

ACCELERATED COMMUNICATION

Are $\alpha 9\alpha 10$ Nicotinic Acetylcholine Receptors a Pain Target for α -Conotoxins?

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ABSTRACT

The synthetic α -conotoxin Vc1.1 is a small disulfide bonded peptide currently in development as a treatment for neuropathic pain. Unlike Vc1.1, the native post-translationally modified peptide vc1a does not act as an analgesic in vivo in rat models of neuropathic pain. It has recently been proposed that the primary target of Vc1.1 is the $\alpha 9\alpha 10$ nicotinic acetylcholine

receptor (nAChR). We show that Vc1.1 and its post-translationally modified analogs vc1a, [P6O]Vc1.1, and [E14 γ]Vc1.1 are equally potent at inhibiting ACh-evoked currents mediated by $\alpha 9\alpha 10$ nAChRs. This suggests that $\alpha 9\alpha 10$ nAChRs are unlikely to be the molecular mechanism or therapeutic target of Vc1.1 for the treatment of neuropathic pain.

The α -conotoxins are a subfamily of peptide toxins, isolated from the venom of snails from the *Conus* genus, that typically range in size from 12 to 16 amino acids, contain two disulfide bonds in a I-III, II-IV connectivity, and have an amidated C terminus (McIntosh et al., 1999). The α -conotoxins interact with both muscle and neuronal nicotinic acetylcholine receptors (nAChRs), which are now implicated in many neurological disorders and play a role in analgesia and addiction (McIntosh et al., 1999; Dutton and Craik, 2001; Livett et al., 2004). A polymerase chain reaction screen of cDNAs from the venom ducts of *Conus victoriae* was used to reveal α -conotoxin Vc1.1 (Sandall et al., 2003). The native peptide, designated vc1a, was subsequently identified in the venom of *C. victoriae* using MS analysis and has the two residues, Pro6 and Glu14, post-translationally modified to hydroxyproline and γ -carboxyglutamate, respectively (Jakubowski et al.,

2004). Synthetic Vc1.1 is a competitive antagonist of neuronal nAChRs in bovine adrenal chromaffin cells (Clark et al., 2006) and is most potent at recombinant $\alpha 9\alpha 10$ nAChRs expressed in *X. laevis* oocytes (Vincler et al., 2006). Vc1.1 also antagonizes the nicotine-induced increase in axonal excitability of unmyelinated C-fiber axons in isolated segments of peripheral human nerves (Lang et al., 2005). Inhibition of nAChRs on unmyelinated peripheral sympathetic and/or sensory axons may alleviate pain associated with small-fiber neuropathies. We were surprised to find that synthetic vc1a was unable to antagonize neuronal nAChRs in chromaffin cells and was also found to be inactive in two rat neuropathic pain assays (Livett et al., 2002). In contrast, it has been reported that Vc1.1 alleviates neuropathic pain in three rat models of human neuropathic pain and accelerates the functional recovery of injured neurons (Satkunanathan et al., 2005). Vc1.1 is currently under development as neuropathic pain treatment without the unequivocal identification of a specific receptor target (Livett et al., 2004, 2006).

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Materials and Methods

Peptide Synthesis and Oxidative Folding. Vc1.1, vc1a, [P6O]Vc1.1, and [E14 γ]Vc1.1 were synthesized by manual solid-

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; MS, mass spectrometry; HF, hydrogen fluoride; TFA, trifluoroacetic acid; RP-HPLC, reversed-phase-high-performance liquid chromatography; ES-MS, electrospray-mass spectrometry; ACh, acetylcholine; PNL, partial ligation of the left sciatic nerve; PWT, paw withdrawal threshold; AChBP, acetylcholine binding protein.

phase peptide synthesis on 4-methylbenzhydryl amine amide resin using the in situ neutralization/*O*-benzotriazole-*N,N,N',N'*-tetramethyluronium protocol for Boc chemistry (Schnölzer et al., 1992). Cleavage of the peptides from the resin was achieved using HF with *p*-cresol and *p*-thiocresol as scavengers [9:0.5:0.5 (v/v) HF:*p*-cresol:*p*-thiocresol]. The reaction was allowed to proceed at -5 to 0°C for 1.5 h. The HF was then removed under vacuum, and the peptides were precipitated with ether, filtered, dissolved in 50% acetonitrile containing 0.05% TFA, and lyophilized. The crude peptides were purified by RP-HPLC on a C_{18} (Phenomenex, Torrance, CA) column using a gradient of 0 to 80% B (buffer A, H_2O and 0.05% TFA; buffer B, 90% CH_3CN , 10% H_2O , and 0.045% TFA) in 80 min, and the eluent was monitored at 230 nm. These conditions were used in subsequent purification steps. Analytical RP-HPLC and ES-MS confirmed the purity and molecular mass of the synthesized peptides. The linear peptides were oxidized by dissolving in 0.1 M NH_4HCO_3 , pH 8.2, at a concentration of 0.3 mg/ml and stirring overnight at room temperature. The oxidized peptides were then purified by RP-HPLC and the molecular mass confirmed by ES-MS. All four peptides, Vc1.1, vc1a, [P6O]Vc1.1, and [E14 γ]Vc1.1, formed almost exclusively a single isomer with a monoisotopic molecular mass of 1809.7, 1866.6, 1821.6, and 1850.9 Da, respectively, as determined by ES-MS (see Clark et al., 2006).

NMR Spectroscopy. NMR data for Vc1.1, vc1a, [P6O]Vc1.1, and [E14 γ]Vc1.1 were recorded on samples dissolved in 90% H_2O /10% D_2O at pH 3.5. ARX 500 and 600 MHz spectrometers (Bruker, Karlsruhe, Germany) were used in the acquisition of data. Two-dimensional NMR experiments included double quantum filtered-correlation spectroscopy, two-dimensional total correlation spectroscopy, and nuclear Overhauser effect spectroscopy, with all spectra recorded at 280 K. All spectra were analyzed on Indigo workstations (SGI, Mountain View, CA) using XWINNMR 1.3 (Bruker) and Sparky software. Chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt at 0 ppm.

Electrophysiological Recordings from nAChRs Exogenously Expressed in *Xenopus laevis* Oocytes. RNA preparation, oocyte preparation, and expression of nAChR subunits in *X. laevis* oocytes were performed as described previously (Clark et al., 2006). Plasmids with cDNA encoding the rat $\alpha 9$ and $\alpha 10$ nAChR subunits were kindly provided by Dr. A. B. Elgoyhen (Universidad de Buenos Aires, Buenos Aires, Argentina). All oocytes were injected with 1 to 5 ng of cRNA and then kept at 18°C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 and 5 mM HEPES at pH 7.4) supplemented with 50 mg/liter gentamicin and 5 mM pyruvic acid 2 to 5 days before recording.

Membrane currents were recorded from *X. laevis* oocytes using an automated workstation with eight channels in parallel, including drug delivery and on-line analysis (OpusXpress 6000A workstation; Molecular Devices, Sunnyvale, CA). Both the voltage recording and current injecting electrodes were pulled from borosilicate glass (Harvard Apparatus Ltd., Edenbridge, UK) and had resistances of 0.3 to 1.5 M Ω 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 300-s incubation times for the conotoxin. Acetylcholine (ACh; 30 μM) was applied for 2 s at 5 ml/min, with 600-s washout periods between applications. Conopeptides were bath applied and coapplied with the agonist. Cells 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 after incubation of the peptide.

Neuropathic Pain Was Assessed Using Partial Ligation of the Left Sciatic Nerve (PNL). (Seltzer et al., 1990). In brief, the left sciatic nerve in the mid-thigh region of male Sprague-Dawley rats (250–300 g) was exposed by blunt dissection through the biceps femoris. A 4-0 silk thread was inserted into the left sciatic nerve trunk approximately 3 mm proximal to the trifurcation of the nerve

at the popliteal fossa and was tightly ligated so that 30 to 50% of the dorsal nerve thickness was trapped in the ligature.

The effects of the conotoxins on withdrawal thresholds and motor function were assessed between 12 and 16 days after PNL in all animals. On the day of drug testing, animals were tested twice before injection (30 min before and immediately before injection) to stabilize preinjection responses and then at 1, 2, and 4 h after injection. Conotoxins were injected in a normal saline vehicle (0.2 ml per animal) intramuscularly in the vicinity of the nerve injury. Mechanical paw withdrawal thresholds (PWT) were measured using a series of von Frey filaments with bending pressures ranging from 0.41 to 15.1 g. Rats were placed in elevated plastic cages with wire mesh bases. All rats were given 30 min to acclimate 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, licking of the paw, or flinching upon removal of the von Frey filament.

The mechanical PWT was calculated using the up-down paradigm (Chaplan et al., 1994). If the animals responded to all or did not respond to any hairs then the mechanical PWT was assigned as 0.2 or 15 g, respectively. The tester was blind to all treatments.

Statistics. Concentration-response curves for antagonists were fitted by unweighted nonlinear regression to the logistic equation

$$E_x = E_{\max} X^{n_H} / (X^{n_H} + \text{IC}_{50}^{n_H})$$

where E_x is the response, X is the antagonist concentration, E_{\max} is the maximal response, n_H is the slope factor, and IC_{50} is the concentration of antagonist that gives 50% inhibition of the agonist response. All electrophysiological data were pooled ($n = 3$ – 7 for each data point) and represent arithmetic means \pm standard error of the fit. Computation was done using SigmaPlot 9.0 (Systat Software, Point Richmond, CA). Statistical comparisons of mechanical PWT over time were made using a two-way analysis of variance (analysis of variance, using time and drug treatment as within- and between-subjects factors), with post hoc comparisons over time for individual drug treatment groups using Sidak's adjustment for multiple comparisons (SPSS Inc., Chicago, IL).

Results

Sequences and Structural Comparison of α -conotoxin Vc1.1 and Its Post-Translationally Modified Analogs. In this study, we synthesized α -conotoxin Vc1.1, the post-translationally modified native peptide vc1a, and the intermediate analogs [P6O]Vc1.1 and [E14 γ]Vc1.1 and compared their structures and selectivity for nAChR subtypes. The sequences and disulfide connectivity are shown in Fig. 1. [P6O]Vc1.1 and

Peptide	Sequence															
	I	II					III						IV			
Vc1.1	G	C	C	S	D	P	R	C	N	Y	D	H	P	E	I	C *
vc1a	G	C	C	S	D	O	R	C	N	Y	D	H	P	γ	I	C *
[P6O]Vc1.1	G	C	C	S	D	O	R	C	N	Y	D	H	P	E	I	C *
[E14 γ]Vc1.1	G	C	C	S	D	P	R	C	N	Y	D	H	P	γ	I	C *

Fig. 1. The sequences of Vc1.1, vc1a, [P6O]Vc1.1, and [E14 γ]Vc1.1. Positions subject to modification are highlighted in light gray; hydroxyproline and γ -carboxyglutamic acid are represented in bold. The CysI-CysIII and CysII-CysIV disulfide bonds are indicated, and the loops between CysII and CysIII and CysIII and CysIV are named Loops 1 and 2, respectively. *, C-terminal amidation.

[E14 γ]Vc1.1, which contain either a hydroxyproline in position 6 or a γ -carboxyglutamate at position 14, respectively, together with the unmodified peptide Vc1.1 and the fully modified vc1a, provide the full complement of analogs to determine the role of the post-translationally modified residues on activity.

Vc1.1, vc1a, [P6O]Vc1.1, and [E14 γ]Vc1.1 were found to fold efficiently into the predominant "globular" isomer, which has a I-III, II-IV disulfide connectivity common to all α -conotoxins (Fig. 2A). Figure 2B shows a comparison of the NMR α H secondary shifts for the four peptides. This analysis reveals that they are almost identical in structure; the only chemical shift changes occur where there is a change in residue type at positions 6 and/or 14. This similarity in structure is reinforced by the fact the α H secondary shift for [P6O]Vc1.1 matches vc1a at position 6 (because both share a

hydroxyproline at this position) and matches Vc1.1 at position 14 (both Glu), and the reverse is the case for [E14 γ]Vc1.1. In addition, the chemical shift differences between the H β protons of the side chains of comparable residues in each molecule are also similar, suggesting that the side chain orientations in all four peptides are also very similar (see Fig. 2C). Therefore, any differences in the biological targets of these peptides are not due to major changes in the three-dimensional shape of the molecules.

Inhibition of rat $\alpha 9\alpha 10$ nAChRs Expressed in *X. laevis* Oocytes by Vc1.1 and its Post-Translationally Modified Analogs. Vc1.1 has been shown previously to inhibit $\alpha 3$ -containing nAChRs but only at micromolar concentrations, and it was inactive at concentrations up to 10 μ M at $\alpha 7$, $\alpha 4$ -containing, and muscle ($\alpha 1\beta 1\gamma \delta$) nAChRs expressed in oocytes (Clark et al., 2006). The potency and selectivity of Vc1.1, vc1a, [P6O]Vc1.1, and [E14 γ]Vc1.1 were investigated for their effects on ACh-evoked membrane currents in *X. laevis* oocytes expressing various nAChRs subunit combinations. Vc1.1 has been shown to act as a competitive antagonist at nAChRs in bovine chromaffin cells (Clark et al., 2006), and in oocytes, raising the ACh concentration to 100 μ M shifted the Vc1.1 concentration-response curve to the right compared with 30 μ M ACh (data not shown). We examined the effects of Vc1.1, vc1a, [P6O]Vc1.1, and [E14 γ]Vc1.1 on $\alpha 9\alpha 10$ nAChRs expressed in *X. laevis* oocytes. Globular Vc1.1 reversibly inhibited $\alpha 9\alpha 10$ nAChR-mediated currents in a concentration-dependent manner with an IC₅₀ of 64.2 ± 15.0 nM ($n = 12$) (Fig. 3A), whereas the ribbon isomer (i.e., I-IV, II-III disulfide connectivity) was inactive at concentrations up to 10 μ M ($n = 4$). Application of vc1a, [P6O]Vc1.1, and [E14 γ]Vc1.1 also inhibited reversibly $\alpha 9\alpha 10$ nAChRs in a concentration-dependent manner, giving IC₅₀ values of 62.9 ± 5.2 , 99.1 ± 29.7 , and 65.3 ± 14.9 nM ($n = 10$ –11), respectively (Fig. 3B). The inhibition of ACh-evoked currents mediated by $\alpha 9\alpha 10$ nAChRs was complete at peptide concentrations ≥ 1 μ M, in contrast to that observed for other nAChR subunit combinations (Clark et al., 2006), and the Hill coefficients indicate a unitary toxin/receptor stoichiometry (Table 1).

Intramuscular Injection of α -Conotoxin Vc1.1, but Not vc1a or [P6O]Vc1.1, Relieves Mechanical Allodynia in a Nerve Injury Model of Neuropathic Pain. PNL produced a profound reduction in paw withdrawal threshold from a presurgery baseline of 12.9 ± 0.7 to 0.7 ± 0.1 g ($n = 33$) 12 to 16 days after surgery (see Fig. 4). As reported previously (Satkunanathan et al., 2005), intramuscular injection of 60 μ g of Vc1.1 produced significant partial reversal of allodynia associated with nerve injury (Fig. 4). By contrast, injections of high doses of vc1a or [P6O]Vc1.1 (60 μ g/rat each) had no effect on mechanical allodynia. The small apparent (nonsignificant) increase in withdrawal threshold observed 4 h after administration of [P6O]Vc1.1 suggests that this analog may produce a delayed antiallodynic effect compared with Vc1.1. At present, we cannot exclude the possibility that a delayed effect of this analog of Vc1.1 is due to reduced bioavailability.

Discussion

Although the analgesic conopeptide Vc1.1 has been reported previously to exhibit selectivity for $\alpha 3$ -containing

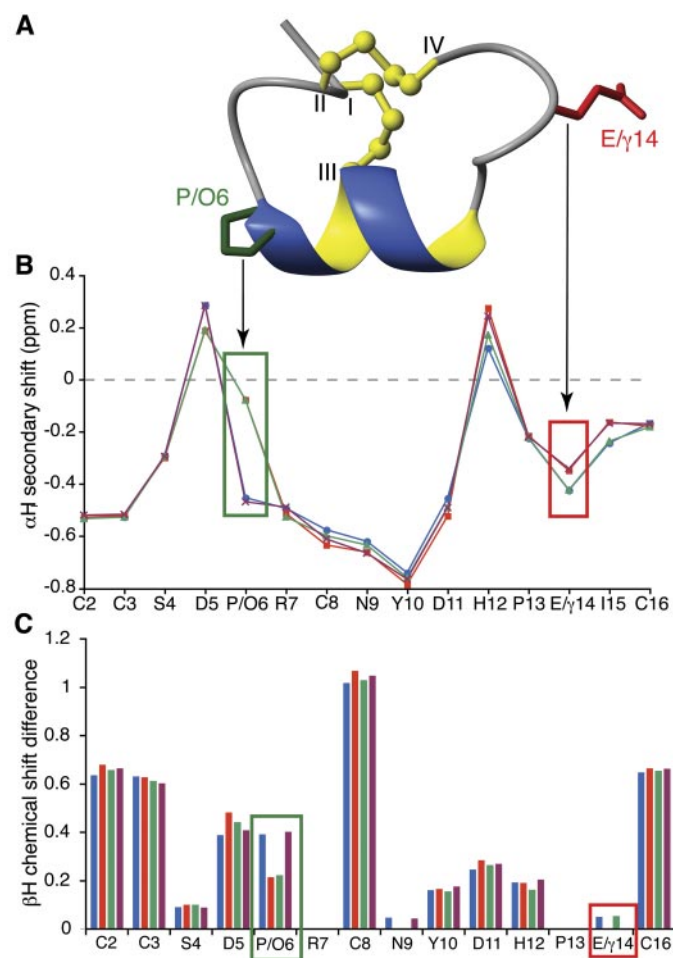


Fig. 2. Structural comparison of Vc1.1 and its post-translationally modified analogs. A, the three dimensional structure of Vc1.1 showing the α -helical region and "globular" disulfide connectivity: Cys^I-Cys^{III} and Cys^{II}-Cys^{IV}. The side chains of Pro6 (green) and Glu14 (red), the two residues subject to modification, are highlighted. B, the α H secondary shifts for Vc1.1 (blue), vc1a (red), [P6O]Vc1.1 (green), and [E14 γ]Vc1.1 (purple). The peptides have almost identical secondary shifts, indicating that the structures are very similar. The changes highlighted by the arrows and boxes for Pro/Hyp6 and Glu/Gla14 are local effects confined to the points of substitution. C, β H chemical shift differences for Vc1.1 (blue), vc1a (red), [P6O]Vc1.1 (green), and [E14 γ]Vc1.1 (purple). Values for Gly1 and Ile15 are not shown because they do not have two β protons. The similarity in most values for all four molecules suggests that the side chains are in comparable orientations. The boxes highlight the β H chemical shift differences for the modified residues. As for the α H secondary shifts, there is a pairing of the trends based on the residue type at positions 6 or 14.

nAChRs (Clark et al., 2006), we demonstrate here that it is approximately 100-fold more potent for $\alpha 9\alpha 10$ nAChRs (see also Vincler et al., 2006) and produces a significant partial reversal of allodynia associated with nerve injury. Likewise, the post-translationally modified peptides vc1a, [P6O]Vc1.1, and [E14 γ]Vc1.1 inhibit $\alpha 9\alpha 10$ nAChRs with potencies equivalent to that of Vc1.1. In contrast, vc1a and [P6O]Vc1.1 at concentrations up to 10 μ M failed to inhibit ACh-evoked currents mediated by $\alpha 1\beta 1\gamma\delta$, $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 4\beta 4$, and $\alpha 7$ nAChRs subunit combinations expressed in oocytes (Clark et al., 2006) and had no effect on mechanical allodynia. The lack of activity of vc1a on these neuronal nAChR subtypes is consistent with findings reported previously in bovine chromaffin cells and other rat models of neuropathic pain (Livett et al., 2002); however, vc1a is equal in potency to Vc1.1 as an antagonist of $\alpha 9\alpha 10$ nAChRs.

A comparison of the NMR chemical shifts for vc1a,

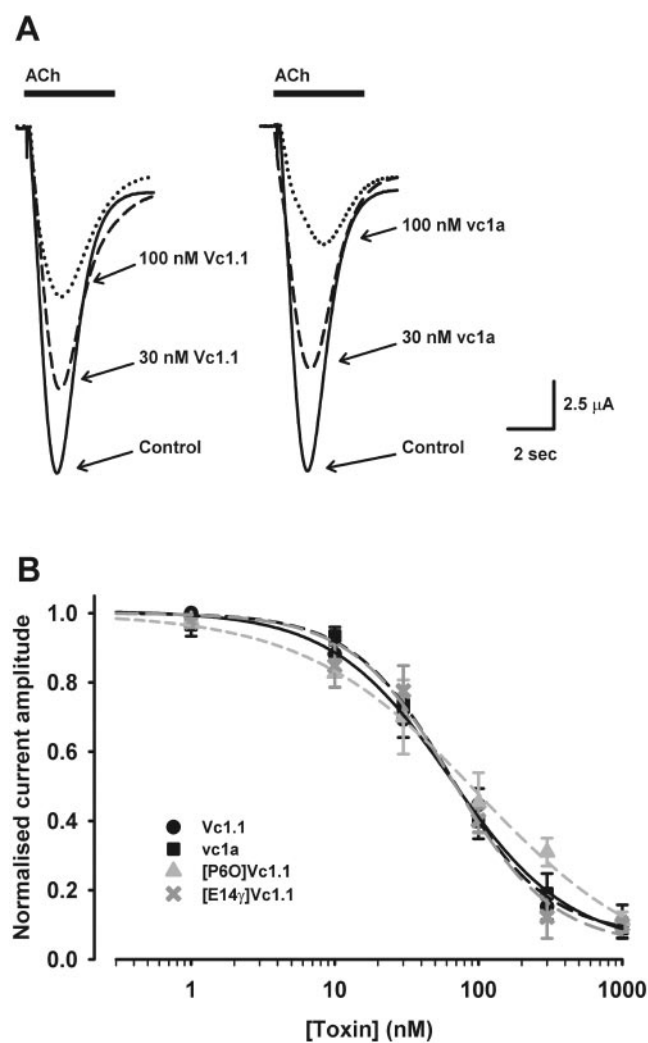


Fig. 3. Inhibition of rat $\alpha 9\alpha 10$ nAChRs expressed in *X. laevis* oocytes by Vc1.1 and its post-translationally modified analogs vc1a, [P6O]Vc1.1, and [E14 γ]Vc1.1. A, representative superimposed traces of ACh-evoked currents in the absence (solid line) and presence Vc1.1 and vc1a (broken lines). Oocytes were voltage-clamped at -80 mV, and membrane currents were evoked with 30 μ M ACh in the absence and presence of the peptide. B, concentration-response curves obtained for the inhibition of ACh-evoked current amplitude after 300-s incubation of Vc1.1 and its analogs, Vc1.1 (\bullet), vc1a (\blacksquare), [P6O]Vc1.1 (gray \blacktriangle), and [E14 γ]Vc1.1 (gray \times). Concentration-response data for the antagonists were fit using the logistic equation from *Materials and Methods*.

[P6O]Vc1.1, and [E14 γ]Vc1.1 with those for Vc1.1 showed that the post-translationally modified analogs are structurally analogous to Vc1.1, and therefore variations in biological activity between these peptides are due to the side-chain modifications and not a structural perturbation. Substitution of Pro6 in Vc1.1 to hydroxyproline in [P6O]Vc1.1 results in a loss of activity at $\alpha 3$ -containing nAChR subtypes (Clark et al., 2006), but the current study shows that activity at the $\alpha 9\alpha 10$ nAChR subtype is not negated by this mutation. The

TABLE 1

Inhibition of recombinant $\alpha 9\alpha 10$ nAChR subunits expressed in *X. laevis* oocytes by Vc1.1 and post-translationally modified peptides
 Membrane currents evoked with 30 μ M ACh; pooled data ($n = 3$ – 7 for each data point) for curve fitting.

	IC ₅₀	n _H	n
	nM		
Vc1.1	64.2 \pm 15.0	1.1 \pm 0.2	12
vc1a	62.9 \pm 5.2	1.3 \pm 0.1	11
[P6O] Vc1.1	99.1 \pm 29.7	0.7 \pm 0.1	10
[E14 γ]Vc1.1	65.3 \pm 14.9	1.2 \pm 0.3	10

IC₅₀, half-maximal inhibitory concentration; n_H, Hill coefficient; n, number of oocytes; γ , γ -carboxyglutamic acid.

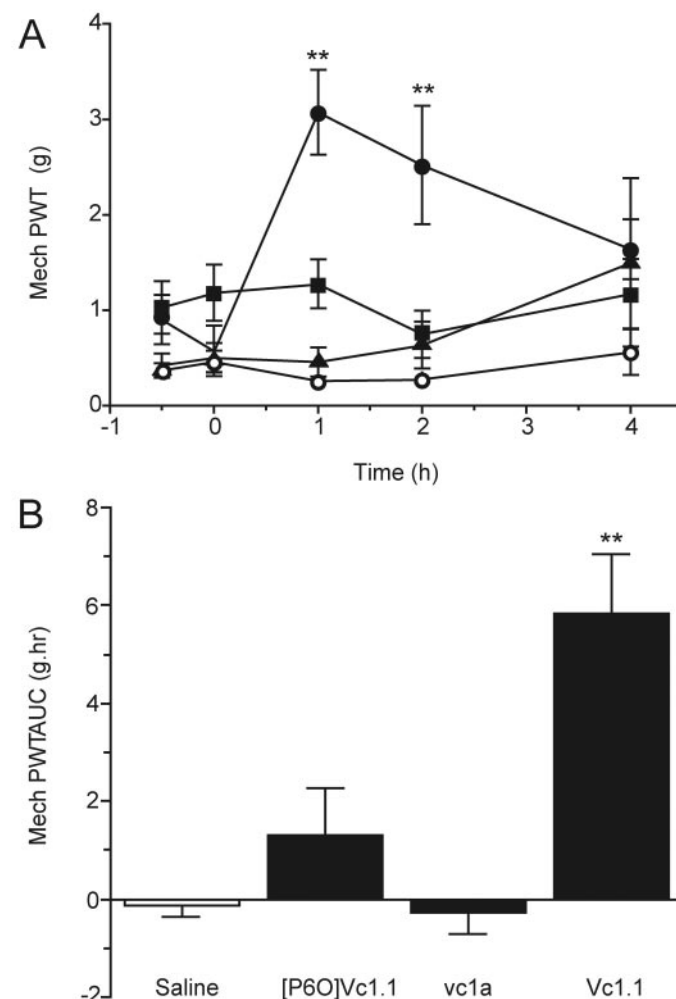


Fig. 4. Intramuscular injection of α -conotoxin Vc1.1 (60 μ g) (\bullet) relieves mechanical allodynia in a nerve injury model of neuropathic pain but vc1a (\blacksquare) and [P6O]Vc1.1 (\blacktriangle) are both inactive at high doses (60 μ g/rat). A, time-response relationship of mechanical paw withdrawal response. **, $p < 0.002$ for Vc1.1 versus baseline, $n = 6$ – 11 animals per group. B, area under the curve for the antiallodynic effect; **, $p < 0.001$ for Vc1.1.

crystal structure of the α -conotoxin ImI, which has a loop 1 identical to that of Vc1.1, in complex with acetylcholine binding protein (AChBP), a soluble homolog of the ligand binding domain of the nAChR, has been reported (Ulens et al., 2006). This structure revealed that Pro6 in ImI makes van der Waals contacts with Tyr53 and Ile116 of the AChBP. The $\alpha 3$ nAChR subunit also has an Ile at the position corresponding to Ile116 in the AChBP. Therefore, it seems likely that a hydroxyproline in position 6 of the conotoxin could disrupt key hydrophobic interactions with the $\alpha 3$ subunit. In contrast, in both the $\alpha 9$ and $\alpha 10$ nAChR subtypes, the residue corresponding to Ile116 is an aspartic acid. Therefore, mutation of Pro6 in Vc1.1 to hydroxyproline may be counteracted by interactions between the new hydroxyl group and the aspartic acid in the $\alpha 9$ and $\alpha 10$ subunits. This is further supported by work that found that substitution of Pro6 with Hyp in α -conotoxin PnIB caused a dramatic reduction in activity on the $\alpha 7$ nAChR subtype (Quiram et al., 2000). The $\alpha 7$ nAChR subtype also has a hydrophobic residue (leucine) at the position corresponding to Ile116 in the AChBP. The mutation of Glu14 to a γ -carboxyglutamic acid is a more conservative residue change, so it is not surprising that it has little effect on activity. In addition, the crystal structures of α -conotoxins with the AChBP show that this residue is more solvent exposed and is less important for receptor interactions.

In conclusion, synthetic vc1a and the partially modified homologs [P6O]Vc1.1 and [E14 γ]Vc1.1 are all active at $\alpha 9\alpha 10$ nAChRs but not at any of the other nAChR subtypes studied (Clark et al., 2006). Recent studies of α -conotoxins Vc1.1 and RgIA have also attributed the acute analgesia produced by these conotoxins to the antagonism of $\alpha 9\alpha 10$ nAChRs (Ellison et al., 2006; Vincler et al., 2006; Vincler and McIntosh, 2007). However, given that Vc1.1, but not vc1a or its analog [P6O]Vc1.1, was able to inhibit a vascular response to pain and reduce chronic pain in several animal models of human neuropathy (Livett et al., 2002, 2006; Lang et al., 2005), it is highly unlikely that $\alpha 9\alpha 10$ nAChRs are the molecular mechanism or therapeutic target of Vc1.1 for the treatment of neuropathic pain.

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