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*Title page

Intrathecal α-conotoxins Vc1.1, AuIB and MII acting on distinct nicotinic receptor subtypes reverse signs of neuropathic pain

Napier IA[a], Klimis H[b], Rycroft BK[b], Jin J[c], Alewood PF[c], Motin L[d], Adams DJ[d,e],Christie MJ[a,b]

[a]Discipline of Pharmacology, The University of Sydney, NSW 2006, Australia

[b]Pain Management Research Institute, The University of Sydney, Royal North Shore Hospital, St Leonards NSW 2065, Australia

[c]Division of Chemistry and Structural Biology, Institute for Molecular Bioscience, The University of Queensland, QLD 4072, Australia

[d]Queensland Brain Institute, The University of Queensland, Brisbane, QLD 4072, Australia

[e]Present address, Health Innovations Research Institute, RMIT University, Melbourne, 3083 Victoria, Australia

Corresponding author: MJ Christie

Email: mac.christie@sydney.edu.au

Postal Address: Discipline of Pharmacology D06 University of Sydney NSW 2006, Australia

Phone: +61 2 9351 0899

Fax: +61 2 9114 4015

Keywords

Conotoxin, pain, nicotinic, nAChR, allodynia, intrathecal

Abbreviations

AUC; area under curve

eEPSC; evoked excitatory post-synaptic current

nAChR; nicotinic acetylcholine receptor

PNL; partial nerve ligation

PWT; paw withdrawal threshold

***Abstract**

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The large diversity of peptides from venomous creatures with high affinity for molecules involved in the development and maintenance of neuropathic pain has led to a surge in venom-derived analgesic research. Some members of the α-conotoxin family from *Conus* snails which specifically target subtypes of nicotinic acetylcholine receptors (nAChR) have been shown to be effective at reducing mechanical allodynia in neuropathic pain models. We sought to determine if three such peptides, Vc1.1, AuIB and MII were effective following intrathecal administration in a rat neuropathic pain model because they exhibit different affinities for the major putative pain relieving targets of α-conotoxins. Intrathecal administration of α-conotoxins, Vc1.1, AuIB and MII into neuropathic rats reduced mechanical allodynia for up to 6 hours without significant side effects. *In vitro* patch-clamp electrophysiology of primary afferent synaptic transmission revealed the mode of action of these toxins was not via a $GABA_B$-dependant mechanism, and is more likely related to their action at nAChRs containing combinations of α3, α7 or other subunits. Intrathecal nAChR subunit-selective conotoxins are therefore promising tools for the effective treatment of neuropathic pain.

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Corresponding author: MJ Christie

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The large diversity of peptides from venomous creatures with high affinity for molecules involved in the development and maintenance of neuropathic pain has led to a surge in venom-derived analgesic research. Some members of the α-conotoxin family from *Conus* snails which specifically target subtypes of nicotinic acetylcholine receptors (nAChR) have been shown to be effective at reducing mechanical allodynia in neuropathic pain models. We sought to determine if three such peptides, Vc1.1, AuIB and MII were effective following intrathecal administration in a rat neuropathic pain model because they exhibit different affinities for the major putative pain relieving targets of α-conotoxins. Intrathecal administration of α-conotoxins, Vc1.1, AuIB and MII into neuropathic rats reduced mechanical allodynia for up to 6 hours without significant side effects. *In vitro* patch-clamp electrophysiology of primary afferent synaptic transmission revealed the mode of action of these toxins was not via a $GABA_B$-dependant mechanism, and is more likely related to their action at nAChRs containing combinations of α3, α7 or other subunits. Intrathecal nAChR subunit-selective conotoxins are therefore promising tools for the effective treatment of neuropathic pain.

1. Introduction

A limitation to effective clinical pain management is the lack of highly specific analgesics that exhibit tolerable side effects. The enormous diversity of peptides from *Conus* snails that target ion channels, receptors, and transporters known to be involved in neuropathic pain has led to the search for better analgesics based on venom-derived peptides (Lewis and Garcia 2003). The α-conotoxins represent one such family that specifically target nicotinic acetylcholine receptor (nAChR) subtypes. Owing to the combination of a large variety of nAChR subunit assemblies and subunit-selective α-conotoxins, many potential novel analgesics have recently been identified (Dutton and Craik 2001; Alonso *et al.* 2003; Sandall *et al.* 2003; Lang *et al.* 2005; Satkunanathan *et al.* 2005; Olivera *et al.* 2008; McIntosh *et al.* 2009) α-Conotoxins, including Vc1.1, RgIA, MII and AuIB, have all been reported to potently reverse signs of neuropathic pain, particularly tactile allodynia, in animal models when administered systemically (Satkunanathan *et al.* 2005; Klimis *et al.* 2011).

Some controversy exists as to the mechanisms of anti-allodynia among α-conotoxins. Early studies suggested that interaction with α3 subunit-containing nAChRs may mediate these actions (Livett *et al.* 2006), but the affinity of Vc1.1 and AuIB for these subtypes is rather weak (Clark *et al.* 2006; Vincler *et al.* 2006). Vc1.1 and RgIA are both potent antagonists of α9α10 nAChRs, suggesting this may be the anti-allodynia target (Vincler *et al.* 2006). However, MII and AuIB are both devoid of activity at α9α10 nAChRs (McIntosh *et al.* 1999; Callaghan *et al.* 2008; Azam and McIntosh 2009; Callaghan and Adams 2010; Klimis *et al.* 2011) and other α-conotoxin analogues that act on these nAChRs fail to inhibit allodynia (Nevin *et al.* 2007). Moreover, α9α10 nAChRs show very limited tissue distribution, being expressed predominantly in the olivochochlear system (Vetter et al., 2007) and their role in sensory nerve function is unclear. We have recently shown that Vc1.1, AuIB and RgIA inhibit N-type calcium channels in dorsal root ganglion (DRG) neurons through a novel $GABA_B$ receptor-dependent mechanism distinct from the well-known modulation of these channels by G-protein βγ subunits (Callaghan *et al.* 2008; Callaghan and Adams 2010; Klimis *et al.* 2011). MII is inactive at this target, although it produces partial reversal of allodynia in nerve injured rats (Klimis *et al.* 2011), suggesting this is not the only mechanism. Taken together, these findings suggest N-type calcium channels and possibly α3 subunit-containing nAChR may both be important, but it is unlikely that α9α10 nAChRs are responsible for pain relief after systemic administration. However, α-conotoxins exhibit varying and incompletely characterized selectivity for nAChR comprising combinations of α3-, α5- and α6 – and α7-subunits together with different β-subunits (Clark *et al.*, 2006; Vincler and McIntosh, 2007). Many of these subunits are expressed by sensory neurons (eg. Khan *et al.* 2003).

The present study was designed to determine if α-conotoxins with distinct activity profiles at α3- (but possibly other α-subunits) or α9α10-containing nAChRs differentially relieve allodynia following intrathecal administration in a neuropathic pain model and, in parallel, if inhibition of N-type calcium channels in primary afferent nerve terminals through a novel $GABA_B$-receptor-dependent mechanism is responsible. The α-conotoxins MII, AuIB and Vc1.1 all displayed long-lasting (up to 6 hours) anti-allodynic activity. In vitro electrophysiological recordings of primary afferent-stimulated evoked excitatory post synaptic currents (eEPSCs) onto superficial dorsal horn neurons revealed that none of these peptides substantially inhibited primary afferent activity, although a conventional $GABA_B$-receptor agonist produced profound presynaptic inhibition. The

findings suggest that neither α9α10 nAChRs nor $GABA_B$-receptor-dependent inhibition of N-type calcium channels in primary afferent synapses is the mechanism of action, but intrathecal delivery of α-conotoxins appears to be a promising therapeutic avenue.

2. Materials and methods

2.1 Rodents and surgical procedures for establishing neuropathic pain

All experiments involving animals were approved by the University of Sydney or Royal North Shore Hospital/University of Technology Animal Ethics Committees. Experiments were performed under the guidelines of the Australian code of practice for the care and use of animals for scientific purposes (National Health and Medical Research Council, Australia, 7th Edition). Great care was taken to minimise animal suffering during these experiments whenever possible. *In vivo* experiments were performed on 59 male Sprague-Dawley rats weighing 200–260 g. Rats were housed four per cage and were maintained on standard 12-h light/dark cycle with free access to food and water. Rats underwent partial ligation of the left sciatic nerve (PNL), as previously described (Seltzer *et al.* 1990). In rats that developed significant mechanical allodynia 7 days after surgery, chronic polyethylene lumbar intrathecal catheters were inserted between vertebrae L5–6, advanced 3 cm rostrally and exteriorized via the occipital region (Storkson *et al.* 1996). All of these procedures were carried out under isoflurane anaesthesia (2.0-2.5% in oxygen).

2.2 Mechanical allodynia testing

Mechanical paw withdrawal threshold (PWT) was measured using a series of von Frey hairs with bending pressures ranging from 0.41 to 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 2 g 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 seven 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 (Dixon 1980). 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), 7, and 12-14 following injury.

2.3 Measurement of motor side effects

To measure motor side effects, ambulation was tested by measuring the latency to fail negotiation of a rotarod device (Ugo Basile, Italy), with a maximal cut-off time of 300 s as previously described (Klimis *et al.* 2011). Each animal was tested immediately prior to intrathecal injection, then 60 and 120 min after injection. Differences (s) between post- and pre-injection latencies (pre-injection ~ 120s) were determined for each animal. Each latency recording consisted of the average of three measurements on the day of testing.

2.4 Drugs and intrathecal conotoxin administration

The α-conotoxins Vc1.1, AuIB and MII were synthesised as previously described (Schnolzer *et al.* 1992; Cartier *et al.* 1996; Luo *et al.* 1998; Clark *et al.* 2006). Briefly, Boc solid phase chemistry was used to synthesise, deprotect and cleave from the resin all peptides as described (Schnolzer *et al.* 1992). 100 mM ammonium bicarbonate (pH 7.5-8.2) was used to oxidise reduced HPLC-purified peptides at room temperature with constant stirring. Finally, each peptide was purified and quantified in triplicate using reverse-phase HPLC and an external reference. Intrathecal injections were made via the exteriorized catheter 10–12 days after PNL surgery using gentle restraint. Peptides were dissolved in 0.9% saline to the desired concentration on the day of the experiment and were injected in a volume of 10 µl, followed by 15 µl of 0.9% saline to wash the drug from the catheter dead-space. Control animals received injections of the corresponding vehicle. In all experiments, the experimenter was blinded to drug treatments. Catheter patency and placement was confirmed by the occurrence of brief bilateral hind limb paralysis following intrathecal lignocaine (20 µL, 2%, (2-diethylamino-*N*[2,6-dimethylphenyl]-acetamide; Sigma-Aldrich, Sydney)) after all experiments.

2.5 In vitro electrophysiology

Spinal cord slices (340 µm) were prepared from isoflurane (4% in air) anaesthetised 12- to 28-day-old male and female rat pups on a Leica VT1200S vibrating blade microtome in ice-cold modified artificial cerebrospinal fluid (ACSF, in mM); choline chloride (120), glucose (11), $NaHCO_3$ (25), KCl (2.5), NaH_2PO_4 (1.4), $CaCl_2$ (0.5), $MgCl_2$ (7), atropine (0.001). Slices were allowed to recover for 1 hour at room temperature in ACSF (in mM); NaCl (125), KCl (2.5), NaH_2PO_4 (1.25), $NaHCO_3$ (25), glucose (11), $MgCl_2$ (1.2), $CaCl_2$ (2.5) before being transferred to a recording chamber (500 µL) where Dodt-contrast optics was used to identify lamina I/II neurons of superficial dorsal horn for patch-clamp electrophysiology. The internal solution of the recording pipette contained (in mM); CsCl (140), EGTA (10), HEPES (5), $CaCl_2$ (2), MgATP (2), NaGTP (0.3), QX314 chloride (5) and had an osmolality of 290 mOsm. Drugs were superfused onto slices at a rate of 2 mL per minute in normal ACSF at a nominal 33^{o}C. All neurons were voltage-clamped at a nominal holding potential of -60 mV (liquid junction potential not corrected). eEPSCs were elicited by stimulating dorsal roots at 0.03Hz with bipolar tungsten stimulating electrodes using a stimulus strength sufficient to evoke reliable submaximal eEPSCs (usually 100 µs, 5-30 V). All reagents were purchased from Sigma Aldrich (Australia) except QX314, which was purchased from Alomone Labs (Israel).

2.6 Data analysis

All data were analysed using Prism(R) (GraphPad version 4 for Windows(TM), San Diego, CA, USA). For PWTs, two-way ANOVA (time, drug) was performed with Bonferroni post-tests if statistically significant effects were found. AUC data were generated using the AUC function in Prism™., with Analyses encompassed the pre-injection (time = 0) to 6 hour time poinst and were analysed by one-way ANOVA with Dunnett's multiple comparison post-tests. $P < 0.05$ was considered significant.

3. Results

3.1 Effects of Vc1.1, AuIB and MII on PNL-induced mechanical allodynia

Partial nerve ligation-induced, ~~rapid and~~ long-lasting mechanical allodynia that was maximal by 10–12 days after surgery (data not shown) as determined by changes to the paw withdrawal threshold

(PWT). At day 12–14 post-PNL, α-conotoxins were administered to conscious rats via a chronically implanted intrathecal catheter. The small apparent differences between animals randomly assigned to the different treatment groups for pre-PNL PWT and post-PNL PWT before injecting conotoxins were not significant. Changes in PWT to mechanical stimuli were then tested at set intervals (1, 2, 4 and 6 hours) to determine anti-nociceptive effects. All three α-conotoxins dose-dependently increased the PWT, with significant anti-allodynia persisting for up to 6 hours for all conotoxins (Figure 1; 2 nmol). Beginning at 1 hour post-injection, i.t. Vc1.1 increased the PWT significantly at a dose of 2 nmol, which lasted for the duration of the experiment ($P < 0.001$, t = 1,2,4,6). A 10-fold lower dose of Vc1.1 (0.2 nmol) also significantly increased PWT beginning at 2 hours ($P < 0.05$) and lasting until 6 hours ($P < 0.01$ for 4 and 6 hours) post-injection, but the lowest dose of Vc1.1 (0.02 nmol) had no significant effect. At no time point post-injection did the PWT for the saline-treated control group increase.

Conotoxin MII produced robust anti-allodynic activity at 2 nmol, significantly increasing the PWT beginning at 1 hour post-injection ($P < 0.01$). This effect persisted for up to 6 hours post-injection at this dose ($P < 0.001$ for t = 2, 4 and 6 hours). Lower doses of MII were also found to be anti-allodynic, with 0.2 nmol and 0.02 nmol both increasing PWT by 2 hours post-injection ($P < 0.05$ and $P < 0.01$, resp.), and the lowest dose tested (0.02 nmol) still significantly elevated at 4 hours ($P < 0.05$).

AuIB was the least potent conotoxin following a single intrathecal injection. At the highest dose tested, AuIB significantly increased PWTs beginning at 1 hour post-injection ($P < 0.05$) and remained elevated for up to 6 hours post-injection (2nmol; $P < 0.001$ t = 2 and 6 hours, $P < 0.01$ t = 4 hours). A lower dose (0.2 nmol) also produced a significant increase in PWT at 4 hours post-injection, but the lowest dose (0.02 nmol) did not produce significant reversal of tactile allodynia.

The area under the curve (AUC) was also calculated for each conotoxin (0–6 hours) to determine dose–response relationships. All three conotoxins displayed a similar increase in AUC as a function of increasing dose. As such, a significant increase in AUC was observed for each conotoxin at 2 nmol ($P < 0.01$; ANOVA with Dunnett's post-hoc test)

3.2 Effects of Vc1.1, AuIB and MII on motor impairment

Rotarod latencies were determined for each dose of intrathecal conotoxin throughout the testing period for mechanical allodynia. No significant change to rotarod latency, and therefore no motor impairment, was observed for the highest dose of each conotoxin tested (2 nmol; repeated measures ANOVA). This suggests motor performance was not impaired and did not confound allodynia testing.

3.3 Effect of Vc1.1, AuIB and MII on evoked EPSCs in spinal cord slices

Primary afferent eEPSCs in whole-cell patch-clamped neurons of spinal lamina I/II were generated by stimulating dorsal roots (0.03Hz) in naive rat spinal cord slices. We superfused 1 μM of each conotoxin onto these slices to determine their influence on primary afferent excitatory neurotransmission. The representative current traces of AMPA receptor-mediated eEPSCs in Figure 3 reveal that for α-conotoxin Vc1.1 (1 μM), no significant change to afferent-evoked eEPSCs were observed (Figure 3Ai and B) in the presence of picrotoxin (100 μM) and strychnine (5 μM) to block

$GABA_A$ and glycine receptor-mediated synaptic currents, respectively. eEPSCs were abolished by the AMPA receptor antagonist CNQX (10 μM, not shown). This finding is summarised in the histogram in Figure 3C. A higher concentration (10 μM) of Vc1.1 was also without effect (97 ± 4% of baseline, N = 9). As Vc1.1 has been shown to interact with somatic $GABA_B$ receptors (Callaghan *et al.* 2008; Callaghan and Adams 2010; Klimis *et al.* 2011), in some experiments the $GABA_B$ agonist baclofen was superfused following washout of Vc1.1 (figure 3Ai, 3B). Baclofen (10 μM) markedly reduced eEPSC amplitude by 81% (Figure 3C, N = 3). For AuIB and MII, a small but significant reduction in the eEPSC was observed (86 ± 4% and 84 ± 3%, resp. N=4 for both). This suggests these two conotoxins may modestly reduce glutamatergic neurotransmission *in vivo*.

4. Discussion

The present study has shown that intrathecal delivery of α-conotoxins differentially targeting α3 subunit-containing, α9α10 nAChR channels and $GABA_B$ receptors/N-type calcium channels are anti-allodynic in rodent models of neuropathic pain. These anti-allodynic effects were not confounded by motor deficits because rotarod performance was not impaired.

Previous studies have shown that nAChR agonists such as nicotine (α4β2) and epibatadine (non-α4β2) display anti-allodynic activity in neuropathic pain models (Rashid and Ueda 2002), and that these effects are likely due to excitatory actions on GABAergic interneurons in the spinal cord dorsal horn (Rashid and Ueda 2002; Genzen and McGehee 2005). α7 (Gao *et al.* 2010) and perhaps α3 subunit-containing (Takeda *et al.* 2003; Gao *et al.* 2010) nAChRs have also been proposed to make a small contribution to nicotinic excitation of dorsal horn GABAergic interneurons. We have previously shown that Vc1.1 is anti-allodynic in neuropathic pain models when administered intramuscularly, and that sustained reversal of allodynia appears due to $GABA_B$-receptor-dependent inhibition of N-type Ca^{2+} channels because it is reversed by a selective $GABA_B$-receptor antagonist (Klimis *et al.* 2011). Furthermore, we observed no reversal of allodynia with peripheral administration of two analogs of Vc1.1, vc1a and [P60]Vc1.1 that exhibited no activity at $GABA_B$ receptor/N-type Ca^{2+} channels but full activity at α9α10 nAChRs, suggesting that antagonism of α9α10 is not a requisite for anti-allodynia (Nevin *et al.* 2007; Callaghan *et al.* 2008). However, Vc1.1, AuIB and MII have markedly different (> 1000-fold) potencies for $GABA_B$ receptors/N-type calcium channels, with MII being nearly inactive. We have therefore tentatively attributed the anti-allodynic activity of MII to its potent antagonism of α3β2 nAChRs (~ 1 nM) (Klimis *et al.* 2011) when administered peripherally. It remains possible that like Vc1.1, AuIB acts either on $GABA_B$ receptors/N-type calcium channels or α3 subunit-containing nAChR channels, or both. Thus multiple pharmacological targets could mediate the anti-allodynic actions of different systemically administered α-conotoxins.

On the basis of the actions of peripherally administered α-conotoxins, we sought to determine whether or not similar actions may be mediated in the spinal cord. A single intrathecal injection of each of the three α-conotoxins produced long lasting (up to 6 hours) anti-allodynia with relative potency that appears to be in the order MII > Vc1.1 > AuIB. This order is based on the significant peak effects (at 2 hours) of all doses of MII compared with smaller effects of Vc1.1, and particularly AuIB at lower doses. This order of potency is not clearly reflected by the AUC calculations, because the duration of action of Vc1.1 and AuIB are longer than found with lower doses (0.02 and 0.2 nmol)

of MII. The finding that MII was the most potent anti-allodynic α-conotoxin after intrathecal administration suggests that α9α10 nAChRs or $GABA_B$ receptors/N-type calcium channels are not the primary targets for this peptide in the spinal cord, because it has little or no activity at these targets (Klimis *et al*., 2011). AuIB also fails to interact with α9α10 nAChRs, suggesting this nAChR is not involved after intrathecal administration. However, we cannot rule out a contribution for Vc1.1, which has high affinity for both α9α10 nAChRs and $GABA_B$ receptors/N-type calcium channels (Klimis *et al*., 2011).

It is possible that the anti-allodynic actions of all three α-conotoxins are mediated by α3- containing nAChRs or other subunit combinations of nAChRs expressed by primary afferent nerve terminals or spinal cord. It is unlikely that MII, Vc1.1 and AuIB all produce their intrathecal anti-allodynic actions via interaction exclusively with α3β2 or α3β4 nAChRs, because the potency of Vc1.1 and AuIB at either of these nAChRs is more than 100-fold weaker than MII (Klimis *et al*., 2011). Vc1.1 has relatively low affinity for α3β2 and α3β4 nAChRs (Clark *et al*., 2006; Vincler and McIntosh, 2007) but may have higher affinity for more complex subunit combinations, e.g. its affinity for α6/α3α2β2β3 nAChRs is 140 nM (Vincler and McIntosh, 2007). It was therefore suggested that Vc1.1 might produce pain relief via inhibition of α3- and/or α5-containing nAChRs on sensory nerves. α3, α4, α5, β2 and β4 subunit transcripts are all found in spinal cord parenchyma and sensory ganglia (Khan *et al.* 2003). Although the specificity of nAChR antibodies has been questioned (Moser *et al.* 2007), the subunits all appear to be expressed on primary afferents that co-label immunohistochemically with IB4, and with synaptophysin in superficial dorsal horn (Khan *et al.* 2003). It therefore remains possible that pain relief may be achieved after intrathecal administration by antagonism of nAChRs comprising complex combinations of α3 with α5, β4 and β2 subunits, perhaps in combination with other less common subunits. However, nAChRs composed of other as yet unidentified subunit combinations may be responsible and it will be important to determine the specific combinations responsible for these actions.

Our findings of highly efficacious anti-allodynic actions (von Frey thresholds) of intrathecal MII appear at odds with the modest increase in responsiveness to strong mechanical stimuli (Randell-Selitto test) following intrathecal injection of a very low dose of intrathecal MII (0.1 pmol) in untreated rats (Young *et al.* 2008). The basis for the discrepancies are unclear, but could be due to dose, different stimuli employed or effects of nerve injury. The lowest intrathecal dose we tested was 20-fold greater than the highest dose examined in rats (Young *et al.* 2008), which could greatly affect distribution of nAChR blockade in the dorsal horn of the spinal cord or primary afferent nerve roots. Young *et al*. (2008) also found no effect of this dose of MII on alldoynia in a nerve injury model, but still noted modest hyperalgesia in the uninjured, contralateral paw. This suggests that the effects of higher doses we observed may be specific for nerve injury-induced allodynia.

The finding that even very high concentrations of Vc1.1, MII and AuIB had little effect on the amplitude of primary afferent eEPSCs in spinal cord slices suggests that the $GABA_B$ receptor-dependent inhibition of N-type Ca^{2+} channels we found for α-conotoxins Vc1.1 and AuIB (Callaghan *et al.* 2008; Klimis *et al.* 2011) in rodent DRG neurons is not present at N-type channels in primary afferent nerve terminals. This is not particularly surprising, because the conventional mechanism of $GABA_B$-receptor agonist inhibition of N-channels via G-protein βγ-subunits in both DRG cell bodies and primary afferent terminals is not responsible for α-conotoxin effect on N-type calcium channels

in DRG neurons. The actual signalling mechanism is more complex, involving c-Src, but is not yet fully understood (Callaghan *et al.* 2008). It is therefore possible that the signalling mechanism is localised to DRG cell bodies, but not their central nerve terminals.

The small reductions in eEPSC amplitudes produced by MII and AuIB may be related to α3 nAChR or other expression of different subunit combinations in primary afferents and/or on post-synaptic NK1-receptor expressing cells (Cordero-Erausquin *et al.* 2004). The small inhibitions of eEPSCs observed here are unlikely to account for the reversal of allodynia produced by all three α-conotoxins after intrathecal administration, presumably because tonic concentrations of ACh in tissue slices are not sufficient to strongly activate nAChRs. Spinal nerve ligation studies have suggested that the α3 subunit, which is confined to neuronal perikarya and expressed mostly by small, bipolar neurons of superficial laminae, increases bilaterally after injury (Vincler and Eisenach 2004). Choline acetyl transferase-expressing neurons are abundant in the dorsal horn of the spinal cord and innervate multiple cell types, including primary afferents (Ribeiro-da-Silva and Cuello 1990). Tonic and phasic ACh concentrations *in vivo* are presumably great enough to activate nAChRs, but it is not known if ACh concentrations are elevated in chronic pain states. On the other hand, GABA/Glycine interneurons are found to preferentially express α4α6β2 nAChRs, whereas NK1-receptor/Calbindin-expressing neurons typically co-label with α3β2α7 subunit-containing nAChRs (Cordero-Erausquin *et al.* 2004). Selective antagonists for α3 subunit-containing nAChRs that could co-express other nAChR subunits may therefore suppress activation by endogenous ACh of primary afferents or pro-nociceptive NK1-receptor/Calbindin expressing neurons. Together, these findings suggest that antagonists acting selectively on α3-subunit containing nAChRsbut not α4 – or α9α10 subunit-containing nAChRs may be promising targets in neuropathic pain. However, it is possible that these nAChR antagonists act on other subunit containing nAChRs, such α7 to relieve neuropathic pain.

5. Conclusions

Here we have shown that α-conotoxins which are applied intrathecally and are known to interact with α3* nAChRs, but not α9α10 nAChRs or $GABA_B$ receptors/N-type calcium channels, display anti-allodynic activity *in vivo* in a neuropathic pain model. This finding implies that drugs (including α-conotoxins) targeting α3-containing nAChRs, or perhaps other nAChR subunit combinations that these conotoxins interact with, may prove to be clinically relevant in the treatment of neuropathic pain.

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Figure captions

Figure 1. Effect of alpha-conotoxins on mechanical allodynia. Time course of the effects of i.t. injection of (A) Vc1.1, (B) MII and (C) AuIB on mechanical PWT (left column). Each point represents the mean +/- SEM for each time point (Vehicle; n = 6, Vc1.1; n = 7 (2 nmol), 6 (0.2 nmol) and 8 (0.02 nmol), AuIB and MII; n=6 (2 nmol), 4 (0.2 nmol) and 6 (0.02 nmol)). Time point t = 0 represents PWT immediately before injection. The right column shows the corresponding dose-response as AUC for each dose of drug between 0 and 6 hours (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, Dunnett's post-hoc tests).

Figure 2. Effect of alpha-conotoxins on motor performance. Time course of the effects of i.t. injection of Vc1.1, MII and AuIB. Each point represents mean +/- SEM change in rotarod latency (s) vs. pre-injection (t = 0) latency for highest dose of each drug tested (2 nmol) (Vehicle, AuIB and MII n = 6, Vc1.1 n = 7). Lower doses were also without effect.

Figure 3. Effect of alpha-conotoxins on synaptic transmission. (A) Example current traces from dorsal-root stimulated dorsal horn neurons in the presence of (i) Vc1.1, (ii) MII and (iii) AuIB. Each trace represents the average of 10 individual episodes for drug (red) vs. baseline (black). In (i) the effect of baclofen (10 µM) is also shown (blue). (B) Example time course of the effects of Vc1.1 and baclofen superfusion onto spinal cord slices (Bac = 10 µM baclofen). (C) Peak effect of each conotoxin and baclofen normalised to baseline eEPSC amplitude (* = $P < 0.05$; One sample t-test, Vc1.1 n= 8, AuIB n = 4, MII n = 4, baclofen n=3).

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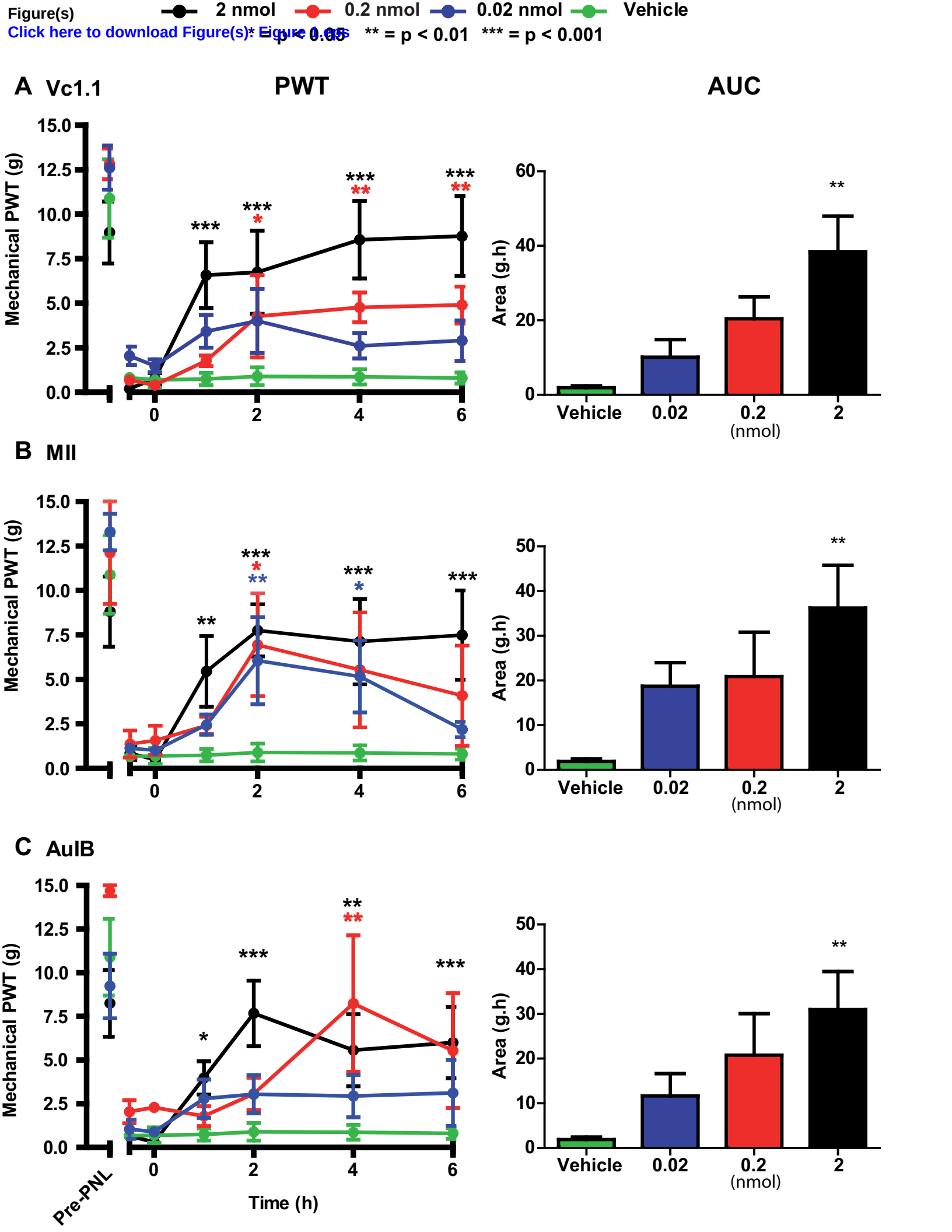

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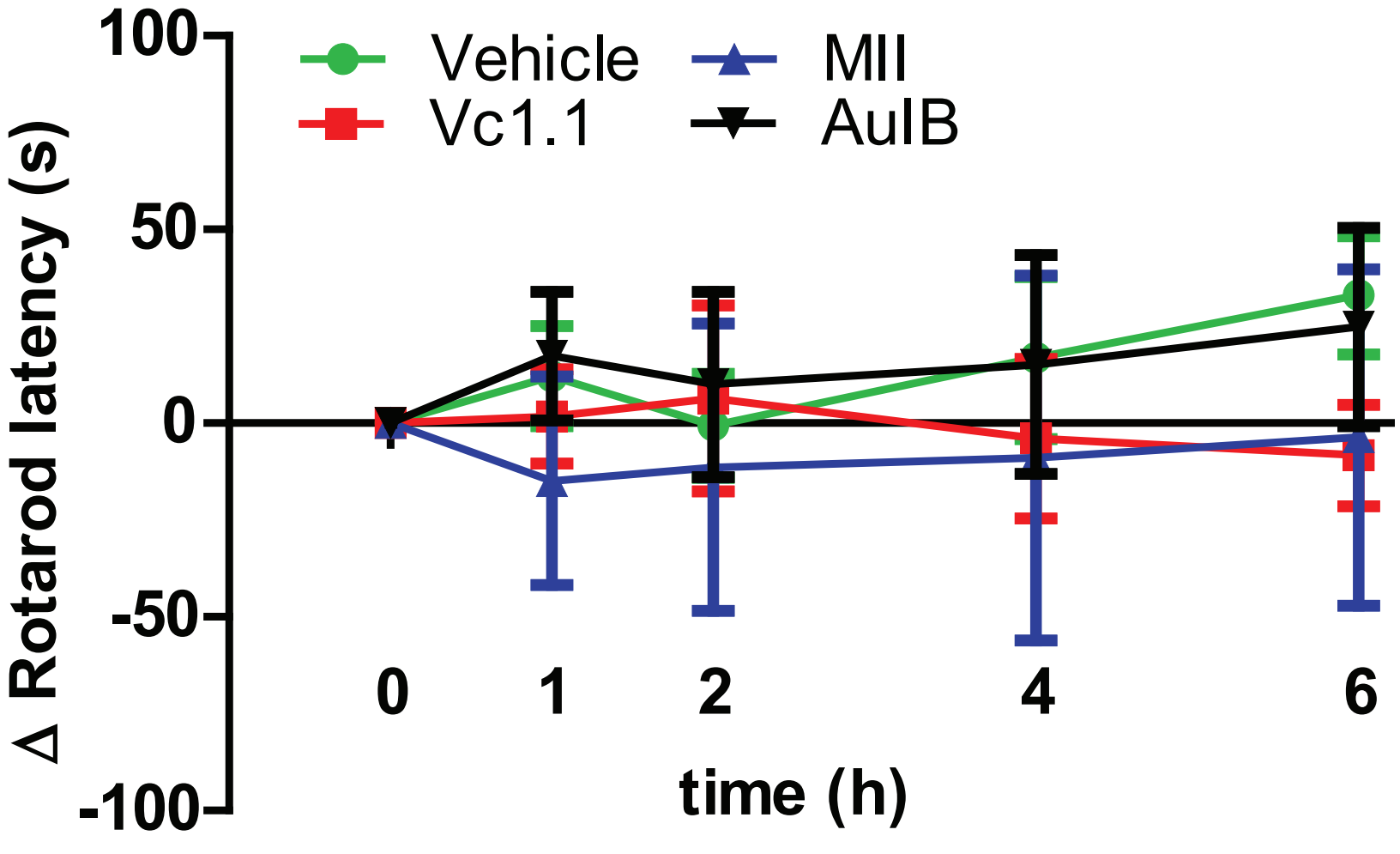

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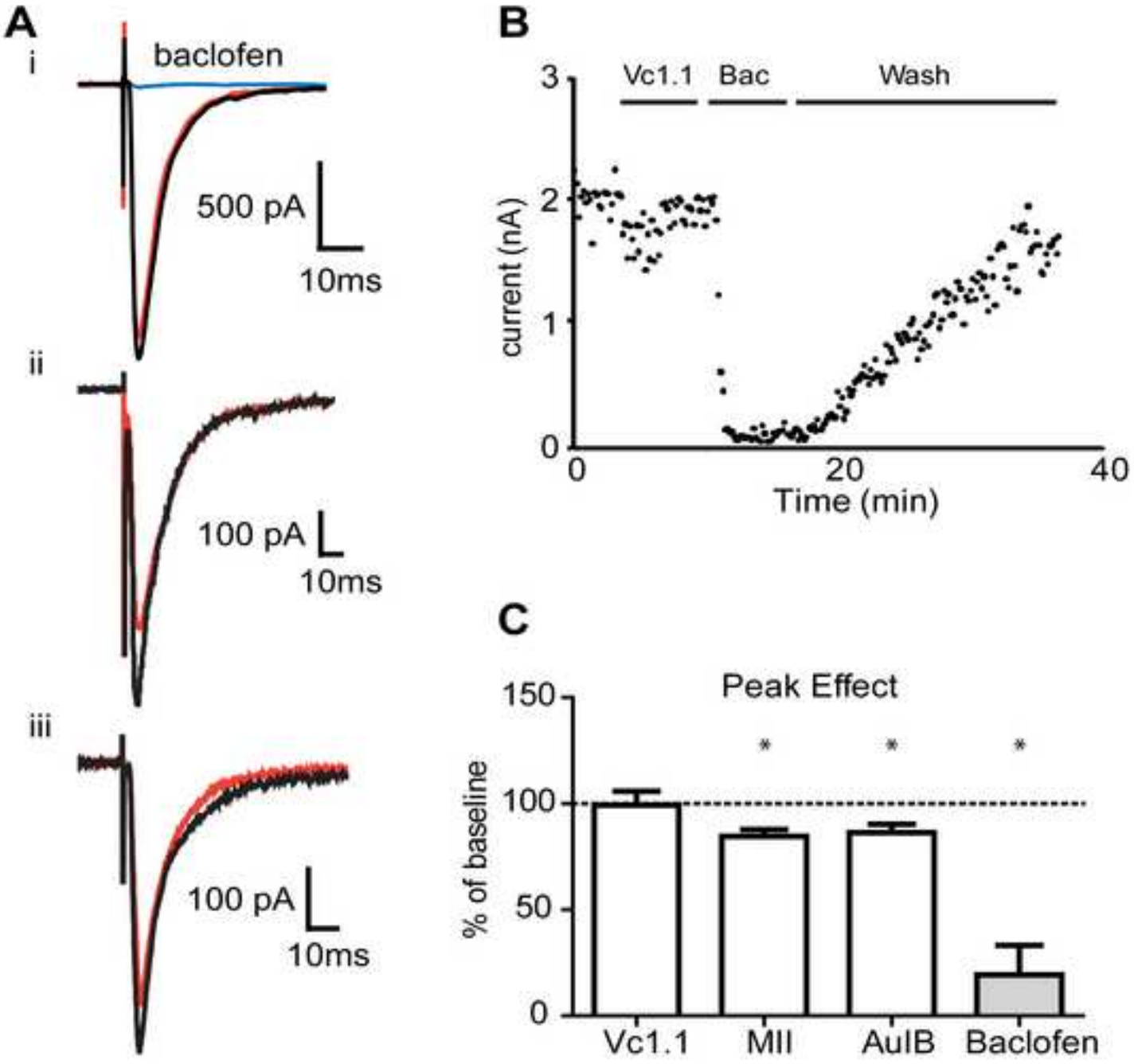

***Highlights**

intrathecal α-conotoxins reduce mechanical allodynia in a neuropathic pain model

primary afferent eEPSCs are not strongly modulated by α-conotoxins MII, AuIB or Vc1.1

actions of intrathecal α-conotoxins not likely related to activity at GABAB receptors

α3-containing nAChR are potential targets for neuropathic pain