC-purified reduced peptides (100 μM) were oxidised in 100 mM ammonium bicarbonate at pH 7.5–8.2 with stirring for 48 h at room temperature. The oxidised peptides were purified by preparative reverse phase-HPLC. Each was quantified in triplicate by RP-HPLC using an external reference standard for each peptide. The effects of the α-conotoxins on withdrawal thresholds and motor function were assessed between 12 and 14 days post-PNL in all animals. On the day of drug testing, animals were tested twice pre-injection (30 min prior to and just before injection) in order to stabilise pre-injection responses. Behavioural testing occurred at 1, 2, 4 and 6 hours post-injection to assess the acute effects of each drug. In addition, every animal was tested 24 hours post-injection in order to assess any potential long-term effects of each peptide. Each dose or vehicle was assigned randomly according to a Latin-square based design to reduce the influence of treatment order and the experimenter was blinded to treatments. CGP 55845 and SCH 50911 were purchased from Tocris Bioscience (UK).

2.7. Data analysis
All data were analysed using Prism software (GraphPad version 4 for Windows, San Diego, CA, USA). Plots of mechanical PWT and bar charts of rotarod latencies were presented as mean ± S.E.M. over time. All other plots of rotarod latencies were presented as mean change ± S.E.M. over time. For treatment groups, a two-way (group x time) repeated measures analysis of variance (ANOVA) was performed. All behavioural data were analysed using a one-way repeated measures ANOVA with time as a within-subjects factor where appropriate to compare the thresholds before and after the induction of pain and administration of drugs. Where one-way ANOVAs were significant, post-hoc comparisons were made against the time 0 hour point at 12-14 days post-PNL (time effects) or against the vehicle-injected group using Dunnett’s adjustment for multiple comparisons. To analyse dose-response relationships the mean changes in mechanical PWT produced by i.m. injection of the α-conotoxins were calculated as the integrated area under the curve (AUC) post-injection relative to pre-injection levels. All AUC data were calculated from 0-6 h after peptide injection using baseline subtraction. Dose-response curves were constructed by fitting a logistic curve to the increase in mechanical PWT. P < 0.05 was considered significant.

3. Results

3.1. Inhibition of α9α10 nAChR channel currents by Vc1.1 but not AuIB or MII

As previously reported [31,43], robust ACh-induced cation currents were expressed in *Xenopus laevis* oocytes following injection of mRNA encoding α9α10 nAChR channel subunits (Figure 1). Superfusion of Vc1.1 (100 nM) potently inhibited α9α10 nAChR currents, whereas in the same series of experiments, neither AuIB nor MII produced any inhibition of α9α10 nAChR currents at concentrations up to 3 µM (Figure 1).
3.2. Inhibition of N-type Ca\textsuperscript{2+} channel currents in DRG neurons by Vc1.1 and AuIB but not MII.

We have previously reported that Vc1.1 potently inhibits (IC\textsubscript{50} = 1.7 nM) high voltage-activated Ca\textsuperscript{2+} channel currents in DRG neurons via a novel mechanism involving GABA\textsubscript{B} receptor-mediated inhibition of N-type Ca\textsuperscript{2+} channel [8]. Using the same procedures, AuIB similarly produced a partial inhibition of high voltage-activated Ca\textsuperscript{2+} channel currents in rat DRG neurons (Figure 2) with an IC\textsubscript{50} of 1.5 ± 0.3 nM (n = 17). As with Vc1.1 [8], inhibition by AuIB did not affect the kinetics of activation of the N-type Ca\textsuperscript{2+} channel currents (Figure 2). By contrast, in the same series of experiments, MII (1 µM) produced no inhibition of depolarization-activated Ca\textsuperscript{2+} channel currents. Application of the selective N-type Ca\textsuperscript{2+} channel inhibitor, ω-conotoxin CVID, confirmed that AuIB targeted the N-type component of the HVA Ca\textsuperscript{2+} channel currents.

AuIB (100 nM) reduced peak Ca\textsuperscript{2+} channel current amplitude to 54.7 ± 6.2% (n = 10) of control. Application of 100 nM AuIB in the presence of CVID (200 nM) produced no further reduction of the HVA Ca\textsuperscript{2+} channel current amplitude (51.6 ± 8.9% of control, n = 10) (Figure 2 C (i)). Furthermore, in the presence of the GABA\textsubscript{B} receptor antagonist CGP 55845, AuIB (100 nM) failed to inhibit the HVA Ca\textsuperscript{2+} channel currents. CGP 55845 (1 µM) alone had no effect on the HVA Ca\textsuperscript{2+} channel current amplitude and following the addition of AuIB the Ca\textsuperscript{2+} channel current amplitude was 87.9 ± 4.0% (n = 12) of control (Figure 2 C (ii)).

The inhibition of HVA Ca\textsuperscript{2+} channel currents by Vc1.1 (100 nM) was antagonized in the presence of the long acting GABA\textsubscript{B} receptor antagonist SCH 50911 (1 µM) [6]. Bath application of 1 µM SCH 50911 alone, increased HVA Ca\textsuperscript{2+} channel current amplitude (115 ± 5.6%, n = 7, P = 0.046), the current amplitude was 117 ± 6 % of control following application of Vc1.1 in the presence of SCH50911. In a control batch of cells, application of Vc1.1 alone (100 nM) reduced HVA Ca\textsuperscript{2+} channel currents to 61 ± 6.0 % of control (n = 5). (Figure 2 C (iii)).
3.3. Effects of Vc1.1, MII and AuIB on PNL-induced mechanical allodynia

PNL produced allodynia that was maximal 12-14 days after surgery. Mechanical PWT decreased from 9.8 ± 0.4 g prior to surgery (day 0, pre-PNL, n = 115) to 0.7 ± 0.1 g by 12-14 days after PNL. The α-conotoxins Vc1.1, MII and AuIB all produced a significant dose-dependent increase in the mechanical PWT. As shown in Figure 3A, i.m. injection of Vc1.1 produced a significant, dose-dependent increase in mechanical PWT, which, for the highest dose tested (36 µg), was significant at the earliest time point tested. The anti-allodynic activity of Vc1.1 was maintained throughout the initial test period up to 6 hours post-injection at all doses tested. As previously reported [26], the anti-allodynic action of Vc1.1 persisted for at least 24 h after injection. PWT was significantly elevated 24 h after injection at both the 3.6 and 36 µg doses. As shown in Figure 3B, AuIB also produced a significant, dose-dependent increase of mechanical PWT, which was significant soon after injection and persisted at the highest dose for at least 24 h after injection. As shown in Figure 3C, i.m. injection of MII also dose-dependently increased the mechanical PWT but this was only significantly different from vehicle-treated animals 4 h after injection (3.6 and 36 µg doses) and had returned to baseline after 24 h.

3.4 Dose-response relationships
The area under the curve (AUC) from 0-6 hours post-injection was calculated for each animal to construct dose-response curves of the data represented in Figures 3A, 3B and 3C (Figure 4). A logistic curve was then fitted to estimate the EC$_{50}$ for each drug. A two-way ANOVA (drug x dose) indicated significant dose (P < 0.0001) and type of α-conotoxin (P < 0.01) effects, indicating that the three α-conotoxins had significantly different potencies. Vc1.1 was clearly the most potent of the α-conotoxins, followed by AuIB and then MII. Vc1.1 had an estimated EC$_{50}$ for increasing mechanical PWT of 0.34 µg (95% CI = 0.06-1.89 µg). By contrast, AuIB had an EC$_{50}$ of 1.88 µg (95% CI = 0.04-8.75 µg) and MII had an EC$_{50}$ of 9.16 µg (95% CI = 2.75-30.54 µg).

(Insert Figure 4 about here)

3.5. Effects of Vc1.1, MII and AuIB on motor performance

Rotarod latencies were 155 ± 8 s (n = 114) at baseline 12-14 days following PNL. Injection of the α-conotoxins Vc1.1, MII and AuIB at any dose did not produce any significant changes in rotarod latency over time (P>0.05, two-way repeated measures ANOVA). For clarity the effects on rotarod performance are shown only for the highest dose of each α-conotoxin in Figure 5 but lower doses were also without significant effects. No other behavioural disturbances were noted after all doses of the α-conotoxins.

(Insert Figure 5 about here)

3.6. Effects of Vc1.1 and baclofen are prevented by a GABA$_B$ receptor antagonist
As previously reported the GABA\textsubscript{B} receptor agonist, baclofen (4 mg/kg, s.c.) produced significant reversal of alldynia [33], as did Vc1.1. Both actions were significantly and almost completely antagonised by pre-treatment (15 min prior to baclofen or Vc1.1) with the long-acting GABA\textsubscript{B} receptor antagonist SCH 50911 [6] (20 mg/kg, s.c.) but not vehicle (isotonic phosphate buffered saline) (Figure 6).

(Insert Figure 6 about here)

4. Discussion

The present study establishes that intramuscular injections of a range of \(\alpha\)-conotoxins with diverse spectra of target selectivity can all reverse signs of alldynia in a nerve injury model of chronic pain, albeit with differing potencies and durations of action. The interactions of \(\alpha\)-conotoxins Vc1.1, MII and AuIB with their potential analgesic targets as identified from the present and previous studies are summarised in Table 1. The large, often more than 1,000-fold differences in potencies of Vc1.1, AuIB and MII at their known molecular targets provide suggestive evidence for which target(s) are important for reversal of alldynia. The results presented in Table 1 suggest that GABA\textsubscript{B} receptor-dependent inhibition of N-type Ca\textsuperscript{2+} channels could be a major mechanism of sustained anti-alldynic actions of \(\alpha\)-conotoxins. Inhibition of \(\alpha9\alpha10\) nAChR is not required though \(\alpha3\beta2\) nAChRs may contribute to the reversal of alldynia.

(Insert Table 1 about here)

Focusing only on the most potent of the three \(\alpha\)-conotoxins suggests that Vc1.1 could potentially reverse alldynia via either \(\alpha9\beta10\) nAChRs [43] or the novel GABA\textsubscript{B} receptor-mediated inhibition
of N-type Ca\(^{2+}\) channels in sensory neurons [8]. As previously proposed [43], antagonism of α3β2 or α3β4 nAChRs is not a likely mechanism of action of Vc1.1 because moderately effective doses (<0.1 nmol/kg in this study) are several orders of magnitude lower than Vc1.1’s affinity for heterologously expressed α3β2 or α3β4 nAChRs (>1 µM, Table 1) and the ability of Vc1.1 to antagonise the nicotine-induced increase in axonal excitability of isolated unmyelinated human C-fiber axons (>1 µM) [24].

Resolution of which of these targets mediate the anti-allodynic actions of α-conotoxins (either α9β10 nAChRs or GABA\(_B\) receptor/N-type Ca\(^{2+}\) channels, or both) can potentially be achieved using peptides highly selective for one or the other target. Given that Vc1.1 and another previously examined anti-allodynic α-conotoxin, Rg1A, have high affinity for both targets [8,43], they cannot be utilised to resolve this issue. The present results show that AuIB, which has high potency for GABA\(_B\) receptor/N-type Ca\(^{2+}\) channels (low nanomolar range) but does not interact with α9α10 nAChRs, has potent, long-acting anti-allodynic actions. These findings suggest that inhibition of α9α10 nAChRs is not necessary for long-term relief of alldynia. Furthermore, we have reported previously that two analogues of Vc1.1, vlca and [P6O] Vc1.1 that retain full activity at α9α10 nAChRs [31] but have little or no activity GABA\(_B\) receptor/N-type Ca\(^{2+}\) channels [8], produce no reversal of nerve injury-induced allodynia [31]. Therefore antagonism of α9α10 nAChRs is neither necessary nor sufficient to reverse alldynia after nerve injury. Thus the most parsimonious mechanism for the anti-allodynic actions of Vc1.1 and AuIB is the GABA\(_B\) receptor-dependent inhibition of N-type Ca\(^{2+}\) channels in sensory neurons [8] although an as yet unidentified mechanism mediated by the same subset of α-conotoxins cannot be ruled out. Prevention of anti-allodynic actions of both Vc1.1 and baclofen by pre-treatment with the long-acting GABA\(_B\) receptor antagonist, SCH 50911 is consistent with this interpretation.
N-type Ca\(^{2+}\) channels are an appealing target for chronic pain treatment as they are well known to play a central role in the detection and transmission of nociceptive stimuli in DRG neurons [3,28]. Several studies have highlighted the importance of N-type Ca\(^{2+}\) channels in neuropathic pain: N-type α\(_{1B}\) channel knock-out mice have a decreased response to neuropathic pain [2023,35], there is an up-regulation of N-type α\(_{1B}\) as well as α\(_{2δ}\) subunits in rat nerve injury models [1,11,33,44], and currently used treatments, or treatments being developed, for pain relief include direct (α-conotoxin MVIIA, aka Prialt®) and indirect (eg. Gabapentin) inhibitors of N-type Ca\(^{2+}\) channels [28]. We have previously reported a novel mechanism by which some anti-allodynic α-conotoxins, including Vc1.1 and Rg1A, modulate N-type Ca\(^{2+}\) channel currents in DRG neurons, requiring GABAB receptors [8]. The activation of GABAB receptors by agonists such as baclofen is well established as producing antinociceptive and antiallodynic actions in chronic pain models [4,32,33,39]. Therefore, the Vc1.1, AuIB and Rg1A inhibition of Ca\(_{v2.2}\) (N-type) channels that depends on GABAB receptors via a novel transduction process is the most likely the mechanism mediating their anti-allodynic properties. As discussed above, the mechanism of N-type calcium channel inhibition by α-conotoxins has yet to be fully elucidated but is not mediated by a conventional GPCR signalling mechanism that involves direct channel modulation by agonist-mobilized G-protein βγ subunits [8].

The basis for the long duration of action (>24 h) of both Vc1.1 and AuIB (but not MII) after a single, systemic injection is still unknown. It is therefore possible that the sustained anti-allodynic actions of these α-conotoxins result from a long-term effect of N-type Ca\(^{2+}\) channel inhibition on peripheral nerves that also express GABAB receptors. Indeed, reversal of alldynia has recently been reported with the peripherally-restricted, N-type selective ω-conotoxin, CVID [22]. It is not yet known whether inhibition of N-type Ca\(^{2+}\) channel currents by Vc1.1 and AuIB persists for the duration of anti-allodynic activity but it is irreversible during patch-clamp experiments (<1 h) [8].
is also unknown whether differential biodistribution and metabolism of the distinct peptides contributes to the long duration of action.

The present study also suggests that antagonism of α3β2 nAChRs can weakly contribute to reversal of allodynia after nerve injury. MII is a potent (nanomolar range) antagonist of α3β2 nAChRs (Table 1) but does not inhibit either N-type calcium channels or α9α10 nAChRs. Attempts to ascribe either the acute or longer term anti-allodynic actions of all α-conotoxins to a single combination of subunits [eg. 43] are therefore probably futile. Conotoxin MII was found to reverse allodynia more weakly than either Vc1.1 or AuIB although its action was not sustained 24 hours after injection. This might suggest that α3β2 nAChRs are not of primary importance but can contribute to reversal of allodynia. Indeed, nicotinic agonists excite nociceptive primary afferents and α3-subunit containing nAChRs are functionally expressed in at least some nociceptors in rodents [34] and unmyelinated nerves in humans [25]. By contrast, there is some evidence that antagonism of α3β2 nAChRs in the spinal cord has pro-nociceptive actions [45], but this effect is probably restricted to spinal neurons as we observed only anti-alldynic actions after peripheral administration of MII.

In conclusion, the present study demonstrates that novel GABA_B receptor-mediated inhibition of N-type Ca^{2+} channels in sensory neurons is a likely mechanism underlying the anti-allodynic actions of α-conotoxins and should be further investigated as a potential therapeutic target for persistent pain. Furthermore, antagonism of α9α10 nAChRs is neither necessary nor sufficient to reverse allodynia after nerve injury. Because antagonism of α3-subunit containing nAChRs may also contribute to reversal of allodynia, it may be premature to rule out investigation of this target in further development of α-conotoxins as potential therapies for persistent pain states.
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Figure 1

**Effect of α-conotoxins on α9α10 nAChRs expressed in Xenopus oocytes.**

(A) Superimposed traces of ACh-evoked currents mediated by α9α10 nAChRs obtained in the absence (Control) and presence of 100 nM Vc1.1 (i), 1 μM AuIB (ii), and 1 μM MII (iii). Oocytes were voltage clamped at −80 mV and membrane currents evoked with 2 sec application of 30 μM ACh co-applied with the toxin following 300 s incubation. (B) Bar graph of the inhibition of ACh-evoked peak current amplitude by α-conotoxins Vc1.1, AuIB and MII. ACh-evoked currents were inhibited 35 ± 5% (n = 9) and 89 ± 5% (n = 6) of control by 100 nM and 1 μM Vc1.1, respectively, whereas neither AuIB nor MII inhibited the ACh-evoked currents at concentrations up to 3 μM (n = 3-9). All data were pooled and represented as mean ± SEM.

Figure 2

**Effect of α-conotoxins on HVA calcium channel currents in rat DRG neurons.**

(A) Superimposed depolarization-activated Ba²⁺ currents elicited by voltage steps from a holding potential of -80 mV to -10 mV in the absence (control) and presence of 10 nM AuIB (i) and 1 μM MII (ii), respectively. (B) Concentration-response relationships obtained for inhibition of high voltage-activated Ca²⁺ channel currents in DRG neurons by AuIB (○) (n = 4-19), Vc1.1 (---) and MII (■, 1 μM) (n = 6). Data points represent mean ± SEM of normalized peak current amplitude. The IC50 for inhibition of Ca²⁺ channel currents by AuIB was 1.5 nM compared to 1.7 nM for Vc1.1 [7]. (C) (i) Bar graph of the relative inhibition of HVA Ca²⁺ channel currents by the N type Ca²⁺ channel blocker CVID (200 nM) alone and following application of 100 nM AuIB in the presence of CVID. (ii) Bar graph of relative inhibition of HVA Ca²⁺ channel currents by 100 nM AuIB alone, in the presence of 1 μM CGP55845A alone and after application of 100 nM AuIB in the presence of
CGP 55845A. (iii) Bar graph of relative inhibition of HVA Ca\textsuperscript{2+} channel currents by 100 nM Vc1.1 alone, in the presence of 1 μM SCH 50911 alone and after bath application of 100 nM Vc1.1 + 1 μM SCH 50911. Numbers in parentheses reflect numbers of cells.

Figure 3

Effect of α-conotoxins on PNL-induced mechanical allodynia

Time course of the effects of an i.m. injection of different doses of (A) Vc1.1 (n = 6 in each group) and vehicle (n = 6), (B) AuIB (n = 6 in each group) and vehicle (n = 6 ) and (C) MII (n = 6 for 0.36μg and 36μg, n = 9 for 3.6μg) and vehicle (n = 6) on mechanical PWT. Each point represents the mean ± S.E.M. of the mechanical PWT at the indicated times. Time point 0 h represents the time of drug injection. Pre-PNL values for each group are shown. ● – vehicle, ▼ - 0.36 μg, ■- 3.6 μg and ▲ – 36 μg of each α-conotoxin. (*-P < 0.05 and **-P < 0.01, Dunnett’s post hoc test vs 0 h values).

Figure 4

Dose-response relationships of α-conotoxins for reversal of mechanical allodynia.

Dose-response curves (logistic curve fitted) depicting the area under the curve (AUC) of the mechanical PWT to non-noxious mechanical stimuli following i.m. injection with α-conotoxins. Each point represents the mean AUC ± S.E.M. of the mechanical PWT integrated from 0 to 6 hours post-injection.● – Vc1.1, ● - AuIB and ○- MII. Asterisks represent significant difference from the vehicle treatment group (*denotes P < 0.05 and ** denotes P < 0.01, Dunnett’s post hoc test).
Figure 5

**Effect of Vc1.1, MII and AuIB on motor performance**

Time course of the effects of i.m. injection of different doses of Vc1.1, AuIB and MII (n = 6 in each group) and vehicle (n = 6) on rotarod latency. Each point represents the mean change ± S.E.M. of the rotarod latency at the indicated times. Time point 0 h represents the time of drug injection. ▲ – Vc1.1, ▲ - AuIB and Δ- MII and ○ - vehicle.

Figure 6

**Reversal of mechanical allodynia by Vc1.1 and baclofen are antagonised by SCH 50911**

Area under the curve (AUC) of the mechanical PWT to non-noxious mechanical stimuli following injection with Vc1.1 (50 µg i.m.) following pretreatment 15 min earlier with SCH50911 (20 mg/kg, s.c.) or vehicle. Each bar represents the mean AUC ± S.E.M. of the mechanical PWT integrated from 0 to 6 hours post-injection (n = 8 animals per group, *denotes P < 0.05 and ** denotes P < 0.01, Bonferroni post hoc tests).
Table 1

**Target selectivity of α-conotoxins Vc1.1, MII and AuIB.**

<table>
<thead>
<tr>
<th>α-conotoxin</th>
<th>N-type Ca(^{2+}) channel</th>
<th>α9α10 nAChR</th>
<th>α3β2 nAChR</th>
<th>α3β4 nAChR</th>
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<tbody>
<tr>
<td>Vc1.1</td>
<td>1.7</td>
<td>19 - 64</td>
<td>7,300</td>
<td>4,200</td>
</tr>
<tr>
<td></td>
<td>[6]</td>
<td>[31,43]</td>
<td>[12,43]</td>
<td>[12,43]</td>
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<td>AuIB</td>
<td>1.5</td>
<td>&gt;&gt; 1,000</td>
<td>&gt;&gt; 1,000</td>
<td>750</td>
</tr>
<tr>
<td></td>
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<td>[this study]</td>
<td>[27]</td>
<td>[27]</td>
</tr>
<tr>
<td>MII</td>
<td>&gt;&gt; 1,000</td>
<td>&gt;&gt; 1,000</td>
<td>0.5 – 3.7</td>
<td>&gt;&gt; 1,000</td>
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<td></td>
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<td>[19]</td>
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α-conotoxins selective for GABA\textsubscript{B} receptor dependent inhibition of N-type Ca\textsuperscript{2+} channels rather than other identified mechanisms relieve allodynia in a nerve injury pain model.
Figure 3

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