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Review

Alpha9 nicotinic acetylcholine receptors and the treatment of pain

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ABSTRACT

Chronic pain is a vexing worldwide problem that causes substantial disability and consumes significant medical resources. Although there are numerous analgesic medications, these work through a small set of molecular mechanisms. Even when these medications are used in combination, substantial amounts of pain often remain. It is therefore highly desirable to develop treatments that work through distinct mechanisms of action. While agonists of nicotinic acetylcholine receptors (nAChRs) have been intensively studied, new data suggest a role for selective antagonists of nAChRs. α-Conotoxins are small peptides used offensively by carnivorous marine snails known as *Conus*. A subset of these peptides known as α-conotoxins RgIA and Vc1.1 produces both acute and long lasting analgesia. In addition, these peptides appear to accelerate the recovery of function after nerve injury, possibly through immune mediated mechanisms. Pharmacological analysis indicates that RgIA and Vc1.1 are selective antagonists of α9α10 nAChRs. A recent study also reported that these α9α10 antagonists are also potent GABA-B agonists. In the current study, we were unable to detect RgIA or Vc1.1 binding to or action on cloned GABA-B receptors expressed in HEK cells or *Xenopus* oocytes. We review the background, findings and implications of use of compounds that act on α9* nAChRs.[1]

Contents

Abbreviations: nAChRs, nicotinic acetylcholine receptors; ACh, acetylcholine; CCI, chronic constriction injury; PSNL, partial sciatic nerve ligation; STZ, streptozotocin; GPCR, G-protein coupled receptor; GIRK, G-protein-activated inwardly rectifying K^+ channel; CGP54626, [S-(R*,R*)]-[3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid.

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[1] * indicates the possible presence of additional subunits.

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1. Introduction

Chronic pain is estimated to affect millions of people worldwide and is one of the most common reasons for physician visits [1]. Nociception is the neural processes of encoding and processing noxious stimuli. Mechanical, chemical and thermal insults stimulate nerve endings referred to as nociceptors. The stimulated nociceptors transmit signals, via the dorsal horn of the spinal cord to the brainstem, midbrain and cerebral cortex. Descending pathways may further modulate nociceptive activity [2]. Inflammation may cause direct painful stimuli as well as sensitize nociceptors to stimulation [3]. Thus, there are multiple points along the pain pathway that represent opportunities for therapeutic intervention. Despite this, there are only a limited number of mechanisms through which current pain medications work. Major classes of analgesics include opioids, non-steroidal anti-inflammatory drugs, antidepressants, and anticonvulsants. Although these treatments provide relief, the effects are often incomplete and complicated by serious side effects and/or tolerance. Thus, therapeutics with novel mechanisms of actions are desperately needed [4].

Chronic pain is that which persists beyond the healing phase following an injury. Cytokines and chemokines released from immune cells are thought to play a pivotal role not only in inflammatory pain, but also in neuropathic pain following damage to neuronal tissue [3]. α-Conotoxins RgIA and Vc1.1 have recently come to attention not only for their ability to produce acute analgesia, but, of particular interest, for their ability to produce long lasting analgesia and restoration of nerve function possibly through immune mediated mechanisms [5,6].

2. Overview of α9* nAChR pharmacology

Nicotinic acetylcholine receptors (nAChRs) are allosteric transmembrane proteins assembled from one or more α subunits (α1–α10) either alone or together with one or more non-α subunits (β1–β4) [7,8]. Individual subtypes of nAChRs have unique pharmacological and biophysical properties as well as expression patterns that enable the possibility that subtype selective compounds may have distinct therapeutic applications with a restricted set of side effects [9–12]. nAChR subunits may be divided into 2 broad classes. Alpha subunits have a defining "cysteine loop" that contains two vicinal cysteine residues, whereas non-alpha subunits lack this cysteine loop. Alpha subunits may be further divided into two clades based on phylogenetic analysis. In mammals, one clade consists of α7, α9 and α10 subunits [13,14]. α7, α9 and α9α10 nAChRs are all potently blocked by α-bungarotoxin [15–17]. Both the α7 and α9 subunits can assemble into a functional homopentamer [16]. In contrast the α10 subunit has only been functionally co-expressed with an α9 subunit [17]. In *Xenopus* oocytes, the co-injection of α9 and α10 subunits boosts functional nAChR expression 100-fold or more compared to injection of α9 alone [17,18]. α9α10 nAChRs have a number of notable characteristics (see Table 1). While Acetylchoine (ACh) activates these receptors, the classic nicotinic agonist nicotine does not. Moreover, nicotine blocks ACh-evoked currents [16,17]. Thus, although α9 and α10 are members of the nicotinic family based on gene homology, nicotine is an antagonist of α9 and α9α10 nAChRs. Cytisine and epibatine, other well-characterized agonists of other nAChR subtypes are also antagonists of α9 nAChRs [19]. In contrast the muscarinic agonist oxotremorine is a partial agonist of α9 and α9α10 nAChRs [17]. The classic muscarinic agonist, muscarine, as well as the classic antagonist atropine, block α9 and α9α10 nAChRs [16,17]. Choline, often referred to as an α7-selective agonist is also a potent partial agonist of α9 and α9α10 nAChRs [18]. α-Bungarotoxin and methylycaconitine are antagonists of α9* nAChRs [17,18,20]. In addition a number of non-cholinergic antagonists also potently block α9α10 nAChRs; these include strychnine (glycine receptor antagonist), bicuculline (GABA-A antagonist) and ICS-205, 930 ($5HT_3$ receptor antagonist) [17]. Thus, α9α10 nAChRs have a pharmacological profile unknown for any other nicotinic or muscarinic cholinergic receptor subtype.

3. α-Conotoxins that block α9α10 nAChRs

α-Conotoxins are small disulfide rich peptides, 13–20 amino acids in length, which are found in the venom of carnivorous marine snails known as *Conus*. The 500–700 different species of cone snails comprise perhaps the richest natural sources of ligands targeted to nAChRs [21]. Cone snails prey on organisms from five different phyla that utilize cholinergic neurotransmission. Thus, there has been intense evolutionary pressure to develop peptides targeted to different molecular forms of nAChRs. α-Conotoxins have historically been purified from venom. More recently the ability to predict toxin sequences by PCR of α-conotoxin genes has led to a substantial increase in the number of these ligands for characterization [21,22]. Three α-conotoxins have now been reported to be antagonists at α9α10 nAChRs. The first recognized peptide was α-conotoxin PeIA from the venom of *Conus pergrandis*. α-Conotoxin PeIA potently blocks α9α10 nAChRs but not α7 or α1* nAChRs [23]. In contrast, the snake toxin α-bungarotoxin,

Table 1
Antagonists and agonists of α9α10 nAChRs.

Antagonists	IC_{50} (nM)
Nicotine	3900
Atropine	1000
Muscarine	41,000
Strychnine	20
Bicuculline	1000
ICS-205,930	20
d-Tubocurarine	110
Methylycaconitine	7.5[a]
α-Bungarotoxin	14[b]
PeIA	6.9
Vc1.1	19
RgIA	5.2
Agonists	**EC_{50} (nM)**
Acetylcholine	13,800
DMPP	Partial agonist
Oxotremorine	Partial agonist
Choline	Partial agonist[a]

Values are for rat α9α10 except where noted.
[a] Human.
[b] Rat α9χα10χ where χ denotes a subunit chimera composed of the N-terminal ligand binding domain of the nicotinic subunit and the C-terminal domain of the 5-hydroxytryptamine 3A subunit. Data are from [17,18,6,23,111].

Table 2
Amino acid sequences of α-conotoxins that block α9α10 nAChRs.

α-Conotoxin	Sequence
RgIA	GCCSDPRCRYRCR
Vc1.1	GCCSDPRCNYDHPEIC[a]
PeIA	GCCSHPACSVNHPELC[a]

[a] Indicates amidated C-terminus.

blocks α9α10, α7 and α1 nAChRs. However, among non-α-bungarotoxin-sensitive receptors, α-conotoxin PeIA is also active on α3β2 and α6β2β3 nAChRs. We therefore used a phylogenetic approach to identify other α-conotoxin sequences that might be more selective. α-Conotoxin RgIA was isolated from *Conus regius* and found to be both potent and selective for heterologously expressed as well as native α9α10 nAChRs [24]. α-Conotoxin RgIA is the most selective α9α10 antagonist yet reported. One additional peptide that blocks α9α10 nAChRs is α-conotoxin Vc1.1, also known as ACV1. α-Conotoxin Vc1.1 from *Conus victoriae* was originally identified by gene sequencing and identified as a neuronal nAChR antagonist based on its ability to block nicotine and ACh-evoked norepinephrine release from bovine adrenal chromaffin cells [25]. It was subsequently shown to block heterologously expressed nAChRs in *Xenopus* oocytes with highest potency for α9α10 nAChRs [5,26]. Vc1.1 also blocks α6* nAChRs with lower potency [5]. Each of the three α-conotoxins, PeIA, RgIA and Vc1.1 have been synthesized by solid phase methods [23–25,27]. The sequences of these peptides are shown in Table 2 and the specificities in Table 3. Three-dimensional structures of these peptides have been determined by nuclear magnetic resonance [27–29] enabling receptor docking studies [30].

4. Acute antinociceptive effects of α9α10 nAChR antagonists

The subcutaneous or intramuscular administration of α9α10 selective antagonists acutely alleviates pain resulting from traumatic, inflammatory, or metabolic neuronal injury. The chronic constriction nerve injury model of neuropathic pain (CCI), which involves both a traumatic (loose constriction of the sciatic nerve) and inflammatory (chromic gut suture) component [31], has been utilized repeatedly to assess the efficacy of α9α10 nAChR antagonists. This model of neuropathic pain results in painful response to a normally non-painful stimulus (allodynia) and an exaggerated response to a painful stimulus (hyperalgesia) within 7 days post-injury. Subcutaneous administration of Vc1.1 in doses of 24, 80, 160, 240, or 800 μg/kg in rat dose-dependently reverse CCI-induced mechanical allodynia by approximately 54–80%, with a peak analgesic effect of 1 h; the highest concentrations have an extended effect lasting up to 24 h post-administration [5,32,33]. CCI-induced mechanical hyperalgesia, measured by paw withdrawal to pressure, is also reduced dose-dependently following the systemic administration of α9α10-selective antagonists. Administration of Vc1.1 (0.036, 0.36, or 3.6 μg/200 μl) or RgIA (0.02 or 0.2 nmol/200 μl) into the musculature overlying the constricted sciatic nerve alleviates mechanical hyperalgesia from 1 to 4 h [6,34,35]. This analgesic response to intramuscular administrations of Vc1.1 (0.36 or 3.6 μg/200 μl) or RgIA (0.2 nmol/200 μl) is consistent across 7 days without the development of tolerance [34,6]. Similar administration of the N-type Ca^{2+} channel blocker, ω-conotoxin MVIIA (0.53 μg/200 μl), has no effect [35]. Others have reported efficacy with topical or local application of MVIIA [36,37]. The effect of Vc1.1 is not necessarily a localized, direct effect on the peripheral nerves in the hind leg because the administration of Vc1.1 (0.36 μg and 3.6 μg) in the contralateral hind leg is also capable of reversing mechanical hyperalgesia [34].

Table 3
IC_{50}s of α-conotoxins and α-bungarotoxin.

nAChR subtype	PeIA	RgIA	Vc1.1	α-BgTx
α9α10	6.9	5.2	19	14
α7	1,800	4,700	>30,000	0.5
α1β1δε		16,000		
α1β1δγ			>30,000	4.9
α2β2	>10,000	>10,000	>10,000	
α2β4	>10,000	>10,000	>10,000	
α3β2	23	>10,000	7,300	
α3β4	480	>10,000	4,200	
α4β2	11,600	>10,000	>30,000	
α4β4	>10,000	>10,000	>30,000	
α6/α3β2β3	<100	>10,000	140	
α6/α3β4		>10,000	980	

Values shown are IC_{50}s in nM at mammalian nAChRs expressed in *Xenopus* oocytes and are from [6,24,27,23,18,15]. α-BgTx, α-bungarotoxin.

α9α10 nAChR antagonists show acute analgesic efficacy in an additional model of neuropathic pain, the partial sciatic nerve ligation model (PSNL) [38]. In this model of traumatic nerve injury, 1/3 to 1/2 of the sciatic nerve is ligated with silk suture to produce stable and long lasting mechanical allodynia and hyperalgesia. However, in the PSNL model, compared to the loose ligation of the sciatic nerve in the CCI model, the injury to the nerve itself evokes the immune response which contributes to axonal degeneration and the development of neuropathic pain [39]. α-Conotoxin Vc1.1 (1 μg/kg, s.c.; 0.36 μg/200 μl, i.m.) significantly reverses mechanical hyperalgesia in this model at 1 and 3 h post-administration [34]. A higher concentration of Vc1.1 (60 μg, i.m.) is also efficacious at reducing mechanical allodynia over the same time course [26].

Vc1.1 is also an effective analgesic against pain resulting from a purely inflammatory insult. Intraplantar administration of Complete Freund's Adjuvant (CFA) produces profound acute mechanical hyperalgesia within 4 h which is alleviated by subcutaneous administration of Vc1.1 in concentrations ranging from 8 μg/kg to 2.4 mg/kg [33]. Peak analgesic effects of the highest concentration of Vc1.1 (2.4 mg/kg) were observed at 1 and 1.5 h and paw withdrawal thresholds remained elevated 3 h post-Vc1.1 administration [33].

In a rat model of diabetic neuropathy, pancreatic β-cells are destroyed following the injection streptozotocin (STZ) resulting in pronounced hyperglycemia and glucosuria within 24 h and the development of mechanical allodynia and hyperalgesia within 5–7 days [40,41]. Vc1.1 (300 μg/kg, s.c.) administration significantly reduces mechanical allodynia in diabetic rats for a prolonged period of at least 6 h [33]. However, an acute analgesic effect on mechanical hyperalgesia is not observed with this dose in this model [33].

Vc1.1 (known as ACV1) was tested in human clinical trials [42]. In a phase 1 safety study, there was no evidence of systemic drug-related adverse effects from single or multiple doses [43]. ACV1 progressed to phase 2A trials, but development was halted after *in vitro* data indicated that Vc1.1 was ~100-fold less potent on human α9α10 vs. rat nAChRs [44]. Required dosage adjustment for humans was judged to be cost-prohibitive [45].

5. Cumulative antinociceptive effects of α9α10 nAChR antagonists

As mentioned above, the analgesic effects of the higher concentrations of α9α10 nAChR antagonists often exhibit efficacy as long as 24 h post-administration [34,6], a time point at which serum levels of Vc1.1 are negligible [33]. This prolonged analgesic effect is compounded by repeated, once daily administration of α9α10 nAChR antagonists and manifests as a gradual reduction of

injury-induced mechanical allodynia and hyperalgesia over time. This reduction in injury-induced behavioral hypersensitivity is supported by biochemical, physiological, and immunohistochemical studies that suggest α9α10 nAChR antagonists effect the underlying pathology of these pain models [6,34,46].

Repeated, once daily administration of Vc1.1 (0.36 and 3.6 μg/200 μl, i.m.) or RgIA (0.36 and 3.6 μg/200 μl, i.m.) produces a significant decrease in mechanical hyperalgesia in CCI rats across 5–7 days when paw withdrawal thresholds are measured 24 h post-antagonist administration [6,35]. Similar intramuscular administration of the N-type Ca^{2+} channel blocker, MVIIA, has no effect on mechanical hyperalgesia in CCI rats [35]. Vc1.1 (0.36 μg/200 μl, i.m.; 1 μg/kg, s.c.) and RgIA (0.36 μg/200 μl, s.c.) also exhibit cumulative analgesia in the PSNL model of neuropathic pain [33,34] [M. Vincler, unpublished observations].

In addition to the cumulative effects of repeated Vc1.1 or RgIA administration on mechanical hypersensitivity, changes in the underlying disease pathology of nerve injury and repair are observed. Intramuscular administration of 0.2 nmol RgIA once daily for 5 days significantly decreases the number of lymphocytes and macrophages at the site of injury in CCI rats [6]. Repeated intramuscular administration of Vc1.1 (0.36 and 3.6 μg) either in the ispilateral or contralateral hind limb of CCI rats, significantly accelerates the functional recovery of peripheral nerves distal to the ligation [34] whereas the N-type Ca^{2+} channel blocker, MVIIA, was without effect [35].

The cumulative analgesic effects of Vc1.1 are most pronounced in the STZ diabetic neuropathic pain model. Repeated once daily administration of Vc1.1 (300 μg/kg, s.c.) produces a significant alleviation of tactile allodynia and mechanical hyperalgesia at the 24 h post-administration time point following the third administration of Vc1.1 [46]. A continuing and increasing alleviation of mechanical allodynia is observed over a 4 week time course of repeated Vc1.1 administration at both 30 and 300 μg/kg [46]. Concomitant with this reversal of mechanical allodynia, a significant decrease in markers of oxidative stress is observed. Levels of lipid hydroperoxide in the sciatic nerve and nitrotyrosine in systemic blood of diabetic rats treated with Vc1.1. (300 μg/kg) for 4 weeks are significantly lower than in diabetic rats treated with vehicle [46].

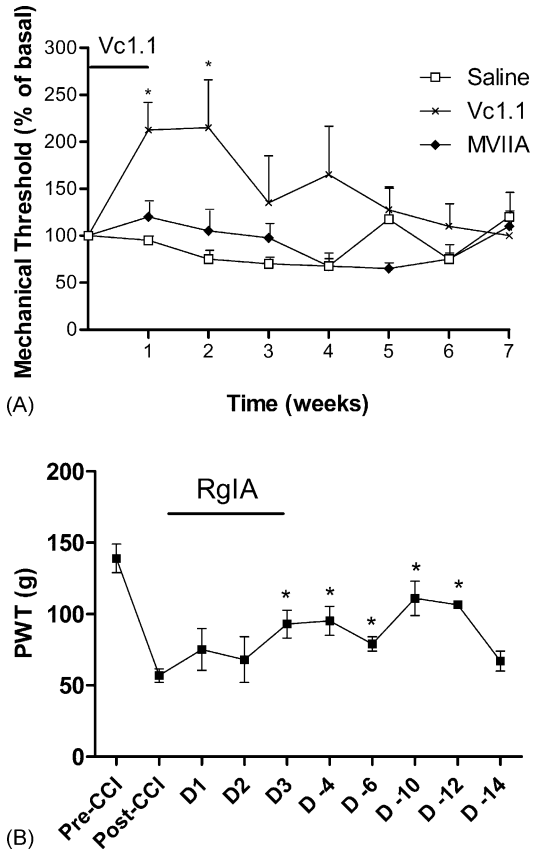

Fig. 1. Extended analgesic effect of α-conotoxins Vc1.1 and RgIA. (A) CCI rats ($n = 6$/group) treated intramuscularly with saline, 0.36 μg/200 μl Vc1.1, or 0.53 μg/200 μl MVIIA for 7 days. Mean percent changes in post-injury mechanical hyperalgesia are shown across weeks. *$p < 0.05$. Data are from [35]. (B) CCI rats ($n = 8$) treated s.c. with 0.31 μg RgIA for 3 days. Mean paw withdrawal thresholds (PWT) in grams are shown prior to injury (Pre-CCI), 7 days post-injury (Post-CCI), across RgIA treatment days (D1, D2, D3), and 14 days following cessation of treatment (D-4 to D-14). * $p < 0.05$.

6. Extended antinociceptive effects of α9α10 nAChR antagonists

Further support for the disease modifying impact of α9α10 nAChR antagonists can be provided by the extended analgesic effects of Vc1.1 and RgIA that are observed once the antagonists are no longer administered. The cumulative analgesic effects of 7 days of once daily Vc1.1 (0.36 and 3.6 μg, i.m.) on mechanical hyperalgesia in CCI and PSNL rats are measurable 1 week after the cessation of treatment [34,35]. A similar effect has been observed with repeated RgIA administration (0.31 μg, s.c, see Fig. 1). A significant reduction of tactile allodynia and mechanical hyperalgesia remains 7–10 days following the cessation of 5 days of once daily Vc1.1 administration (300 μg/kg, s.c.) to STZ diabetic rats (Fig. 2) [35,46]. The detailed molecular mechanism of prolonged analgesia is unknown. The off-rate kinetics for both RgIA and Vc1.1 are rapid ($\tau < 1$ min, see [24] and (unpublished observations)) and thus would not account for sustained effects.

7. α-Conotoxins and GABA-B receptors

Recently there was a report indicating that α-conotoxins Vc1.1 and RgIA inhibit N-type Ca^{2+} channels via activation of GABA-B receptors [47]. Blockade of Ca^{2+} channel currents was observed in rat dorsal root ganglion neurons. However Vc1.1 did not directly inhibit cloned N-type Ca^{2+} channels heterologously expressed in *Xenopus* oocytes suggesting an indirect mechanism for antagonism. Subsequent analysis in dorsal root ganglion neurons showed that block of Ca^{2+} current was pertussis toxin sensitive. Blockade of N-type Ca^{2+} channels was also prevented by co-incubation with GABA-B antagonists. Based on these observations it was proposed that RgIA and Vc 1.1 modulate N-type Ca^{2+} channels by activating G-protein coupled GABA-B receptors and that agonism of GABA-B receptors, rather than blockade of α9α10 nAChRs is responsible for the α-conotoxin analgesic effects. An analog of Vc1.1 known as vc1a blocks α9α10 nAChRs but is not analgesic lending support to this hypothesis [26,42]. However, vc1a does not stimulate GABA-B receptors [47], yet retains the ability of Vc1.1 to accelerate functional recovery of injured nerve [48].

8. Does GABA-B activation account for α-conotoxin analgesic activity?

Activation of GABA-B receptors by baclofen has been shown to be analgesic. In addition, activation of GABA-B receptors modulates activity of ion channels including N-type Ca^{2+} channels [49]. Block of N-type Ca^{2+} channels is analgesic and is the basis of the antinociceptive properties of another well known *Conus* derived compound, ω-conotoxin MVIIA, an FDA approved drug known as Prialt [50,51]. Thus, activation of GABA-B receptors by RgIA or Vc1.1 could be analgesic via modulation of N-type Ca^{2+} channels.

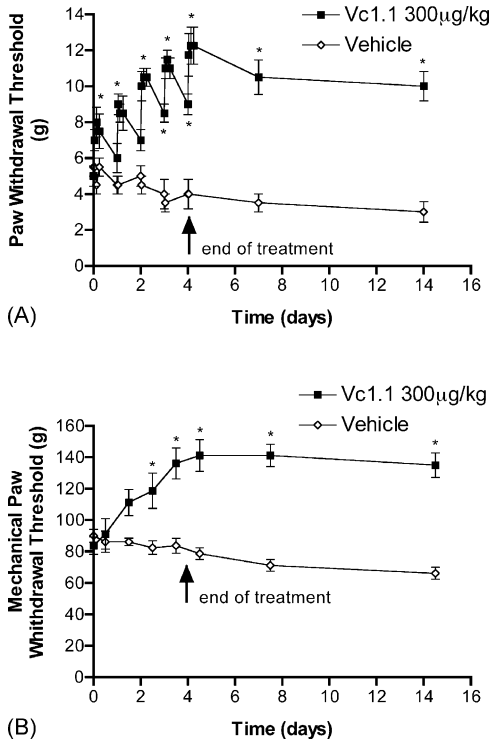

Fig. 2. Vc1.1 produces acute and extended analgesic effects in a rat model of diabetic neuropathic pain. Hyperglycemic rats were produced by treatment with streptozotocin [110]. Six weeks later, rats were treated with s.c. Vc1.1, 300 μg/kg/day for 5 days. (A) *Relief from allodynia*. Mean paw withdrawal thresholds to von Frey filaments applied to the plantar surface of the hindpaw at 1, 3, 6 and 24 h post-injection are shown across treatment days and 3 and 10 days post-Vc1.1 treatment. Note the acute analgesic effect on each treatment day that is partially maintained 24 h post-treatment. Note that analgesia appears cumulative over the 5 days of injection and that analgesia is partially maintained for 10 days following cessation of Vc1.1. (B) *Relief from hyperalgesia*. Hyperalgesia was assessed using an Ugo Basile analgesia meter applied to the dorsal surface of the hind paw. Responses were measured 12 h following each injection. Two measurements were taken between the metatarsals 2 and 3 and metatarsals 4 and 5 and the data averaged. Note that analgesia over treatment days appears cumulative over the 5 days of injection and is partially maintained for 10 days after cessation of Vc1.1. $^{*}p < 0.05$, $n = 4$. Data are provided by Zeinab Khalil and Bruce Livett, Department of Biochemistry and Molecular Biology, The University of Melbourne, Parkville, Victoria 3010, Australia.

GABA-B receptors are members of the G-protein coupled receptor (GPCR) family and are heterodimers composed of B1 and B2 subunits. GABA-B1 is responsible for GABA recognition and GABA-B2 couples to the G-protein. The composite receptor mediates slow synaptic inhibition [52,53]. Thus, from both structural and functional standpoints, GABA-B receptors and nAChRs are quite dissimilar. We are not aware of previous reports of a potent nAChR antagonist also acting as a potent GABA-B receptor agonist. Thus, there is no other literature to evaluate as to what analgesic effects might be expected from a pure α9α10 antagonist vs. a mixed α9α10 antagonist/GABA-B agonist.

In contrast, GABA-B agonists have been well studied. Baclofen, a prototypical GABA-B agonist has been evaluated for over 25 years as an antinociceptive agent. Some characteristics of baclofen-mediated analgesia substantially differ from the analgesia shown by α-conotoxin α9α10 antagonists. First, use of GABA-B agonists leads to tolerance to the antinociceptive effects which limits its use in the clinic [54]. A common property of GPCRs is that following agonist activation, the receptor internalizes or rapidly recycles. Likewise, the state of GABA-B receptor activation affects GABA-B receptor turnover in recombinant cells and neurons [55]. In dorsal root ganglion, activation of GABA-B by baclofen leads to clathrin-dependent internalization and recycling to the plasma membrane [56] (but see [57]). By contrast, use of Vc1.1 and RgIA have thus far not been associated with tolerance (see above).

Second, the primary analgesic effects of baclofen appear to be centrally mediated (see [52] for review). GABA-B receptors are present in the cerebral cortex, thalamus and dorsal horn of the spinal cord consistent with a largely central site of action (though GABA-B receptors also are present in dorsal root ganglia). CNS effects of baclofen include sedation, asthenia and confusion, side effects that have hindered clinical development of other GABA-B agonists [54]. In contrast, α-conotoxins as charged peptides are unlikely to cross the blood–brain-barrier in significant quantity. Thus, the mechanism of action of Vc1.1 and RgIA is almost certainly peripheral. Indeed intrathecal administration of 0.2 nmol/10 μl α-conotoxin RgIA is not analgesic in spinal nerve ligated rats (M. Vincler, unpublished data).

9. Lack of activity of α-conotoxins on cloned GABA-B receptors

We sought to further investigate the effects of α-conotoxins Vc1.1 and RgIA on GABA-B receptors. We first examined the ability of the conotoxins to displace binding of the competitive antagonist [^{3}H]CGP-54626 to GABA-B(1b,2) receptors transiently expressed in HEK cells. Surprisingly, as seen in Fig. 3, K_is for both compounds were greater than 10 μM. This is in contrast to the 1.7 nM IC_{50} GABA-B mediated block of N-type Ca^{2+} channels previously reported in dorsal root ganglion [47]. α-Conotoxins Vc1.1 and RgIA were also previously reported to block N-type Ca^{2+} channels via activation of endogenous *Xenopus* oocytes GABA-B receptors [47]. However, other investigators have failed to detect endogenous GABA-B receptors in oocytes [58]. GABA-B receptors are well known to couple to G-protein-activated inwardly rectifying K^+ (GIRK) channels providing a way to readily observe GABA-function in oocytes [58]. In the report detailing that heterodimerization is required for the formation of functional GABA-B receptors, White and co-workers failed to find GABA-B responses in oocytes that were not injected with both GABA-B(1a) and GABA-B2 subunits, along with GIRK channels. In contrast, large inward currents were seen in 21/21 oocytes injected with both GABA-B(1a) and GABA-B2 along with GIRK [58]. Similar results have been reported by other groups [59–61]. We also failed to detect inward currents in defolliculated oocytes injected with GIRK(1,4) in the absence of co-injection of GABA-B(1b) and GABA-B2 subunits in 10/10 oocytes (data not shown). We next tested α-conotoxins Vc1.1 and RgIA for their ability to activate heterologously expressed human GABA-B receptors in *Xenopus* oocytes. As seen in Fig. 4, both conotoxins

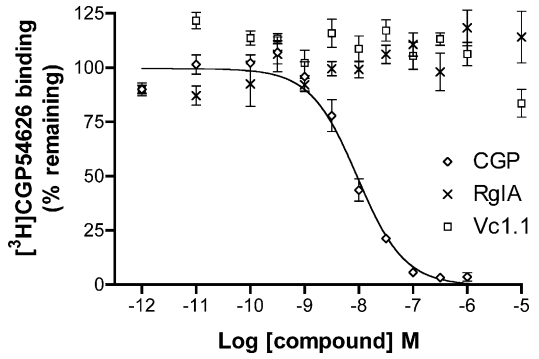

Fig. 3. α-Conotoxins Vc1.1 and RgIA do not potently displace [^{3}H]CGP54626 binding. HEK293T cells were transiently transfected with GABA-B(1b) and GABA-B(2) subunits. α-Conotoxins Vc1.1 and RgIA (concentrations ranging from 10 pM to to 10 μM) were tested for their ability to displace the binding of the competitive antagonist [^{3}H]CGP54626 as described in Section 13. The K_i for both α-conotoxins was greater than 10 μM. Experiments with unlabeled CGP54626 were used to define total and non-specific binding. $n = 3$–6 for each data point. Error bars are S.E.M.

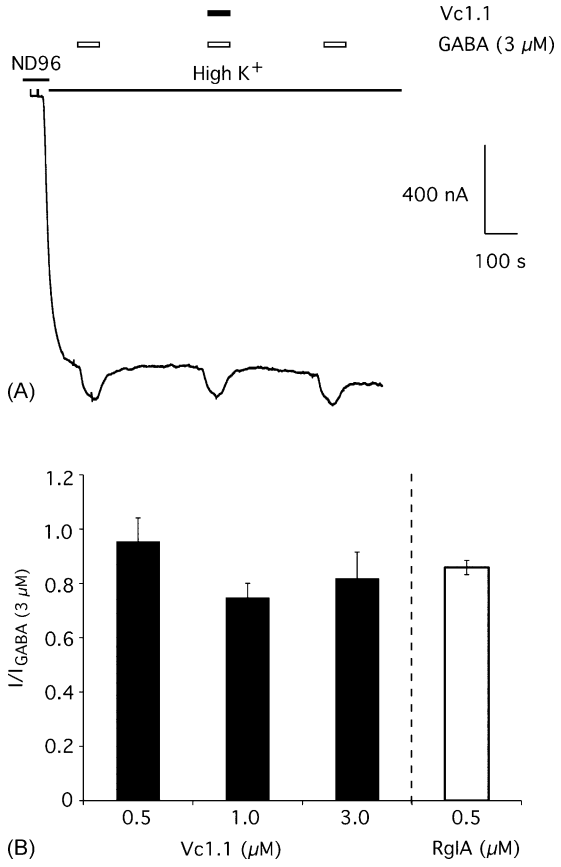

Fig. 4. α-Conotoxins Vc1.1 and RgIA have no effect on GABA-B nAChRs. (A) Oocytes expressing human GABA-B(1b,2) receptors coupled to GIRK1/4 were clamped at −60 mV as described in Section 13. In the presence of GABA (3 μM; duration indicated by open bar), Vc1.1 (0.5 μM; duration indicated by closed bar) had no effect as a positive or negative modulator of GABA (3 μM). (B) Bar graph showing the effect of three concentrations of Vc1.1 (0.5, 1.0 and 3.0 μM) and RgIA (0.5 μM) in the presence GABA (3 μM). There was no significant inhibitory or potentiating effect on the GABA response. Data are the mean ± S.E.M. (n = 3–6 oocytes).

failed to either activate or block GABA-B receptors. Thus, we were unable to demonstrate functional activity of conotoxins on cloned human GABA-B receptors expressed in oocytes. We were also unable to confirm conotoxin activation of endogenous *Xenopus* oocyte GABA-B receptors (because we did not observe any response to GABA-B in the absence of cloned human receptors but in the presence of GIRK channels).

We are unsure how to reconcile the present findings with those of Callaghan et al. There are, however, several differences between the experiments. Callagahan et al. reported agonist effects of conotoxins Vc1.1 and RgIA on rat and *Xenopus* GABA-B receptors. Our binding and functional experiments were carried out with human GABA-B clones. Callaghan et al. assessed GABA-B as a function of block of N-type Ca^{2+} channels. We assessed GABA-B as a function of its ability to activate GIRK channels. In the latter case, one might speculate that the coupling mechanism could differ between GABA-B receptors and GIRK channels vs. GABA-B receptors and N-type Ca^{2+} channels and that RgIA and Vc1.1 activate only GABA-B receptors coupled to N-type Ca^{2+} channels. Such specificity would be most interesting but is, as far as we know, unprecedented. Experiments with co-expressed GABA-B receptors and N-type Ca^{2+} channels should resolve this possibility.

10. Expression pattern of α9 and α10 nAChR subunits

In oocytes, α9 and α10 nAChRs co-assemble to form a receptor. Mutagenesis studies of α9 and α10 subunits indicate that in oocytes, the stoichiometry of the major functional nAChR is α9(2)α10(3) [62]. The molecular composition and subunit stoichiometry of native α9* nAChRs remains to be determined.

The function of native α9 and α10 nAChR subunits is best known in the auditory system. These subunits assemble to form the receptor that mediates synaptic transmission between efferent olivocochlear cholinergic fibers which descend from the brainstem and hair cells of the cochlea [16,17]. For an extended review of this receptor in cochlear hair cells see Elgoyhen et al. this issue. The α9α10 nAChR of outer hair cells inhibits amplification of sound brought about by the active mechanism of these cells [63]. For a short period of time, before the onset of hearing, inner hair cells are also innervated by efferent fibers and the receptor mediating synaptic transmission at this synapse is again the α9α10 nAChR [64–66]. Although, after the onset of hearing, inner hair cells continue to express α9 but not α10, no ACh-mediated responses are observed when assessed by electrophysiological techniques [16,17,66,67]. This result indicates that, in spite of the fact that α9 can form homomeric receptors *in vitro* [16], α10 subunits are required for functional receptors in inner hair cells. Moreover, although in α10 knockout mice a small percentage of outer hair cells exhibit small ACh-mediated responses, most likely due to the activation of α9 homomeric receptors, these remnant cholinergic responses are insufficient to drive normal olivocochlear inhibition of cochlear mechanics [17]. Thus, taken together these results indicate that in the cochlea both α9 and α10 are strictly required in order to assemble into a functional nAChR. Although both α9 and α10 nAChR subunits have been described in the vestibular end organs, the function of the efferent system and of this receptor subtype in these organs is still obscure [16,68–72].

Both α9 and α10 nAChR subunits are expressed in skin [18,73–75]. The non-neuronal cholinergic system of human epidermis includes the keratinocyte ACh axis composed of the enzymes mediating ACh synthesis and degradation, and two classes of ACh receptors, the nicotinic and muscarinic receptors, mediating biological effects of the cutaneous cytotransmitter ACh. Regulation of keratinocyte cell–cell and cell–matrix adhesion is one of the important biological functions of cutaneous ACh [75,76]. A series of nAChR subunits (e.g. α3, α5, α7, β1, β2 and β4) in addition to α9 and α10 are expressed in skin [75]. Inactivation of α9 signaling by pharmacologic antagonism and RNA interference in keratinocyte cultures and null mutation in knockout mice delayed wound reepithelialization *in vitro* and *in vivo*, respectively, and diminished the extent of colony scattering and cell outgrowth from the megacolony. α9-Containing nAChRs seem critical for completion of the very early stages of epithelialization. By activating α9-containing nAChRs, ACh can control the dynamics and strength of cell–cell cohesion, disabling of a trailing uropod and disassembly and reassembly of focal adhesions, thus facilitating crawling locomotion [77]. Moreover, α9 autoantibodies are present in patients with pemphigus vulgaris, a potentially fatal autoimmune mucocutaneous blistering disease [74], thus suggesting the participation of this nAChR subunit in disease. Whether α9 receptors signal as homomers or as α9α10 heteromers in skin has not been established.

Expression sites of α9 and α10 nAChR subunits further include the nasal epithelium and the pars tuberalis of the pituitary gland [16,18], human and rat urothelium [78,79], human and rat placenta [80–83], rat heart [84] and dorsal root ganglia [85–87]. Furthermore, α9 (α10 not investigated) is expressed in retina [88], sperm [89] and olfactory bulb [90], whereas α10 is expressed in arteries [91,92] and rat sympathetic ganglia [93]. An oral epithelial cell line [94] and the NT2-N neuronal phenotype cell line [95] only express α9. At sites where α9 is expressed in the absence of α10, the possibility exists that it signals via the assembly of homomeric α9 nAChRs, as has been reported in *Xenopus* oocytes [16]. However, the functional significance of α10 at sites in which α9 is not

expressed is unknown, since it has been reported that α10 does not form functional ACh-gated channels in pairwise combination with other neuronal nAChR subunits and does not modify the known properties of α7 nAChRs when expressed in *Xenopus laevis* oocytes [17,18]. We note that a recent study has shown that for several nAChRs, the available antibodies are not suited for immunolocalization under commonly used conditions, since staining is similar in wild-type and subunit specific knockout mice [96]. Therefore, caution should be taken when drawing conclusions using α9 and/or α10 nAChR antibodies until their specificity is verified in, for example, their respective knockout mice.

Finally, both α9 and α10 are expressed in a variety of immune cells [97–101]. The α9 and α10 subunits were identified in Jurkat, MT2 and CEM T-cell lines, purified populations of CD3+, CD4+ and CD8+ T-cells, CD19+ and CD80+ B cells, monocytes, macrophages and in tonsil by various techniques including RT-PCR, single cell RT-PCR, Northern and Western blot analysis and immunohistochemistry [97–100]. The expression of these subunits in blood indicates that caution should be taken when concluding expression of these subunits in non-leukocytes based on PCR experiments. The presence of functional cholinergic receptors in leukocytes is expected given that an "extra neuronal" cholinergic system appears operational in these cells. Choline acetyltransferase, an enzyme used to synthesize ACh, has been detected in a variety of immune cells and T-cells have been shown to synthesize ACh [102,103].

11. Immune cell function and chronic pain

The cumulative analgesic and restorative effects of Vc1.1 and RgIA may be due to immunological effects. RgIA and Vc1.1 administration in rats significantly reduces the number of choline acetyltransferase positive lymphocytes and macrophages in the neural and perineural area of chronic constriction nerve sciatic nerve injury [5,6]. Immune cells release inflammatory mediators which produce pain and hyperalgesia. In addition, immune and inflammatory mechanisms are operative in nerve-injury (neuropathic) pain [104,105]. Rats that lack mature T-cells (athymic nude) have reduced mechanical hypersenstivity after nerve injury [106]. Resident macrophages act as sentinels against invasion and inflammatory macrophages are recruited to the site of inflammation after injury. Depletion of macrophages reduces hyperalgesia and Wallerian degeneration after nerve injury [39]. Mast cells may also play a role in neuropathic pain states [107]. Alpha9 and α10 subunits are present in a variety of immune cells (see above) and block of the formed receptor may modulate the response of these immune cells. For a detailed review of the role of immune cells in chronic pain, see [105].

12. Conclusions

α-Conotoxins are nicotinic antagonists that show acute analgesic efficacy, and intriguingly also show an ability to accelerate functional recovery from nerve injury. α-Conotoxins RgIA and Vc1.1 are the only known compounds that selectively block α9α10 vs. other nAChR subtypes. Thus, there is no prior literature on non-conotoxin compounds to compare with the analgesic effects of RgIA and Vc1.1. In contrast, there is a large pre-existing literature on GABA-B agonists and analgesia. Analgesic α9α10 antagonist α-conotoxins do not have a therapeutic or side effect profile that closely matches that of GABA-B agonists. It is conceivable, though, that α-conotoxins act on an unknown allosteric site to activate GABA-B receptors and this leads to an atypical *in vivo* response. Thus far, we have been unable to further study this possibility as experiments to date with cloned GABA-B receptors have not demonstrated GABA-B activation by α-conotoxins Vc1.1 or RgIA. The resolution of the analgesic mechanism of action of RgIA and Vc1.1 must await further studies, perhaps involving novel small molecule α9α10 antagonists or receptor subunit knockout mice. In the mean time, α-conotoxins Vc1.1 and RgIA represent novel chemical entities with unique pharmacological actions and analgesic effects that may serve as prototypes for the development of additional therapeutic agents.

13. Materials and methods

13.1. Competition binding

Binding to GABA-B receptors was assessed by a radioligand competition binding assay using HEK293T cells transiently transfected with 10 μg each of human GABA-B(1b) and GABA-B2 (Origene, Rockville, MD (NM_001470.1 and NM_005458.5)). Reactions contained [^{3}H]CGP54626 (American Radiolabelled Chemicals Inc., St. Louis, MO) (2 nM final), CGP54626 (Tocris, Ellisville, MO) or α-conotoxins at various concentrations (ranging from 10 pM to 10 μM), and membrane fractions in assay buffer (50 mM Tris, pH 7.4, 2.5 mM $CaCl_2$). After a 1.5-h incubation, reactions were harvested onto filtermats (Filtermate A, PerkinElmer, Waltham, MA) using a PerkinElmer Filtermate harvester. Filters were dried using microwave radiation (~30 s per filter), then a scintillant sheet (Meltilex, Perkin-Elmer) was melted onto each. Filtermats with dried scintillant were sealed in sample bags and counted on a Trilux Microbeta counter. Remaining bound radioactivity (in cpm) was normalized, with [^{3}H]CGP54626 binding in the absence of competitor defined as 100% and [^{3}H]CGP54626 binding in the presence of 10 μM unlabeled CGP54626 defined as 0%. Data were analyzed by non-linear regression using the "one-site competition" model built into Graphpad Prism 4.0. K_i values were calculated from IC_{50} values using the Cheng–Prusoff approximation.

13.2. GABA-B receptors expressed in Xenopus oocytes

Human GABA-B(1b), GABAB2 cDNAs and rat G-protein-coupled inwardly rectifying potassium channels (GIRK) 1 and 4 were kindly provided by Dr. Andrew Green (GlaxoSmithKline, Uxbridge, Middlesex, UK). Human GABAB(1b) was encapsulated in the pcDNA3.1(−) (Invitrogen, Carlsbad, CA), GABAB2 and rat GIRK1 were encapsulated in the pcDNA3 (Invitrogen), whereas the rat GIRK4 was encapsulated in pBluescript KS(−) (Stratagene, La Jolla, CA). Human GABAB(1b) and GABAB2 plasmids were linearized using EcoRI. Rat GIRK1 and GIRK4 were linearized using XbaI. For GABAB and GIRK receptor expression, human GABA-B(1b), GABAB2, rat GIRK1 and rat GIRK4 mRNA in the ratio of 1:2:1:1 was used. mRNAs were transcribed *in vitro* using T7 mMessage mMachine™ transcription kit (Ambion Inc., Austin, TX, USA). α-Conotoxins were synthesized as previously described [108].

The experiments were performed with Animal Ethics approvals from The University of Sydney. Female *X. laevis* was anesthetized with tricaine (850 mg/500 mL). Several ovarian lobes were surgically removed by a small incision on the abdomen of the *X. laevis*. The lobes were cut into small pieces and were rinsed thoroughly with oocyte releasing buffer 2 (OR2; 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES (hemi-Na)). The lobes were digested with collagenase A (2 mg/mL in OR2; Boehringer Manheim, Germany) at room temperature. The oocytes were further washed with OR2 and stored in Frog Ringer buffer or ND96 wash solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES (hemisodium salt) supplemented with 2.5 mM sodium pyruvate and 0.5 mM theopylline until ready for injection. Stage V–VI oocytes were selected and microinjected with 2 ng mRNA. After injection, the oocytes were maintained at

18 °C in the presence of ND96 wash solution augmented with 2.5 mM sodium pyruvate, 0.5 mM theopylline and gentamicin at 50 mg/mL.

Whole-cell currents were measured using a two-electrode voltage clamp with a Digidata 1200, Geneclamp 500B amplifier together with a Powerlab/200 (AD Instruments, Sydney, Australia) and Chart version 3.5 for PC as previously described [109]. The recording microelectrodes were filled with 3 M KCl and had resistance between 0.2 and 1 MΩ. Three to 5 days post-injection, oocytes held at −60 mV were used for recording. While recording, oocytes were initially superfused with Frog Ringer (ND96) wash for 5 min before switching to 45 mM K^+ buffer (45 mM NaCl, 45 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES (hemisodium salt)) and cells perfused with this buffer until a stable base current was reached. Vc1.1 (500 nM, 1 μM and 3 μM) or RgIA (0.5 μM) was evaluated in the absence and presence of a submaximal dose of GABA (3 μM), respectively, until maximal current was reached, at which time the oocyte was washed for 5–10 min to allow complete recovery of response to GABA (3 μM).

Conflict of interest statement

JMM has received financial support or served as a consultant for Metabolic, Bristol-Meyers Squibb, Cognetix, GlaxoSmithKline, Mead Johnson, Pfizer and Sandoz. MC has received financial support from Metabolic, Circadian and Pfizer.

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