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1  
2 **A novel mechanism of inhibition of high-voltage activated calcium channels by  $\alpha$ -conotoxins**  
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4 **contributes to relief of nerve injury-induced neuropathic pain**  
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10 Harry Klimis<sup>1,2</sup>, D.J. Adams<sup>3,4¶</sup>, B. Callaghan<sup>3,4¶</sup>, S. Nevin<sup>3</sup>, P.F. Alewood<sup>5</sup>, C.W. Vaughan<sup>1</sup>, **C.**

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13 **A.Mozar<sup>2</sup>** and M.J. Christie<sup>2\*</sup>  
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15  
16 <sup>1</sup>Pain Management Research Institute, University of Sydney, Royal North Shore Hospital, St  
17  
18 Leonards NSW 2065, Australia  
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20  
21 <sup>2</sup>Brain and Mind Research Institute. University of Sydney, NSW 2006, Australia  
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23  
24 <sup>3</sup>Queensland Brain Institute, The University of Queensland, Brisbane, Queensland 4072, Australia  
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26  
27 <sup>4</sup>Health Innovations Research Institute, RMIT University, Melbourne, Victoria 3083, Australia  
28

29  
30 <sup>5</sup>Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland 4072,  
31  
32 Australia  
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34  
35 \*Corresponding author  
36

37  
38 MacDonald J Christie – Brain & Mind Research Institute M02G, The University of Sydney, NSW  
39  
40 2006, Australia, telephone - +61 2 9351 0899, FAX - +61 2 9214 4015, email –  
41  
42 [mac.christie@sydney.edu.au](mailto:mac.christie@sydney.edu.au)  
43  
44

45 <sup>¶</sup>Present address: Health Innovations Research Institute, RMIT University, PO Box 71, Bundoora,  
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47 Vic 3083, Australia  
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52 *Keywords:*  
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57 mechanical allodynia; neuropathic pain  
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## Abstract

1  
2  $\alpha$ -Conotoxins that are thought to act as antagonists of nicotinic acetylcholine receptors (nAChRs)  
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4 containing  $\alpha 3$ -subunits are efficacious in several preclinical models of chronic pain. Potent  
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6 interactions of Vc1.1 with other targets have suggested that the pain relieving actions of  $\alpha$ -  
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8 conotoxins might be mediated by either  $\alpha 9\alpha 10$  nAChRs or a novel GABA<sub>B</sub> receptor-mediated  
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10 inhibition of N-type calcium channels. Here we establish that three  $\alpha$ -conotoxins, Vc1.1, AuIB and  
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12 MII, have distinct selectivity profiles for these three potential targets. Their potencies after  
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14 intramuscular administration were then determined for reversal of allodynia produced by partial  
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16 nerve ligation in rats. Vc1.1, which potently inhibits  $\alpha 9\alpha 10$  nAChRs and GABA<sub>B</sub>/Ca<sup>2+</sup> channels but  
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18 weakly blocks  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  nAChRs, produced potent, long-lasting reversal of allodynia **that were**  
19  
20 **prevented by pretreatment with the GABA<sub>B</sub> receptor antagonist, SCH50911.**  $\alpha$ -Conotoxin AuIB, a  
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22 weak  $\alpha 3\beta 4$  nAChR antagonist, inhibited GABA<sub>B</sub>/Ca<sup>2+</sup> channels but did not act on  $\alpha 9\alpha 10$  nAChRs.  
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24 AuIB also produced reversal of allodynia. These findings suggest that GABA<sub>B</sub> receptor-dependent  
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26 inhibition of N-type Ca<sup>2+</sup> channels can mediate the sustained anti-allodynic actions of some  $\alpha$ -  
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28 conotoxins. However, MII, a potent  $\alpha 3\beta 2$  nAChR antagonist but inactive on  $\alpha 9\alpha 10$  and  $\alpha 3\beta 4$   
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30 nAChRs and GABA<sub>B</sub>/Ca<sup>2+</sup> channels, was demonstrated to have short-acting anti-allodynic action.  
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32 This suggests that  $\alpha 3\beta 2$  nAChRs may also contribute to reversal of allodynia. Together, these  
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34 findings suggest that inhibition of  $\alpha 9\alpha 10$  nAChR is neither necessary nor sufficient for relief of  
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36 allodynia and establish that  $\alpha$ -conotoxins selective for GABA<sub>B</sub> receptor dependent inhibition of N-  
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38 type Ca<sup>2+</sup> channels relieve allodynia, and could therefore be developed to manage chronic pain.  
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## 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  $\alpha$ -conotoxins that competitively inhibit neuronal nicotinic acetylcholine receptors (nAChRs) with varying degrees of subtype selectivity [2,15,24,29,36,37]. One  $\alpha$ -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  $\alpha$ -conotoxins relieve neuropathic pain remains controversial. Vc1.1 more selectively, but weakly, antagonises peripherally expressed nAChR subtypes ( $\alpha3\beta2$ ,  $\alpha3\beta4$  and  $\alpha3\alpha5\beta2$ ) than those more abundantly expressed in the CNS ( $\alpha4\beta2$ ,  $\alpha4\beta4$ , and  $\alpha7$ ) or skeletal muscle ( $\alpha1\beta1\gamma\delta$ ) [12]. More recently, Vc1.1 was shown to have high potency as an antagonist at  $\alpha9\alpha10$  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  $\alpha9\alpha10$  but not other nAChR subtypes [31] suggests that targets other than  $\alpha9\alpha10$  contribute to the pain-relieving activity of  $\alpha$ -conotoxins.

We have recently reported that Vc1.1 inhibits N-type calcium channels in rat sensory neurons via a novel GABA<sub>B</sub> 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

1 because it is voltage-independent [8], requires src-kinase activity [8], does not modulate GABA<sub>B</sub>  
2 receptor-activated GIRK channels expressed in *Xenopus* oocytes [29] and does not directly displace  
3 binding of GABA<sub>B</sub> receptor ligands [29]. N-type calcium channel inhibition also persists in sensory  
4 neurons from  $\alpha 9$  nicotinic receptor knockout mice [7]. To better understand the potential analgesic  
5 mechanisms of  $\alpha$ -conotoxins, the present study employed  $\alpha$ -conotoxins with vastly differing  
6 selectivities for  $\alpha 3$ -containing nAChR,  $\alpha 9\alpha 10$  nAChRs and GABA<sub>B</sub>/Ca<sup>2+</sup> channels to determine  
7 whether one or several of these targets contributes to relief of neuropathic pain. Intramuscular Vc1.1,  
8 which interacts potently with both  $\alpha 9\alpha 10$  nAChRs [31,42,43] and GABA<sub>B</sub>/Ca<sup>2+</sup> channels [8] but  
9 quite weakly with  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  nAChRs [11], produced potent, long-lasting relief from allodynia  
10 after partial nerve ligation. Another  $\alpha$ -conotoxin, AuIB also produced potent, long-lasting relief from  
11 allodynia. AuIB, which is a relatively weak but selective  $\alpha 3\beta 4$  nAChR antagonist [14,27], potently  
12 inhibited N-type Ca<sup>2+</sup> channels but did not interact with  $\alpha 9\alpha 10$  nAChRs. We found that a third  $\alpha$ -  
13 conotoxin, MII, produced weak, short-lived reversal of allodynia. MII did not interact with  $\alpha 9\alpha 10$   
14 nAChRs or GABA<sub>B</sub>/Ca<sup>2+</sup> channels but is a potent inhibitor of  $\alpha 3\beta 2$  nAChRs [7]. Significantly, these  
15 results suggest that GABA<sub>B</sub> receptor-dependent inhibition of N-type Ca<sup>2+</sup> channels may be the major  
16 mechanism of sustained anti-allodynic actions of  $\alpha$ -conotoxins. As reported previously, we confirm  
17 inhibition of  $\alpha 9\alpha 10$  nAChR is not required for the anti-allodynic actions of  $\alpha$ -conotoxins [31].  
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## 44 **2. Materials and Methods**

### 45 *2.1. Electrophysiological recordings in oocytes*

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49 All experiments adhere to the guidelines of the Committee for Research and Ethical Issues of  
50 IASP published in Pain, 1983;16:109–110. Procedures for harvesting *Xenopus laevis* oocytes were  
51 approved by the University of Queensland Animal Ethics Committee, which complies with National  
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1 Health & Medical Research Council of Australia (NHMRC) guidelines. RNA preparation, oocyte  
2 preparation and expression of nAChR subunits in *Xenopus* oocytes were performed as described  
3 previously [31]. Briefly, plasmids with cDNA encoding the rat  $\alpha 9$  and  $\alpha 10$  nAChR subunits were  
4 provided by Dr. A.B. Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina). 5 ng of  
5 cRNA was injected into each oocyte which was then kept at 18°C in ND96 buffer (96 mM NaCl, 2  
6 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 5 mM HEPES at pH 7.4) supplemented with 50 mg/L  
7 gentamycin and 5 mM pyruvic acid for 2-5 days before recording.  
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19 Membrane currents were recorded from the *Xenopus* oocytes using an OpusXpress™ 6000A  
20 workstation (Molecular Devices, Sunnyvale, CA). Electrodes had resistances of 0.3-1.5 MΩ when  
21 filled with 3 M KCl. All recordings were conducted at room temperature (20-23°C) using a bath  
22 solution of ND96 as described above. During recordings, the oocytes were perfused continuously at  
23 a rate of 1.5 ml/min, with 5 min incubation times for the conotoxins. Acetylcholine (ACh; 30 μM)  
24 was applied for 2 s at 5 ml/min, with 10 min washout periods between applications. Conotoxins were  
25 bath applied and co-applied with the agonist. Oocytes were voltage clamped at a holding potential of  
26 -80 mV. Data were sampled at 500 Hz and filtered at 200 Hz. Peak current amplitude was measured  
27 before and following incubation of the peptide.  
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## 44 2.2. Electrophysiological recordings from DRG neurons

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49 Dorsal root ganglion (DRG) neurons were enzymatically dissociated from ganglia of 7-14 day old  
50 Wistar rats. All procedures were approved by the University of Queensland Animal Ethics  
51 Committee, which complies with NHMRC guidelines. Briefly, rats were killed by cervical  
52 dislocation, the spinal column was hemi-segmented and the spinal cord removed. Ganglia were  
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1 removed and rinsed in cold Hanks' balanced salt solution (HBSS; MultiCel), minced and incubated  
2 in 1 mg/ml collagenase (Type 2; 405U/mg, Worthington Biochemical Corp., Lakewood NJ) in  
3 HBSS at 37°C for ~30 min. Following incubation, ganglia were rinsed three times with warm (37°C)  
4 Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal calf serum  
5 and 1% penicillin/streptomycin and gently triturated with a fire-polished Pasteur pipette. Cells were  
6 plated on glass cover slips, incubated at 37°C in 95% O<sub>2</sub>:5% CO<sub>2</sub> and used within 4-24 hrs.  
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17 The external recording solution for rat DRG neurons contained (in mM): 150 TEACl, 2 BaCl<sub>2</sub>, 10  
18 D-glucose, 10 HEPES, pH 7.4. Recording electrodes were filled with an internal solution containing  
19 (in mM): 140 CsCl, 1 MgCl<sub>2</sub>, 5 MgATP, 0.1 NaGTP, 5 BAPTA-Cs<sub>4</sub>, 10 HEPES, pH 7.3 with CsOH  
20 and had resistances of 1.0-2.5 MΩ. Membrane currents were recorded using the whole-cell  
21 configuration of the patch clamp technique with an Axopatch 200B amplifier (Molecular Devices,  
22 Sunnyvale, CA). A voltage protocol using step depolarizations from -80 mV to 0 mV was used  
23 when examining high voltage-activated (HVA) Ca<sup>2+</sup> channel currents. Test potentials 150 ms in  
24 duration were applied every 20 s. Leak and capacitative currents were subtracted using a -P/4 pulse  
25 protocol. Currents were generated by a computer using pClamp 9.2 software (Molecular Devices),  
26 filtered at 2 kHz and sampled at 8 kHz by the Digidata 1322A (Molecular Devices). Sampled data  
27 were stored digitally on a computer for further analysis.  
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### 46 2.3. Surgery: partial nerve ligation model 47 48 49 50

51 Male Sprague-Dawley rats weighing 200-250g were housed in groups of 3 in a constant  
52 temperature room (22 ± 1°C), under a 12/12 h light/dark cycle (lights were on between 05:30-17:30  
53 h) with access to food and water ad libitum. Animals were anaesthetised for all surgical procedures  
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1 using isoflurane and were killed by anaesthetic overdose at the end of the experiment. All  
2 experiments were approved by the Royal North Shore Hospital and University of Technology  
3 Animal Ethics Committee, which complies with NHMRC guidelines.  
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9 Signs of neuropathic pain were produced using partial ligation of the left sciatic nerve (PNL)  
10 under isoflurane anaesthesia [40]. Briefly, the left sciatic nerve in the mid-thigh region of the rat was  
11 exposed by blunt dissection through the biceps femoris at a site near the trochanter just distal to the  
12 posterior biceps semitendinosus nerve branches off the common sciatic nerve. A 4-0 silk suture was  
13 inserted into the left sciatic nerve trunk approximately 3mm proximal to the trifurcation of the nerve  
14 at the popliteal fossa and was tightly ligated so that the dorsal 30-50% of the nerve thickness was  
15 trapped in the ligature.  
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#### 29 *2.4. Mechanical allodynia testing*

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

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

#### 40 41 42 43 *3.1. Inhibition of $\alpha 9\alpha 10$ nAChR channel currents by Vc1.1 but not AuIB or MII*

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48 As previously reported [31,43], robust ACh-induced cation currents were expressed in *Xenopus*  
49 *laevis* oocytes following injection of mRNA encoding  $\alpha 9\alpha 10$  nAChR channel subunits (Figure 1).  
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51 Superfusion of Vc1.1 (100 nM) potently inhibited  $\alpha 9\alpha 10$  nAChR currents, whereas in the same  
52 series of experiments, neither AuIB nor MII produced any inhibition of  $\alpha 9\alpha 10$  nAChR currents at  
53 concentrations up to 3  $\mu$ M (Figure 1).  
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7 *3.2. Inhibition of N-type Ca<sup>2+</sup> channel currents in DRG neurons by Vc1.1 and AuIB but not MII.*  
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11 We have previously reported that Vc1.1 potently inhibits (IC<sub>50</sub> = 1.7 nM) high voltage-activated Ca<sup>2+</sup> channel  
12 currents in DRG neurons via a novel mechanism involving GABA<sub>B</sub> receptor-mediated inhibition of N-type  
13 Ca<sup>2+</sup> channel [8]. Using the same procedures, AuIB similarly produced a partial inhibition of high voltage-  
14 activated Ca<sup>2+</sup> channel currents in rat DRG neurons (Figure 2) with an IC<sub>50</sub> of 1.5 ± 0.3 nM (n = 17). As with  
15 Vc1.1 [8], inhibition by AuIB did not affect the kinetics of activation of the N-type Ca<sup>2+</sup> channel currents  
16 (Figure 2). By contrast, in the same series of experiments, MII (1 μM) produced no inhibition of  
17 depolarization-activated Ca<sup>2+</sup> channel currents. Application of the selective N-type Ca<sup>2+</sup> channel inhibitor, ω-  
18 conotoxin CVID, confirmed that AuIB targeted the N-type component of the HVA Ca<sup>2+</sup> channel currents.  
19 AuIB (100 nM) reduced peak Ca<sup>2+</sup> channel current amplitude to 54.7 ± 6.2% (n = 10) of control. Application  
20 of 100 nM AuIB in the presence of CVID (200 nM) produced no further reduction of the HVA Ca<sup>2+</sup> channel  
21 current amplitude (51.6 ± 8.9% of control, n = 10) (Figure 2 C (i)). Furthermore, in the presence of the  
22 GABA<sub>B</sub> receptor antagonist CGP 55845, AuIB (100 nM) failed to inhibit the HVA Ca<sup>2+</sup> channel currents.  
23 CGP 55845 (1 μM) alone had no effect on the HVA Ca<sup>2+</sup> channel current amplitude and following the  
24 addition of AuIB the Ca<sup>2+</sup> channel current amplitude was 87.9 ± 4.0% (n = 12) of control (Figure 2 C (ii)).  
25 The inhibition of HVA Ca<sup>2+</sup> channel currents by Vc1.1 (100 nM) was antagonized in the presence of the long  
26 acting GABA<sub>B</sub> receptor antagonist SCH 50911 (1 μM) [6]. Bath application of 1 μM SCH 50911 alone,  
27 increased HVA Ca<sup>2+</sup> channel current amplitude (115 ± 5.6 %, n = 7, P = 0.046), the current amplitude was  
28 117 ± 6 % of control following application of Vc1.1 in the presence of SCH50911. In a control batch of cells,  
29 application of Vc1.1 alone (100 nM) reduced HVA Ca<sup>2+</sup> channel currents to 61 ± 6.0 % of control (n = 5).  
30 (Figure 2 C (iii)).  
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10 *3.3. Effects of Vc1.1, MII and AuIB on PNL-induced mechanical allodynia*  
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14 PNL produced allodynia that was maximal 12-14 days after surgery. Mechanical PWT decreased  
15 from  $9.8 \pm 0.4$  g prior to surgery (day 0, pre-PNL, n = 115) to  $0.7 \pm 0.1$  g by 12-14 days after PNL.  
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17 The  $\alpha$ -conotoxins Vc1.1, MII and AuIB all produced a significant dose-dependent increase in the  
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19 mechanical PWT. As shown in Figure 3A, i.m. injection of Vc1.1 produced a significant, dose-  
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21 dependent increase in mechanical PWT, which, for the highest dose tested (36  $\mu$ g), was significant at  
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23 the earliest time point tested. The anti-allodynic activity of Vc1.1 was maintained throughout the  
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25 initial test period up to 6 hours post-injection at all doses tested. As previously reported [26], the  
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27 anti-allodynic action of Vc1.1 persisted for at least 24 h after injection. PWT was significantly  
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29 elevated 24 h after injection at both the 3.6 and 36  $\mu$ g doses. As shown in Figure 3B, AuIB also  
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31 produced a significant, dose-dependent increase of mechanical PWT, which was significant soon  
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33 after injection and persisted at the highest dose for at least 24 h after injection. As shown in Figure  
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35 3C, i.m. injection of MII also dose-dependently increased the mechanical PWT but this was only  
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37 significantly different from vehicle-treated animals 4 h after injection (3.6 and 36  $\mu$ g doses) and had  
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39 returned to baseline after 24 h.  
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56 *3.4 Dose-response relationships*  
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1 The area under the curve (AUC) from 0-6 hours post-injection was calculated for each animal to  
2 construct dose-response curves of the data represented in Figures 3A, 3B and 3C (Figure 4). A  
3 logistic curve was then fitted to estimate the EC<sub>50</sub> for each drug. A two-way ANOVA (drug x dose)  
4 indicated significant dose (P < 0.0001) and type of  $\alpha$ -conotoxin (P < 0.01) effects, indicating that the  
5 three  $\alpha$ -conotoxins had significantly different potencies. Vc1.1 was clearly the most potent of the  $\alpha$ -  
6 conotoxins, followed by AuIB and then MII. Vc1.1 had an estimated EC<sub>50</sub> for increasing mechanical  
7 PWT of 0.34  $\mu$ g (95% CI = 0.06-1.89  $\mu$ g). By contrast, AuIB had an EC<sub>50</sub> of 1.88  $\mu$ g (95% CI =  
8 0.04-8.75  $\mu$ g) and MII had an EC<sub>50</sub> of 9.16  $\mu$ g (95% CI = 2.75-30.54  $\mu$ g).  
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22 *(Insert Figure 4 about here)*  
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### 26 *3.5. Effects of Vc1.1, MII and AuIB on motor performance*

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31 Rotarod latencies were 155  $\pm$  8 s (n = 114) at baseline 12-14 days following PNL. Injection of the  
32  $\alpha$ -conotoxins Vc1.1, MII and AuIB at any dose did not produce any significant changes in rotarod  
33 latency over time (P>0.05, two-way repeated measures ANOVA). For clarity the effects on rotarod  
34 performance are shown only for the highest dose of each  $\alpha$ -conotoxin in Figure 5 but lower doses  
35 were also without significant effects. No other behavioural disturbances were noted after all doses of  
36 the  $\alpha$ -conotoxins.  
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### 53 *3.6. Effects of Vc1.1 and baclofen are prevented by a GABA<sub>B</sub> receptor antagonist*

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1 As previously reported the GABA<sub>B</sub> receptor agonist, baclofen (4 mg/kg, s.c.) produced significant  
2 reversal of allodynia [33], as did Vc1.1. Both actions were significantly and almost completely  
3 antagonised by pre-treatment (15 min prior to baclofen or Vc1.1) with the long-acting GABA<sub>B</sub>  
4 receptor antagonist SCH 50911 [6] (20 mg/kg, s.c.) but not vehicle (isotonic phosphate buffered  
5 saline) (Figure 6).  
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#### 19 **4. Discussion**

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24 The present study establishes that intramuscular injections of a range of  $\alpha$ -conotoxins with diverse  
25 spectra of target selectivity can all reverse signs of allodynia in a nerve injury model of chronic pain,  
26 albeit with differing potencies and durations of action. The interactions of  $\alpha$ -conotoxins Vc1.1, MII  
27 and AuIB with their potential analgesic targets as identified from the present and previous studies  
28 are summarised in Table 1. The large, often more than 1,000-fold differences in potencies of Vc1.1,  
29 AuIB and MII at their known molecular targets provide suggestive evidence for which target(s) are  
30 important for reversal of allodynia. The results presented in Table 1 suggest that GABA<sub>B</sub> receptor-  
31 dependent inhibition of N-type Ca<sup>2+</sup> channels could be a major mechanism of sustained anti-  
32 allodynic actions of  $\alpha$ -conotoxins. Inhibition of  $\alpha$ 9 $\alpha$ 10 nAChR is not required though  $\alpha$ 3 $\beta$ 2 nAChRs  
33 may contribute to the reversal of allodynia.  
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51 *(Insert Table 1 about here)*  
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56 Focusing only on the most potent of the three  $\alpha$ -conotoxins suggests that Vc1.1 could potentially  
57 reverse allodynia via either  $\alpha$ 9 $\beta$ 10 nAChRs [43] or the novel GABA<sub>B</sub> receptor-mediated inhibition  
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1 of N-type  $\text{Ca}^{2+}$  channels in sensory neurons [8]. As previously proposed [43], antagonism of  $\alpha 3\beta 2$  or  
2  $\alpha 3\beta 4$  nAChRs is not a likely mechanism of action of Vc1.1 because moderately effective doses  
3 ( $<0.1$  nmol/kg in this study) are several orders of magnitude lower than Vc1.1's affinity for  
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5 ( $<0.1$  nmol/kg in this study) are several orders of magnitude lower than Vc1.1's affinity for  
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7 heterologously expressed  $\alpha 3\beta 2$  or  $\alpha 3\beta 4$  nAChRs ( $>1$   $\mu\text{M}$ , Table 1) and the ability of Vc1.1 to  
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9 antagonise the nicotine-induced increase in axonal excitability of isolated unmyelinated human C-  
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11 fiber axons ( $>1$   $\mu\text{M}$ ) [24].  
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17 Resolution of which of these targets mediate the anti-allodynic actions of  $\alpha$ -conotoxins (either  
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19  $\alpha 9\beta 10$  nAChRs or  $\text{GABA}_B$  receptor/N-type  $\text{Ca}^{2+}$  channels, or both) can potentially be achieved using  
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21 peptides highly selective for one or the other target. Given that Vc1.1 and another previously  
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23 examined anti-allodynic  $\alpha$ -conotoxin, Rg1A, have high affinity for both targets [8,43], they cannot  
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25 be utilised to resolve this issue. The present results show that AuIB, which has high potency for  
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27  $\text{GABA}_B$  receptor/N-type  $\text{Ca}^{2+}$  channels (low nanomolar range) but does not interact with  $\alpha 9\alpha 10$   
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29 nAChRs, has potent, long-acting anti-allodynic actions. These findings suggest that inhibition of  
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31  $\alpha 9\alpha 10$  nAChRs is not necessary for long-term relief of allodynia. Furthermore, we have reported  
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33 previously that two analogues of Vc1.1, vc1a and [P6O] Vc1.1 that retain full activity at  $\alpha 9\alpha 10$   
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35 nAChRs [31] but have little or no activity  $\text{GABA}_B$  receptor/N-type  $\text{Ca}^{2+}$  channels [8], produce no  
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37 reversal of nerve injury-induced allodynia [31]. Therefore antagonism of  $\alpha 9\alpha 10$  nAChRs is neither  
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39 necessary nor sufficient to reverse allodynia after nerve injury. Thus the most parsimonious  
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41 mechanism for the anti-allodynic actions of Vc1.1 and AuIB is the  $\text{GABA}_B$  receptor-dependent  
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43 inhibition of N-type  $\text{Ca}^{2+}$  channels in sensory neurons [8] although an as yet unidentified mechanism  
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45 mediated by the same subset of  $\alpha$ -conotoxins cannot be ruled out. **Prevention of anti-allodynic  
46  
47 actions of both Vc1.1 and baclofen by pre-treatment with the long-acting  $\text{GABA}_B$  receptor  
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49 antagonist, SCH 50911 is consistent with this interpretation.**  
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1 N- type  $\text{Ca}^{2+}$  channels are an appealing target for chronic pain treatment as they are well known to  
2 play a central role in the detection and transmission of nociceptive stimuli in DRG neurons [3,28].  
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4 Several studies have highlighted the importance of N-type  $\text{Ca}^{2+}$  channels in neuropathic pain: N-  
5 type  $\alpha_{1B}$  channel knock-out mice have a decreased response to neuropathic pain [2023,35], there is  
6  
7 an up-regulation of N-type  $\alpha_{1B}$  as well as  $\alpha_2\delta$  subunits in rat nerve injury models [1,11, 33,44], and  
8  
9 currently used treatments, or treatments being developed, for pain relief include direct ( $\omega$ -conotoxin  
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11 MVIIA, aka Prialt®) and indirect (eg. Gabapentin) inhibitors of N-type  $\text{Ca}^{2+}$  channels [28]. We  
12  
13 have previously reported a novel mechanism by which some anti-allodynic  $\alpha$ -conotoxins, including  
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15 Vc1.1 and Rg1A, modulate N-type  $\text{Ca}^{2+}$  channel currents in DRG neurons, requiring  $\text{GABA}_B$   
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17 receptors [8]. The activation of  $\text{GABA}_B$  receptors by agonists such as baclofen is well established as  
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19 producing antinociceptive and antiallodynic actions in chronic pain models [4,32, 33,39]. Therefore,  
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21 the Vc1.1, AuIB and Rg1A inhibition of  $\text{Ca}_v2.2$  (N-type) channels that depends on  $\text{GABA}_B$   
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23 receptors via a novel transduction process is the most likely the mechanism mediating their anti-  
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25 allodynic properties. As discussed above, the mechanism of N-type calcium channel inhibition by  
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27  $\alpha$ -conotoxins has yet to be fully elucidated but is not mediated by a conventional GPCR signalling  
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29 mechanism that involves direct channel modulation by agonist-mobilized G-protein  $\beta\gamma$  subunits [8].  
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42 The basis for the long duration of action (>24 h) of both Vc1.1 and AuIB (but not MII) after a  
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44 single, systemic injection is still unknown. It is therefore possible that the sustained anti-allodynic  
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46 actions of these  $\alpha$ -conotoxins result from a long-term effect of N-type  $\text{Ca}^{2+}$  channel inhibition on  
47  
48 peripheral nerves that also express  $\text{GABA}_B$  receptors. Indeed, reversal of allodynia has recently been  
49  
50 reported with the peripherally-restricted, N-type selective  $\omega$ -conotoxin, CVID [22]. It is not yet  
51  
52 known whether inhibition of N-type  $\text{Ca}^{2+}$  channel currents by Vc1.1 and AuIB persists for the  
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54 duration of anti-allodynic activity but it is irreversible during patch-clamp experiments (<1 h) [8]. It  
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1 is also unknown whether differential biodistribution and metabolism of the distinct peptides  
2 contributes to the long duration of action.  
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7 The present study also suggests that antagonism of  $\alpha 3\beta 2$  nAChRs can weakly contribute to  
8 reversal of allodynia after nerve injury. MII is a potent (nanomolar range) antagonist of  $\alpha 3\beta 2$   
9 nAChRs (Table 1) but does not inhibit either N-type calcium channels or  $\alpha 9\alpha 10$  nAChRs. Attempts  
10 to ascribe either the acute or longer term anti-allodynic actions of all  $\alpha$ -conotoxins to a single  
11 combination of subunits [eg. 43] are therefore probably futile. Conotoxin MII was found to reverse  
12 allodynia more weakly than either Vc1.1 or AuIB although its action was not sustained 24 hours  
13 after injection. This might suggest that  $\alpha 3\beta 2$  nAChRs are not of primary importance but can  
14 contribute to reversal of allodynia. Indeed, nicotinic agonists excite nociceptive primary afferents  
15 and  $\alpha 3$ -subunit containing nAChRs are functionally expressed in at least some nociceptors in rodents  
16 [34] and unmyelinated nerves in humans [25]. By contrast, there is some evidence that antagonism  
17 of  $\alpha 3\beta 2$  nAChRs in the spinal cord has pro-nociceptive actions [45], but this effect is probably  
18 restricted to spinal neurons as we observed only anti-allodynic actions after peripheral administration  
19 of MII.  
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41 In conclusion, the present study demonstrates that novel GABA<sub>B</sub> receptor-mediated inhibition of  
42 N-type Ca<sup>2+</sup> channels in sensory neurons is a likely mechanism underlying the anti-allodynic actions  
43 of  $\alpha$ -conotoxins and should be further investigated as a potential therapeutic target for persistent  
44 pain. Furthermore, antagonism of  $\alpha 9\alpha 10$  nAChRs is neither necessary nor sufficient to reverse  
45 allodynia after nerve injury. Because antagonism of  $\alpha 3$ -subunit containing nAChRs may also  
46 contribute to reversal of allodynia, it may be premature to rule out investigation of this target in  
47 further development of  $\alpha$ -conotoxins as potential therapies for persistent pain states.  
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## Figure 1

### Effect of $\alpha$ -conotoxins on $\alpha 9\alpha 10$ nAChRs expressed in *Xenopus* oocytes.

(A) Superimposed traces of ACh-evoked currents mediated by  $\alpha 9\alpha 10$  nAChRs obtained in the absence (Control) and presence of 100 nM Vc1.1 (i), 1  $\mu$ M AuIB (ii), and 1  $\mu$ M MII (iii). Oocytes were voltage clamped at  $-80$  mV and membrane currents evoked with 2 sec application of 30  $\mu$ M ACh co-applied with the toxin following 300 s incubation. (B) Bar graph of the inhibition of ACh-evoked peak current amplitude by  $\alpha$ -conotoxins Vc1.1, AuIB and MII. ACh-evoked currents were inhibited  $35 \pm 5\%$  ( $n = 9$ ) and  $89 \pm 5\%$  ( $n = 6$ ) of control by 100 nM and 1  $\mu$ M Vc1.1, respectively, whereas neither AuIB nor MII inhibited the ACh-evoked currents at concentrations up to 3  $\mu$ M ( $n = 3-9$ ). All data were pooled and represented as mean  $\pm$  SEM.

## Figure 2

### Effect of $\alpha$ -conotoxins on HVA calcium channel currents in rat DRG neurons.

(A) Superimposed depolarization-activated  $\text{Ba}^{2+}$  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  $\mu$ M MII (ii), respectively. (B) Concentration-response relationships obtained for inhibition of high voltage-activated  $\text{Ca}^{2+}$  channel currents in DRG neurons by AuIB ( $\circ$ ) ( $n = 4-19$ ), Vc1.1 (---) and MII ( $\blacksquare$ , 1  $\mu$ M) ( $n = 6$ ). Data points represent mean  $\pm$  SEM of normalized peak current amplitude. The  $\text{IC}_{50}$  for inhibition of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channel currents by the N type  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channel currents by 100 nM AuIB alone, in the presence of 1  $\mu$ M CGP55845A alone and after application of 100 nM AuIB in the presence of

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CGP 55845A. (iii) Bar graph of relative inhibition of HVA Ca<sup>2+</sup> 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).

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## 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  $\pm$  S.E.M. of the rotarod latency at the indicated times. Time point 0 h represents the time of drug injection.  $\blacktriangle$  – Vc1.1,  $\blacktriangle$  - AuIB and  $\triangle$ - MII and  $\circ$  - 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  $\mu$ g i.m.) following pretreatment 15 min earlier with SCH50911 (20 mg/kg, s.c.) or vehicle. Each bar represents the mean AUC  $\pm$  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).

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**Table 1**

**Target selectivity of  $\alpha$ -conotoxins Vc1.1, MII and AuIB.**

**Potency *in vitro* (IC<sub>50</sub> - nM) [this study or references]**

<b><math>\alpha</math>-conotoxin</b>	<b>N-type Ca<sup>2+</sup> channel</b>	<b><math>\alpha</math>9<math>\alpha</math>10 nAChR</b>	<b><math>\alpha</math>3<math>\beta</math>2 nAChR</b>	<b><math>\alpha</math>3<math>\beta</math>4 nAChR</b>
<b>Vc1.1</b>	1.7 [6]	19 - 64 [31,43]	7,300 [12,43]	4,200 [12,43]
<b>AuIB</b>	1.5 [this study]	>> 1,000 [this study]	>> 1,000 [27]	750 [27]
<b>MI</b>	>> 1,000 [this study]	>> 1,000 [this study]	0.5 – 3.7 [9,17,19]	>> 1,000 [19]

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$\alpha$ -conotoxins selective for GABA<sub>B</sub> receptor dependent inhibition of N-type Ca<sup>2+</sup> channels rather than other identified mechanisms relieve allodynia in a nerve injury pain model.

Figure 1

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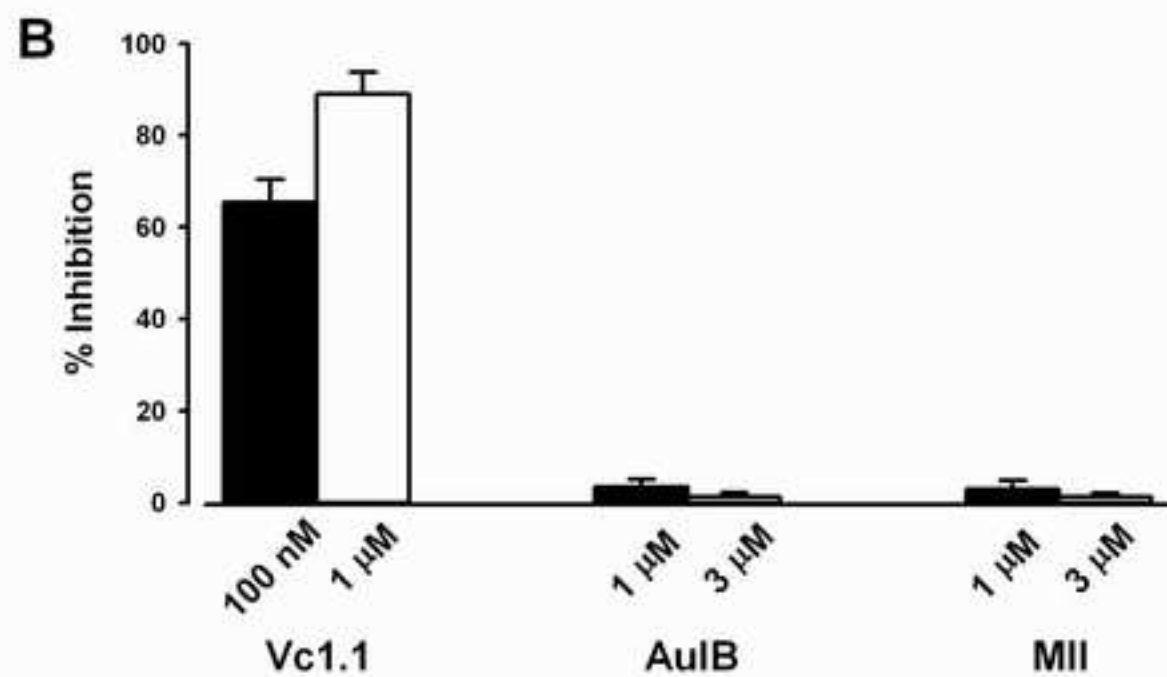
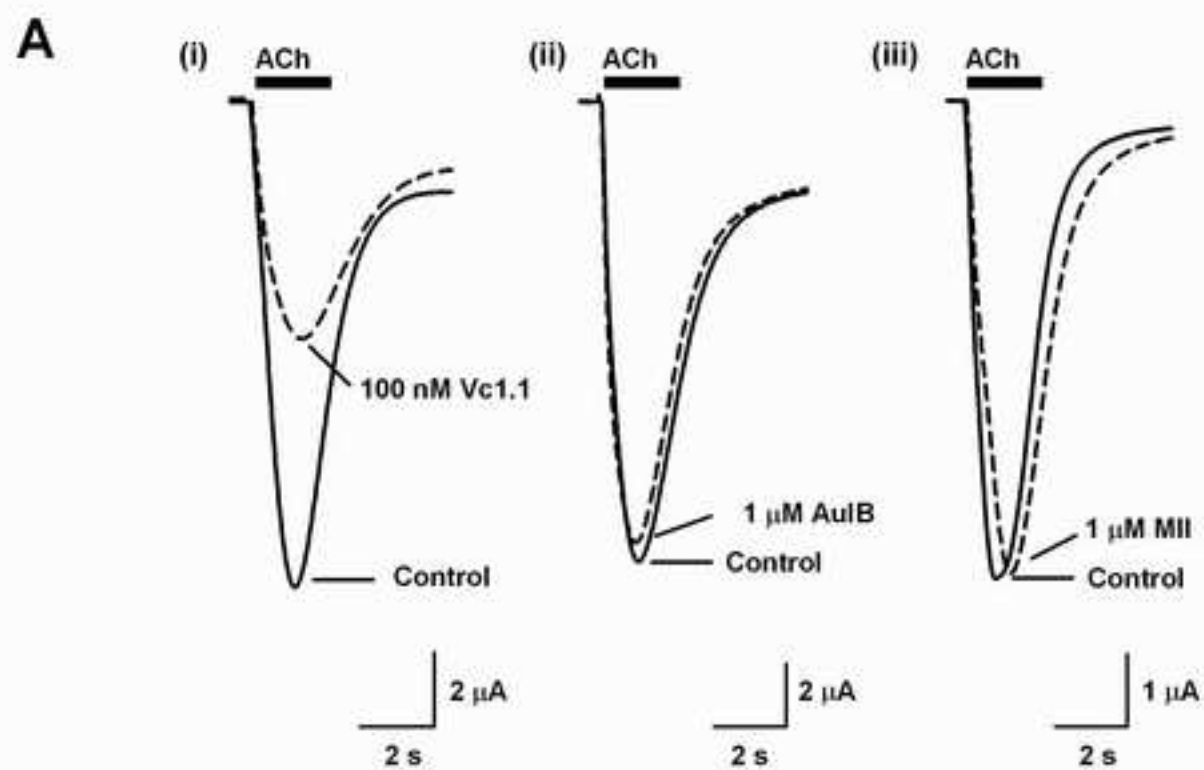


Figure 2

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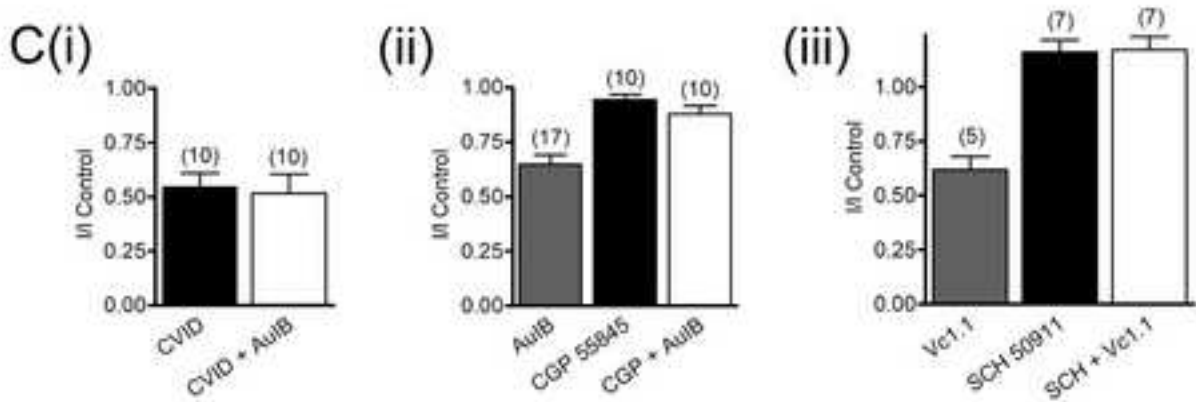
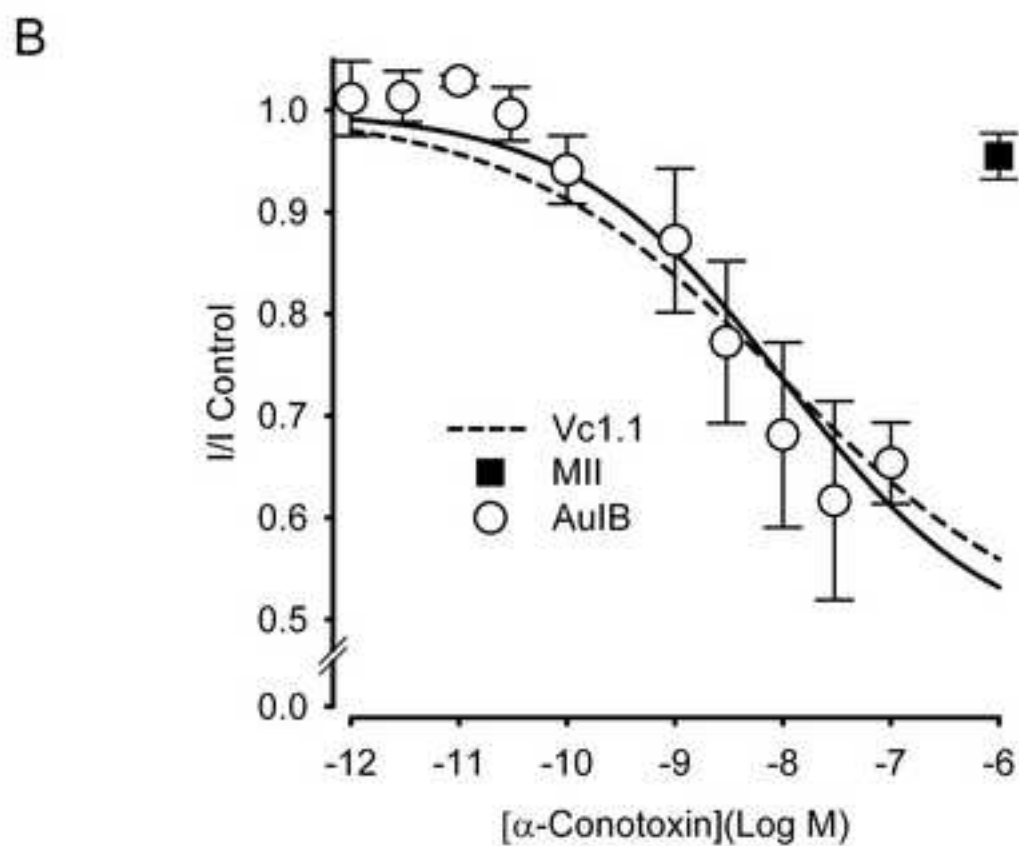
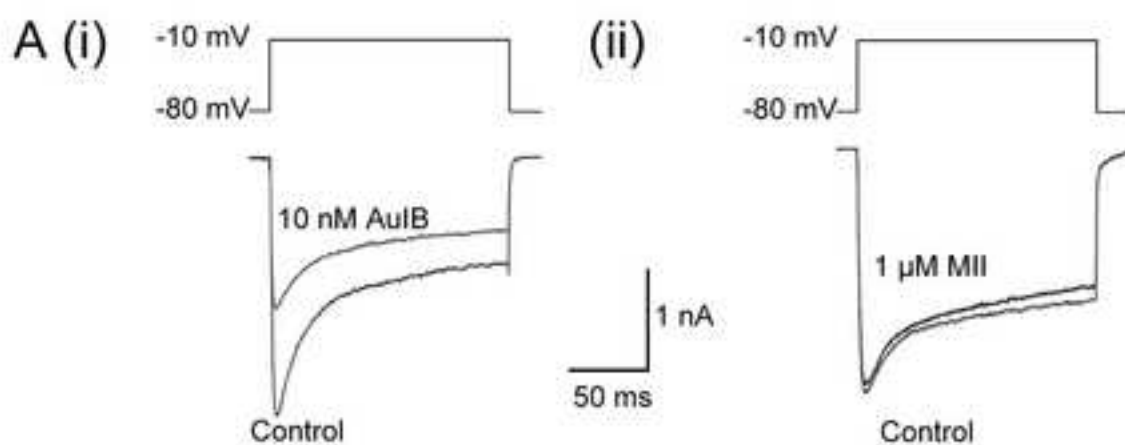




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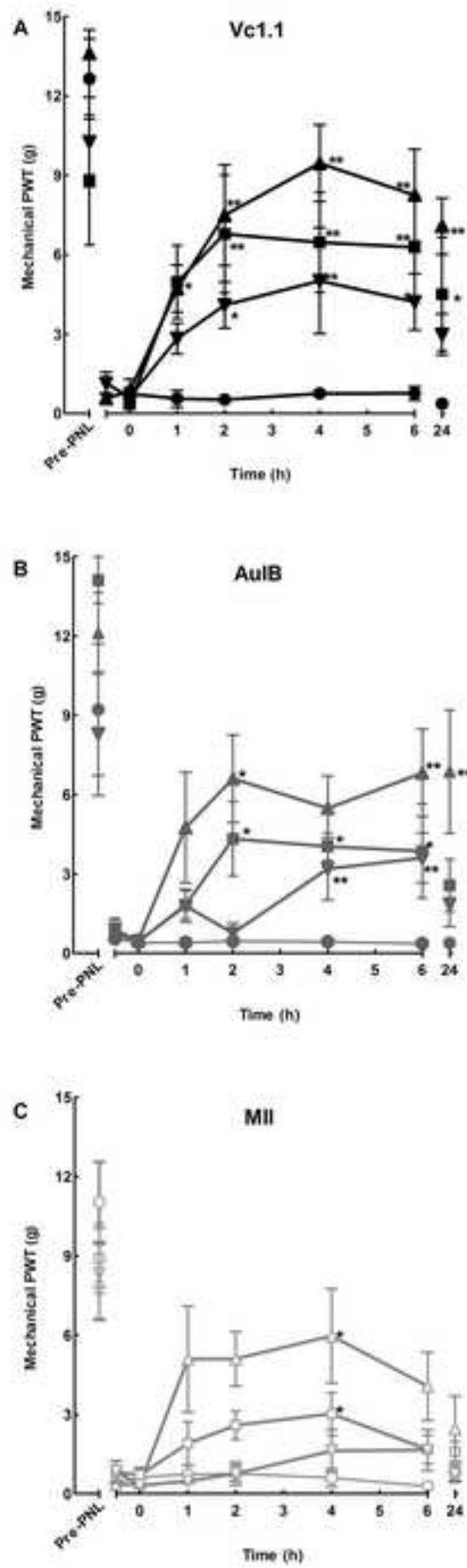


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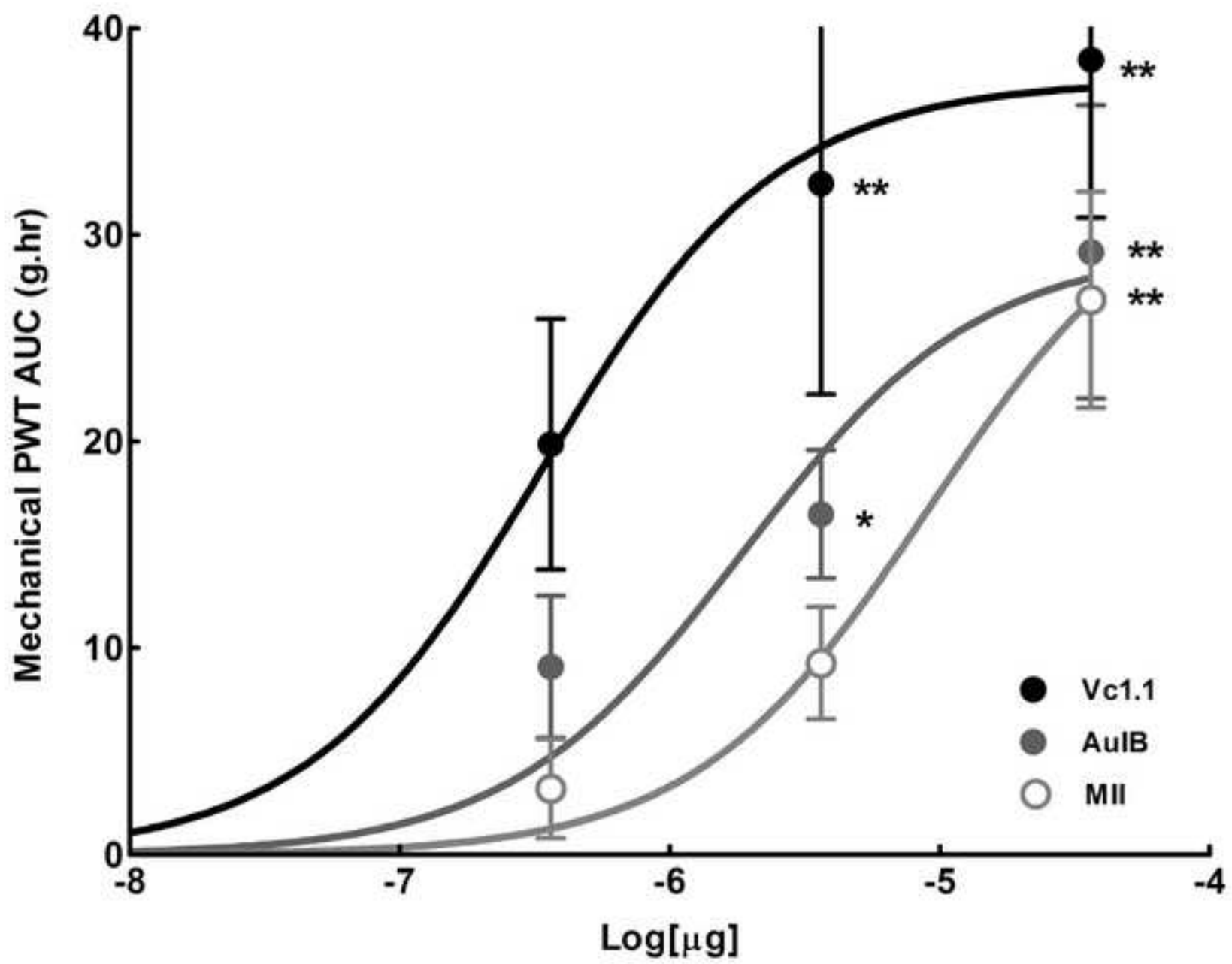


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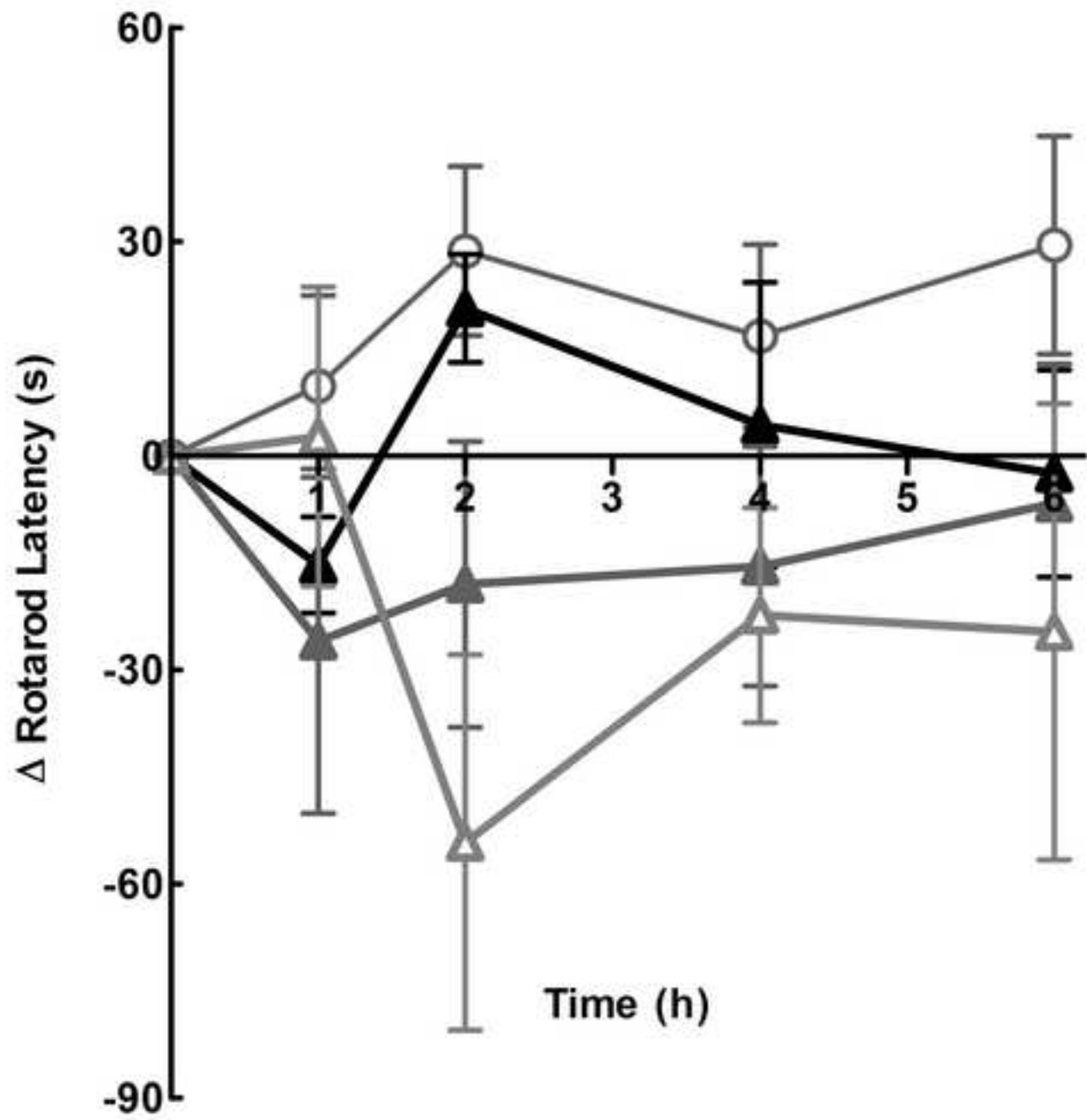


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