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REVIEW ARTICLE

α9-containing nicotinic acetylcholine receptors and the modulation of pain

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Neuropathic pain is a complex and debilitating syndrome for which there are few effective pharmacological treatments. Opioid-based medications are initially effective for acute pain, but tolerance to their analgesic effects quickly develops, and long-term use often leads to physical dependence and addiction. Furthermore, neuropathic pain is generally resistant to non-steroidal anti-inflammatory drugs. Other classes of medications including antidepressants, antiepileptics and voltage-gated calcium channel inhibitors are only partially effective in most patients, may be associated with significant side effects and have few disease-modifying effects on the underlying pathology. Medications that act through new mechanisms of action, and particularly ones that have disease-modifying properties, would be highly desirable. In the last decade, a potential new target for the treatment of neuropathic pain has emerged: the α9-containing nicotinic acetylcholine receptor (nAChR). Recent studies indicate that antagonists of α9-containing nAChRs are analgesic in animal models of neuropathic pain. These nerve injury models include chronic constriction injury, partial sciatic nerve ligation, streptozotocin-induced diabetic neuropathy and chemotherapeutic-induced neuropathy. This review details the history and state of the field regarding the role that α9-containing nAChRs may play in neuropathic pain. An alternative hypothesis that α-conotoxins exert their therapeutic effect through blocking N-type calcium channels via activation of $GABA_B$ receptors is also reviewed. Understanding how antagonists of α9-containing nAChRs exert their therapeutic effects may ultimately result in the development of medications that not only treat but also prevent the development of neuropathic pain states.

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Abbreviations

CCI, chronic constriction injury; DRG, dorsal root ganglia; KO, knockout; nAChR, nicotinic ACh receptor; PSNL, partial sciatic nerve ligation; VGCC, voltage-gated calcium channel; WT, wild type; α-Ctx, α-conotoxin

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Introduction

Compounds that target **nicotinic ACh receptors (nAChRs)** have been investigated for some time as analgesics, and almost all have been agonists or positive allosteric modulators of α4β2 or α7 nAChRs (Umana *et al.*, 2013; Uteshev, 2014; Dineley *et al.*, 2015). Although ligands that activate **α7 nAChRs** are hypothesized to be analgesic and anti-inflammatory (de Jonge and Ulloa, 2007; Medhurst *et al.*, 2008; Feuerbach *et al.*, 2009), positive findings with α7 agonists have not always been observed (Wang *et al.*, 2005; Freitas *et al.*, 2015). The α7- and **α9-containing subtypes** share a similar pharmacological profile, and therefore, one potential complication is that agonists of α7 may not be selective but instead may also activate α9α10 nAChRs. For example, the prototypical agonist of α7 nAChRs, choline, also activates α9-containing subtypes (Verbitsky *et al.*, 2000; Sgard *et al.*, 2002). Intriguingly, new data indicate that nAChR antagonists may also be analgesic, including those that target α9α10 nAChRs (Vincler *et al.*, 2006; Vincler and McIntosh, 2007; Del Bufalo *et al.*, 2014). Accordingly, stimulation of α7 or inhibition of α9α10 nAChRs may produce analgesia and thus agonists that do not discriminate between α7 and α9α10 may have mutually counteracting effects. Studies using such agonists may underestimate potential beneficial effects. Therefore, the functional activity and subtype selectivity of candidate therapeutic compounds may be critical.

nAChRs containing the α9 subunit are a recently identified subtype (Elgoyhen *et al.*, 1994). The first demonstration of a physiological role for α9-containing nAChRs was described in studies of the cochlea where they are expressed in hair cells and are functionally coupled to calcium-activated potassium channels (Vetter *et al.*, 1999; Glowatzki and Fuchs, 2000; Nie *et al.*, 2004). Shortly after the discovery of α9, an additional nicotinic subunit was identified that is now known as α10 (Elgoyhen *et al.*, 2001; Lustig *et al.*, 2001; Sgard *et al.*, 2002). These two nicotinic subunits share a high sequence homology, and their expression patterns overlap considerably. However, in contrast to the α9 subunit, mammalian α10 subunits have not been shown to form functional receptors by themselves but do assemble with α9 subunits to form α9α10 heteromers (Elgoyhen *et al.*, 2001; Sgard *et al.*, 2002).

The presence of α9α10 nAChRs in immune cells has led to the hypothesis that this subtype may be involved in immunological responses. Evidence for such a role has been documented in studies using experimental models of the autoimmune diseases *Pemphigus vulgaris* (Nguyen *et al.*, 2000) and multiple sclerosis (Simard *et al.*, 2013). Curiously, α9α10 nAChRs in immune cells do not appear to function as ionotropic receptors (Peng *et al.*, 2004; Hecker *et al.*, 2015; Richter *et al.*, 2016) in contrast to their known functions in cochlear hair cells. Additionally, some experiments have reported the presence of transcripts for α9 and α10 subunits in rat DRG neurons, and it has been suggested that the expression of α9α10 nAChRs may be a universal feature of all DRG neurons (Lips *et al.*, 2002). However, as in studies of immune cells, patch clamp electrophysiology experiments using cultured DRG neurons have failed to detect currents that could be attributed to α9α10 nAChRs (Rau *et al.*, 2005; Hone *et al.*, 2012). Transcripts for **α10 nAChR** subunits are consistently reported in DRG neurons, but detection of transcripts for α9 subunits is highly variable and, in some cases, absent (Lips *et al.*, 2002; Haberberger *et al.*, 2004; Lips *et al.*, 2006; Callaghan and Adams, 2010; Hone *et al.*, 2012). The significance of α10 subunit expression in the absence of α9 subunits is currently unknown, and therefore, whether or not DRG neurons express α9α10 nAChRs remains unclear. However, one possibility is that a receptor composed of α7 and α10 subunits may be present as reported in sympathetic neurons (Lips *et al.*, 2006).

Discovery of the analgesic α-conotoxin Vc1.1

A PCR screen of mRNAs present in the venom duct of the marine snail *Conus victoriae* identified a sequence encoding a 17 amino acid peptide belonging to a conopeptide subclass called α-conotoxins (α-Ctxs) (Sandall *et al.*, 2003). This α-Ctx, Vc1.1, was shown to be an antagonist of native α3β4*AChRs (asterisk denotes the possible presence of additional subunits) expressed in bovine adrenal chromaffin cells and most potently inhibited heterologously expressed rat **α3-containing nAChRs**, relative to other subtypes tested (Clark *et al.*, 2006). Groundbreaking functional studies in rat models of neuropathic pain demonstrated that Vc1.1 was capable of reversing allodynia and accelerating the recovery of sensory nerves (Satkunanathan *et al.*, 2005). Vc1.1 was also shown to reduce the excitability of human sural nerves via antagonism of α3β4* nAChRs (Lang *et al.*, 2005). It is notable, however, that the concentrations required for inhibition of α3β4 nAChRs in these and other studies were in the μM range (Sandall *et al.*, 2003; Clark *et al.*, 2006), concentrations that are unlikely to be sustained during extended *in vivo* animal studies at the dosages used. This suggested that a molecular target other than α3-containing nAChRs was responsible for the effects observed in rodent models of neuropathic pain. Subsequent studies examining the nAChR subtype selectivity of Vc1.1 revealed that rat α9α10 nAChRs heterologously expressed in oocytes were inhibited at concentrations >2000-fold lower than the α3β4 subtype (Vincler *et al.*, 2006). However, although the higher potency of Vc1.1 for inhibition of α9α10 nAChRs suggested that it could be a molecular target involved in the analgesic effects of Vc1.1, the lack of high nAChR subtype specificity prevented unequivocal assignment of the analgesic effects to the α9α10 subtype.

α-Conotoxins RgIA and Vc1.1 are analgesic in rodent models of neuropathic pain

Advances in molecular cloning strategies rapidly accelerated the discovery of novel peptides from cone snails, and in 2006, a sequence isolated from a genomic DNA library from *Conus regius* was described that encoded an α-Ctx now called **RgIA** (Ellison *et al.*, 2006). Importantly, RgIA was shown to be >1000-fold more potent at inhibiting α9α10 than other nAChR subtypes, and its discovery offered the possibility of selectively studying the role of α9α10 nAChRs in neuropathic pain.

The discovery of RgIA led to the possibility of testing the hypothesis that antagonism of α9α10 nAChRs produced analgesic effects. In a side-by-side comparison of the activities of RgIA and Vc1.1 in rat, both peptides ameliorated neuropathic pain (Vincler *et al.*, 2006). Daily administration of RgIA and Vc1.1 in rats subjected to chronic constriction injury (CCI) dose-dependently inhibited mechanical hypersensitivity. Remarkably, these peptides also reduced the accumulation of immune cells at the site of injury. These studies confirmed and extended previous findings that Vc1.1 possessed analgesic properties (Satkunanathan *et al.*, 2005) and were the first to suggest α9α10 nAChRs as a molecular target for treatment of neuropathic pain. RgIA was subsequently shown to prevent sciatic nerve damage when given to rats following CCI (Di Cesare Mannelli *et al.*, 2014).

α-Conotoxin RgIA is analgesic in oxaliplatin-induced peripheral neuropathy

Oxaliplatin is an anticancer drug used in chemotherapy treatments of certain colorectal, ovarian and pancreatic cancers. A severe complication accompanying the use of this drug is the development of chemotherapy-induced peripheral neuropathy. Currently, there are no effective treatments for the prevention of this type of neuropathic pain. Oxaliplatin and other chemotherapeutics are used in animal models of peripheral neuropathy (Flatters and Bennett, 2004; Xiao *et al.*, 2012). Rats administered oxaliplatin show evidence of peripheral neuropathy indicated by hypersensitivity to mechanical stimuli and a decreased tolerance to non-noxious cold. Oxaliplatin-based drugs have also been shown to produce morphological changes in DRG neurons similar to those observed in the DRG of rats subjected to CCI. In this model, daily intramuscular administration of 3.1 or 15.7 μg of RgIA in rats increased the pain threshold back to control levels and significantly reduced oxaliplatin-induced sensitivity to non-noxious stimuli (Pacini *et al.*, 2016). Additionally, pathological changes in the morphological characteristics of DRG neurons were reduced by administration of RgIA.

Development of an RgIA analogue with high potency at human α9α10 nAChRs

Although RgIA is effective in rodent models of neuropathic pain, its utility in humans is likely to be limited due to the fact that it is orders of magnitude less potent on human compared to rat α9α10 nAChRs (Azam and McIntosh, 2012). However, one advantage of using α-Ctx peptides as a platform for ligand development is that analogues can be generated with increased potency and specificity for particular receptor subtypes of interest. This can be accomplished through various techniques including systematically mutating residues, replacing cysteine residues with non-standard amino acids such as selenocysteine, replacing the sulfur bridges between cysteines with dicarba bridges and by backbone cyclization (McIntosh *et al.*, 2004; Halai *et al.*, 2009; 2011; Clark *et al.*, 2010; Carstens *et al.*, 2011; 2016a,b; Daly *et al.*, 2011; Hone *et al.*, 2013; Chhabra *et al.*, 2014; Yu *et al.*, 2015). A structure–activity relationship study was conducted with RgIA to generate analogues with increased potency for human α9α10 nAChRs. These experiments generated a number of analogues that displayed ~10 000-fold increase in potency for the human receptor (Romero *et al.*, 2017). One of these analogues, RgIA4, was further tested for activity on a panel of receptors, ion channels and transporters and was found to have relatively very low or no activity on non-α9α10 nAChR targets. RgIA4 was also demonstrated to be active in animal models of neuropathic pain. *In vivo* testing in the oxaliplatin model of peripheral neuropathy showed that daily injections of RgIA4 prevented the expression of cold allodynia and mechanical hyperalgesia in rats. RgIA4 also prevented the expression of oxaliplatin-induced cold allodynia in wild-type (WT) (CBA/CaJ) mice but did not attenuate neuropathic pain behaviours in CHRNA9 gene knockout (α9 KO) mice, indicating that α9-containing nAChRs were necessary for the therapeutic effects of RgIA4.

CHRNA9 KO mice show attenuated pain expression in traumatic, inflammatory and chemotherapy-induced pain models

In addition to pharmacological studies demonstrating that antagonism of α9α10 nAChRs reduces symptoms of neuropathic pain and neuropathy induced by physical or chemical nerve injury, studies in α9 KO mice have implicated α9-containing nAChRs in the involvement of neuropathic pain. In a recent study by Mohammadi and Christie (2014), WT (129Sv/Ae) and α9 KO mice were subjected to CCI to model neuropathic pain or injected in the hind paw with complete Freund's adjuvant (CFA) to model inflammatory pain. α9 KO mice displayed normal responses to noxious mechanical and thermal stimuli. Chronic cold mechanical allodynia also developed normally in both models. However, α9 KO mice showed a distinct phenotype in the development of mechanical hyperalgesia. WT mice subjected to CCI continued to display elevated mechanical hyperalgesia 21 days after injury, whereas α9 KO mice showed substantially reduced hyperalgesia by day 14 following CCI surgery. Furthermore, the overall magnitude of hyperalgesia was significantly blunted in α9 KO mice in both CCI and CFA models (Mohammadi and Christie, 2014). Overall, however, the phenotype exhibited by the α9 KO mice significantly differed from the analgesic effects observed after injection of Vc1.1 or RgIA calling into question the mechanistic importance of the α9 subunit (Mohammadi and Christie, 2015).

A recent report utilizing this same mouse strain (129Sv/Ev) also showed behavioural differences in α9 subunit null mice. These alterations included differences in stress-induced arousal, measures of anhedonia- and anxiety-like behaviour, circadian patterns of activity and corticosterone responses (Mohammadi *et al.*, 2017). Such changes could affect pain-related behaviour. Functional α9α10 nAChRs have been demonstrated in rat adrenal medulla, and transcripts have been reported in the pituitary gland (Elgoyhen *et al.*, 1994;

Colomer *et al.*, 2010). Therefore, differences in the hypothalamic–pituitary–adrenal axis may underlie such effects. Behavioural changes were not reported in human clinical trials of the α9α10 nAChR antagonist Vc1.1 (AdisInsight, 2016). However, decreased potency of Vc1.1 for the human α9α10 nAChR could have obscured potential findings. Separate studies, in a different strain of α9 KO mice (CBA/CaJ), have been conducted in the oxaliplatin model of peripheral neuropathy. Oxaliplatin produced robust cold allodynia in WT mice, but the expression of cold-allodynia in α9 KO mice was attenuated in this strain in both magnitude and symptom duration (Romero *et al.*, 2017). By 72 h post oxaliplatin injection, WT mice, but not α9 KO mice, exhibited pronounced cold allodynia.

Together, these studies suggest that germline deletion of α9-containing nAChRs *per se* can attenuate the development and progression of some aspects of neuropathic pain. However, in studies of germline-deleted genes, developmental compensation often alters aspects of the mutant mouse phenotype compared with acute pharmacological silencing of function in adult WT animals. Therefore, a close phenotypic correlation between KO mice and acute pharmacological treatment in WT animals may or may not occur.

Potential disease-modifying effects

A particularly interesting aspect of the compounds discussed in this review is their capacity to produce long-lasting effects. Pivotal work by Khalil and colleagues demonstrated that administration of Vc1.1 not only produced analgesia but also produced long-lasting effects (Satkunanathan *et al.*, 2005). Vc1.1 attenuated mechanical hyperalgesia after CCI or partial sciatic nerve ligation (PSNL), and these analgesic effects persisted for 1 week (last time point measured) following cessation of treatment. In addition, functional recovery of the sciatic nerve has been demonstrated (normalization of vascular response to substance P). Long-lasting reductions in pain responses produced by α-Ctxs have been measured in subsequent studies. Preservation of nerve morphology after CCI was observed and is consistent with disease-modifying effects (Di Cesare Mannelli *et al.*, 2014). Remarkably, the apparent disease-modifying effects of α9α10 antagonists are not limited to traumatic nerve injury. Vc1.1 has also been shown to attenuate established neuropathic pain in streptozotocin-induced diabetic rats. In these studies, Vc1.1 produced a progressive improvement in tactile allodynia and mechanical hyperalgesia that persisted for 10 days (last time points measured) after cessation of drug treatment (Metabolic, 2006; McIntosh *et al.*, 2009). In addition, α9α10 antagonists have been shown to prevent chemotherapy-induced neuropathic pain (Wala *et al.*, 2012; Pacini *et al.*, 2016; Romero *et al.*, 2017). The effects of RgIA4 lasted 72 h post injection (last time point examined), a notable result given that RgIA4 is a peptide (Romero *et al.*, 2017). A common drawback of peptides as drug candidates is their susceptibility to degradation by proteolytic enzymes. Peptide pharmacokinetics are typified by short bloodstream half-lives, often in the order of min, leading to relatively brief biological effects (Diao and Meibohm, 2013). Vc1.1 and RgIA4 have plasma half-lives of 0.5–3 h and <20 min respectively (Metabolic, 2006; Mercado *et al.*, 2014). Thus, the pharmacokinetic profiles of Vc1.1 and RgIA are consistent with the time course of the acute pharmacodynamic effect observed but do not fit with the extended pharmacodynamic effects observed in animal studies, which suggest potential restorative properties. The long-term effects on neuropathic pain produced by Vc1.1 and RgIA might be influenced by acute modulation of immune cell infiltration into the site of injury and the release of inflammatory substances by these cells. Evidence for the acute modulatory effects of α-Ctxs has been observed in the CFA-induced inflammatory pain model where a single dose of Vc1.1 attenuated mechanical hyperalgesia albeit only at a high dose of 2.4 $mg{\cdot}kg^{-1}$ (Metabolic, 2006). These results suggest that Vc1.1 may be less effective in altering CFA-induced cutaneous inflammation than in modifying nerve injury-induced immune responses. The respective immune mediated pathways for inflammatory and neuropathic pain remain active areas of investigation (Pavlov *et al.*, 2003; Ren and Dubner, 2010; McMahon *et al.*, 2015) but probably differ in key elements that might explain the differences in α-Ctx potency in the different pain models.

Rat sciatic nerves subjected to CCI show a number of morphological changes that indicate Wallerian degeneration including oedema, axon demyelination and degeneration, and also show increased infiltration of immune cells into the site of injury (Basbaum *et al.*, 1991; Nuytten *et al.*, 1992; Sommer *et al.*, 1995). Additionally, the cell bodies of these axons, located in the DRG, show signs of damage including oedema and eccentrically located nucleoli. Animals subjected to CCI and treated with RgIA showed largely preserved nerve morphology including a higher number of nerve fibres, increased myelin thickness and axon diameter, and a general reduction in oedema compared with control animals receiving injections of vehicle alone (Di Cesare Mannelli *et al.*, 2014). Furthermore, a significant reduction in the number of activated macrophages (CD86+) was observed in the nerve. Both Vc1.1 and RgIA have been shown to reduce immune cell infiltration into CCI-injured nerves (Vincler *et al.*, 2006). In the oxaliplatin model of peripheral neuropathy, RgIA reduced oxaliplatin-induced morphological changes in nucleoli of DRG neurons. CNS glial cells have been shown to modulate pain. RgIA reduced the number of microglial cells (Iba+) present in the dorsal horn of the spinal column of oxaliplatin-treated rats; RgIA alone increased microglia in brain (Pacini *et al.*, 2016). Additional studies are needed to further elucidate the mechanism of how Vc1.1 and RgIA produce these disease-modifying effects.

Expression of α9α10 nAChRs in immune cells

Accumulating evidence indicates that immune cells play an important role in the development of neuropathic pain following injury to nerves (Grace *et al.*, 2014; Ji *et al.*, 2016; Peng *et al.*, 2016). α9 and α10 subunits have consistently been reported to be expressed by a variety of immune cells (Peng *et al.*, 2004; Biallas *et al.*, 2007; Chernyavsky *et al.*, 2010; Koval *et al.*, 2011; Hecker *et al.*, 2015; Richter *et al.*, 2016). In nerve injury models of neuropathic pain, immune cells

infiltrate the damaged nerve where they release a variety of cytokines, chemokines and growth factors which may promote inflammation and sensitize nerves to nociceptive stimuli (Leskovar *et al.*, 2000; George *et al.*, 2004; Hendriks *et al.*, 2005). A reduction in immune cell infiltrate into nerves is associated with attenuated inflammation as well as disease-modifying effects including delayed or reduced Wallerian degeneration (Liu *et al.*, 2000). Additionally, athymic nude rats that lack the ability to produce mature T-lymphocytes show significantly attenuated neuropathic pain symptoms following CCI injury (Moalem *et al.*, 2004). Vc1.1 and RgIA have been demonstrated to reduce the number of immune cells infiltrating into sciatic nerves and DRG that have been subjected to CCI or PSNL injuries (Vincler *et al.*, 2006; Di Cesare Mannelli *et al.*, 2014). These cell types include ED1+ and CD86+ macrophages, CD2+ lymphocytes and cells positive for choline acetyltransferase (Vincler *et al.*, 2006). Collectively, these observations support the possibility that antagonism of α9α10 nAChRs expressed by immune cells may be a mechanism through which α9α10 nAChR antagonists exert their analgesic and anti-inflammatory properties. However, the function of α9α10 nAChRs in immune cells has been difficult to demonstrate. Patch clamp studies have failed to detect nAChR-mediated currents in these cells (Peng *et al.*, 2004; Hecker *et al.*, 2015). Recently, however, function for immune cell α9α10* nAChRs has been convincingly demonstrated. Stimulation of purine **P2X receptors** in the human monocyte cell line U937 leads to the release of the cytokine **IL-1β**, and nAChR agonists inhibit this release (Hecker *et al.*, 2015; Richter *et al.*, 2016). In these studies, a combination of tools including siRNA, the α9α10 antagonist RgIA4, and α9 or α10 nAChR subunit KO mice indicated that an α9α10* nAChR modulates P2X receptor function and cytokine release in these cells. Interestingly, the pharmacology and biophysical properties of these monocyte-expressed α9α10 nAChRs is distinct from that of heterologously expressed α9α10 nAChRs or those present in cochlear hair cells. In U937 cells, stimulation with nicotinic agonists does not evoke ionic current. Furthermore, nicotine appears to behave as an agonist of α9α10* nAChRs in contrast to the known activity of this ligand in other expression systems (Elgoyhen *et al.*, 1994; Elgoyhen *et al.*, 2001). It is currently not known whether these features of monocyte-expressed α9α10 nAChRs are generalizable to other immune cells. Additional studies examining the functional role of α9α10 nAChRs in other immune cells and in particular those that invade damaged neural tissue during injury are needed to better understand the role of immune cell-expressed α9α10 nAChRs in neuropathic pain.

An alternative hypothesis for the mechanism of α-contoxins and their analogues

Several studies of a subset of α-Ctxs have postulated the analgesic mechanism of action to be dependent on activation of G-protein coupled **$GABA_B$ receptors** (Callaghan *et al.*, 2008; Callaghan and Adams, 2010; Klimis *et al.*, 2011; Cuny *et al.*, 2012; van Lierop *et al.*, 2013; Berecki *et al.*, 2014; Huynh *et al.*, 2015; Castro *et al.*, 2017). Studies examining the activity profile of Vc1.1 and its analogues raised questions as to whether α9α10 nAChRs were the actual target that mediated the analgesic effects of Vc1.1. Vc1.1 is a synthetic version of the natural peptide found in *Conus victoriae*. The natural peptide (vc1a, also known as Vc1.1 ptm) contains two post-translational modifications not present in synthetic Vc1.1 (hydroxyproline at position 6 and γ-carboxyglutamate at position 14). When tested on rat α9α10 nAChRs expressed in oocytes, Vc1.1, the native peptide vc1a and the two Vc1.1 analogues Vc1.1(P6O) and Vc1.1(E14γ) each inhibited ACh-induced responses with similar potencies (Nevin *et al.*, 2007). However, when tested at high doses (60 μg per rat) for their ability to produce analgesia in the PSNL model of neuropathic pain in rats, only Vc1.1 showed effects on mechanical allodynia. These results suggested that activity at α9α10 nAChRs was not necessary for the analgesic effects of Vc1.1 and are consistent with those of Livett *et al.*, who also reported decreased or a lack of effects of vc1a on mechanical thresholds in CCI rats after low-dose (0.37 μg per rat) administration. In contrast, low doses of both Vc1.1 (0.36 μg per animal) and vc1a (0.37 μg per animal) accelerated the functional recovery of injured sensory nerves (Livett *et al.*, 2008). This was demonstrated using an assay that measured the ability of injured sciatic nerve terminals to produce an inflammatory vascular response following exposure to substance P. Vc1.1 showed an 83% recovery compared with controls, while vc1a showed an 85% recovery (Satkunanathan *et al.*, 2005; Livett *et al.*, 2008). Thus, in this vascular response assay, activity at $GABA_B$ receptors was not necessary for effect since vc1a lacks $GABA_B$ receptor agonist activity (Callaghan *et al.*, 2008).

Voltage gated calcium channels (VGCCs) are present in sensory neurons of DRG and are functionally coupled to $GABA_B$ receptors. Activation of $GABA_B$ receptors by the agonist **baclofen** has been shown to inhibit the **$Ca_v2.2$** VGCC subtype via G-protein coupled mechanisms. This inhibition in turn decreases neurotransmitter release and synaptic transmission between DRG neurons and second-order neurons in the spinal column and thus impairs the transmission of nociceptive information to the brain. Evidence supporting the agonist activity of α-Ctxs on $GABA_B$ receptors comes from studies examining their effects on VGCC currents in cultured DRG neurons. In an initial study by Callaghan *et al.*, Vc1.1 and RgIA were shown to be capable of inhibiting VGCC currents in DRG neurons, an effect that was prevented by competitive $GABA_B$ receptor antagonists (Callaghan *et al.*, 2008). These inhibitory effects on VGCC currents were not observed when vc1a, Vc1.1(P6O), or Vc1.1(E14γ) were tested in the same assays. Inhibition of $Ca_v2.2$ channel currents by Vc1.1 could also be prevented by inclusion of a tyrosine kinase inhibitor in the patch pipette or by pertussis toxin, suggesting a G-protein coupled mechanism of VGCC inhibition. Vc1.1 also lacked direct inhibitory activity on VGCCs heterologously expressed in oocytes. Thus, it is possible that acute analgesic effects of some α-Ctxs are mediated by activation of $GABA_B$ receptors. In further support of this mechanism, three α-Ctxs (Vc1.1, **AuIB** and **MII**) with dissimilar pharmacological profiles were compared. α-Ctx Vc1.1 has 1.5 nM potency for partial inhibition of VGCC currents in DRG and 19–64 nM potency for α9α10 nAChRs (see Klimis *et al.*,

2011, and the references therein). Vc1.1 potently reversed mechanical allodynia (rat PSNL model), an effect that was reversed by the $GABA_B$ receptor antagonist **SCH-50911**. AuIB displayed 1.5 nM potency for partial inhibition of VGCC currents in DRG but did not block α9α10 nAChRs expressed in oocytes. AuIB potently reversed mechanical allodynia with an IC_{50} of 1.88 (0.04–8.75) μg, indicating that activity at α9α10 nAChRs was not necessary for this analgesic effect (Klimis *et al.*, 2011). AuIB also inhibits α3β4 (IC_{50} 750 nM) and α6β4 (IC_{50} 7.3 μM) nAChRs, subtypes known to be expressed by DRG neurons (Genzen *et al.*, 2001; Rau *et al.*, 2005; Hone *et al.*, 2012; Smith *et al.*, 2013). However, it seems unlikely that the high nM to low μM concentrations required for inhibition of α3β4 or α6β4 subtypes would be sustained in order to produce analgesia *in vivo*. Surprisingly, MII, which blocks α3β2 and α6-containing nAChRs but does not block α9α10 nAChRs or activate $GABA_B$ receptors, was also analgesic, though the IC_{50}, 9.2 (2.7–30.5) μg, was lower (but note overlapping confidence intervals with IC_{50} for AuIB) and the effects were not significant beyond 4 h. These results indicate that for MII, a mechanism other than activity at α9α10 nAChRs or $GABA_B$ receptors may also produce analgesic effects.

Multiple structure–function studies have been undertaken to further understand the selectivity of α-Ctxs for $GABA_B$ receptors versus α9α10 nAChRs and derive significantly improved ligands (Halai *et al.*, 2009; Carstens *et al.*, 2011; 2016b Daly *et al.*, 2011; Halai *et al.*, 2011; Yu *et al.*, 2015). For instance, dicarba analogues of Vc1.1 show different activity for these receptors (van Lierop *et al.*, 2013). Synthetic cyclization was used to produce the analogue cVc1.1 that retains high potency for inhibiting VGCC currents and is orally active in the CCI model (Clark *et al.*, 2010). In a separate structure–function study, a core peptide motif for inhibiting VGCC currents via $GABA_B$ receptors was identified that enabled development of the Vc1.1 analogue [Ser3]Vc1.1 (1–8) (Carstens *et al.*, 2016b). [Ser3]Vc1.1(1–8) inhibited VGCC currents in rat and mouse DRG neurons, an effect prevented by the $GABA_B$ receptor antagonist **CGP55845**. This analogue failed to inhibit human α9α10 nAChRs at 1 μM yet fully blocked human α7 nAChRs at this same concentration. In a mouse model of visceral pain, intracolonic [Ser3]Vc1.1(1–8) reduced the visceromotor response to colorectal distension (Carstens *et al.*, 2016a), further supporting the idea that $GABA_B$ receptors rather than α9α10 nAChRs produce an analgesic effect. As the parent peptide Vc1.1 is known to be much more potent on rat versus human α9α10 nAChRs, it would be of interest to determine the activity of [Ser3]Vc1.1 (1–8) on rat α9α10 nAChRs.

Separately, studies with conditional KO mice call into question the importance of peripheral versus CNS $GABA_B$ receptors in neuropathic pain. The **$GABA_{B1}$** subunit is required for $GABA_B$ receptor function. Conditional KO mice that specifically lack the $GABA_{B1}$ subunit in sensory nociceptors in DRG but retain $GABA_{B1}$ in spinal cord and brain were generated. Notably, neuropathic pain (spared nerve injury model) developed normally in these animals (Gangadharan *et al.*, 2009), and thus, the pain phenotype seems to differ from that seen after administration of putative α-Ctx $GABA_B$ receptor agonists in WT mice. Therefore, it is unclear whether α-Ctxs agonism of the $GABA_B$ receptors expressed by nociceptive DRG neurons would alone be sufficient for the attenuation of neuropathic pain.

Lastly, although some antagonists of $GABA_B$ receptors prevent α-Ctx inhibition of VGCCs, competition binding studies with Vc1.1 and RgIA failed to demonstrate displacement of the competitive $GABA_B$ receptor antagonist [^{3}H]-CGP54626 (McIntosh *et al.*, 2009). Subsequently, it was proposed that Vc1.1 acts at an allosteric site on the $GABA_B$ receptors to produce agonist activity (Adams *et al.*, 2012), and receptor mutation studies are consistent with this mechanism (Huynh *et al.*, 2015).

A separate difficulty with the $GABA_B$ receptor hypothesis is that the functional effects of the clinically available $GABA_B$ receptor agonist **baclofen** do not closely resemble the effects of α-Ctxs. Baclofen has shown limited efficacy in the treatment of neuropathic pain (Loubser and Akman, 1996). In addition, the effects that are observed are thought to be centrally mediated (Bowery, 2006; Goudet *et al.*, 2009) (necessitating intrathecal administration in humans). In contrast, α-Ctxs are effective following peripheral administration; if they were acting on $GABA_B$ receptors, the tissue site would probably be peripheral, since they are unlikely to efficiently cross the blood-brain-barrier. Furthermore, tolerance to baclofen's therapeutic effects is problematic. In contrast, tolerance to the therapeutic effects of α-Ctxs has not been observed.

Variable agonist effects of α-conotoxins on $GABA_B$ receptors

α-Ctxs Vc1.1 and RgIA have been reported to produce inhibition of VGCCs in rat DRG sensory neurons (Figure 1A, B) (Callaghan *et al.*, 2008; Callaghan and Adams, 2010; Klimis *et al.*, 2011). However, for no obvious reasons, agonist activity at $GABA_B$ receptors has not been observed in a variety of other experimental conditions. In the work by Wright *et al.* (2015), RgIA failed to inhibit VGCC currents in DRG neurons (Wright *et al.*, 2015). Vc1.1, however, produced a variable (on average, small) and reversible inhibition of VGCC currents in a minority of DRG neurons. The magnitude of VGCC current inhibition by Vc1.1 did not correlate with the inhibition produced by baclofen, making it unlikely that the observed effect was mediated by $GABA_B$ receptors. Furthermore, Vc1.1 has also been shown to lack effects on excitatory post-synaptic currents (EPSCs) in spinal cord neurons (Napier *et al.*, 2012). Dorsal horn neurons are excited by glutamate released from DRG afferents that express $GABA_B$ receptors. Activation of presynaptic $GABA_B$ receptors by baclofen inhibits EPSCs and prevents the release of glutamate and, consequently, transmission of nociceptive information from the periphery to the brain. In contrast to the results obtained with baclofen, Vc1.1 failed to inhibit these EPSCs, suggesting a lack of activity on $GABA_B$ receptors (Figure 1C, D). It is difficult to reconcile these findings with the hypothesis that α-Ctxs produce analgesia via inhibition of DRG neuron-expressed VGCCs when synaptic transmission at this synapse is unaffected by Vc1.1.

Agonism of $GABA_B$ receptors is known to produce activation of **G-protein coupled inwardly rectifying potassium channels (K_{ir} also known as GIRK)**

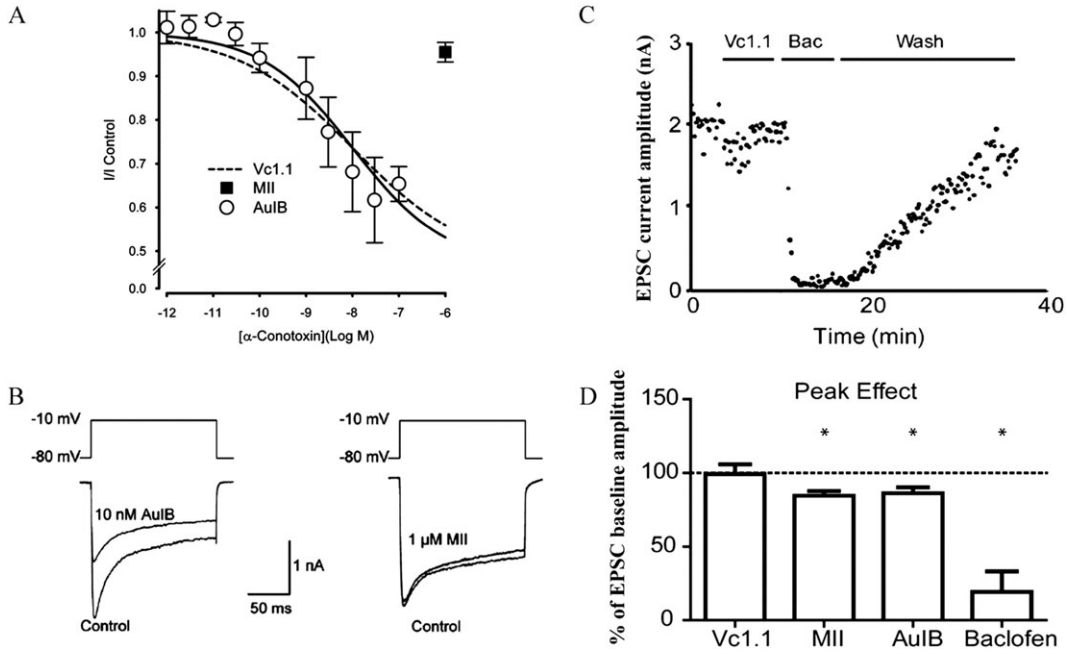

Figure 1

Effects of analgesic α-Ctxs on VGCC currents in DRG neurons and EPSCs in spinal cord neurons. An alternative hypothesis is that α-Ctxs exert their analgesic effects by inhibition of VGCCs via activation of $GABA_B$ receptors rather than by inhibition of α9α10 nAChRs. (A and B) Examples of experiments that demonstrate α-Ctx activity on $GABA_B$ receptors, whereas (C) and (D) show experiments demonstrating a lack of α-Ctx activity on $GABA_B$ receptors. (A) Concentration–response relationship for inhibition of VGCC currents by Vc1.1, AuIB and MII in cultured rat DRG neurons (figures used with permission of Klimis *et al.*, 2011). Similar activity has been reported for analogues of Vc1.1 and for RgIA (see text for full discussion and references). One study did not replicate key findings for Vc1.1 or RgIA in DRG neurons (Wright *et al.*, 2015) (data not shown). (B) AuIB, but not MII, inhibits VGCC currents in DRG neurons. These findings support the hypothesis that the analgesic effects of some α-Ctxs are mediated through activation of $GABA_B$ receptors. However, Vc1.1 failed to inhibit EPSCs in spinal cord neurons via activation of presynaptic $GABA_B$ receptors on DRG afferents. (C) Electrophysiological recordings from rat spinal cord neurons in a slice preparation showing the effect of Vc1.1 and baclofen (Bac) on EPSC amplitudes. (D) Histogram of EPSCs in the presence of α-Ctxs or baclofen compared with baseline controls. Note lack of effect of Vc1.1 and small inhibitory effects of AuIB and MII (the latter attributed to block of α3-containing nAChRs). The $GABA_B$ receptor agonist baclofen is shown as a positive control (figures used with permission, Napier *et al.*, 2012).

via G protein-coupled mechanisms (White *et al.*, 1998). However, GABA, but neither Vc1.1 nor RgIA, activated heterologously expressed $GABA_B$ receptors coupled to K_{ir} channels (McIntosh *et al.*, 2009; Huynh *et al.*, 2015) (Figure 2A, B). Furthermore, in two functional assays of $GABA_B$ receptors, RgIA4 failed to activate $GABA_B$ receptors (Figure 2C, D) (Romero *et al.*, 2017). We also observed that Vc1.1, AuIB or RgIA had no agonist effects on $GABA_B$ receptors expressed in cell lines (unpublished observations).

Finally, extended duration of action and disease modification of several compounds should be considered in the context of the $GABA_B$ receptor hypothesis. The mechanism for producing the extended pharmacodynamics effects is unknown. N-type calcium channel blockade is caused by agonist action on $GABA_B$ receptors (Zamponi and Currie, 2013) and has been proposed as the therapeutic mechanism for Vc1.1, Vc1.1 analogues, RgIA and AuIB (Klimis *et al.*, 2011; Adams *et al.*, 2012; Huynh *et al.*, 2015). In this regard, it is noteworthy that in side-by-side trials with Vc1.1, peripheral administration of the N-type calcium blocker **MVIIA** did not alter short-term or long-term mechanical pain thresholds in CCI rats and did not accelerate functional recovery of neurons (Livett *et al.*, 2008). To our knowledge, in humans, neither the FDA-approved MVIIA (ziconotide) nor the FDA-approved $GABA_B$ receptor agonist baclofen has been associated with disease-modifying effects in neuropathic pain conditions.

As reviewed above, a striking characteristic of ligands that inhibit α9α10 nAChRs is the capacity to prevent or modify the development of chronic neuropathic pain (Sandall *et al.*, 2003; Satkunanathan *et al.*, 2005; Livett *et al.*, 2006; Vincler *et al.*, 2006; Vincler and McIntosh, 2007; McIntosh *et al.*, 2009; Di Cesare Mannelli *et al.*, 2014; Luo *et al.*, 2015; Li *et al.*, 2016; Pacini *et al.*, 2016; Romero *et al.*, 2017). α-Ctx derivatives that lack $GABA_B$ receptor activity show effects consistent with the modification of disease progression (Romero *et al.*, 2017). In addition, small-molecule antagonists of α9α10 nAChRs reverse or prevent the development of neuropathic pain (Holtman *et al.*, 2011; Zheng *et al.*, 2011; Wala *et al.*, 2012). Furthermore, a new set of αO family Ctxs that inhibit α9α10 nAChRs was recently discovered (Luo *et al.*, 2015). These peptides are structurally unrelated to α-Ctxs and lack $GABA_B$ receptor activity yet are analgesic. Furthermore, the analgesia produced by the αO-Ctx lasts at least 2 weeks after cessation of drug, consistent with possible disease-modifying effects (Li *et al.*, 2016). In contrast, known $GABA_B$ receptor agonists have not been shown to have similar properties. Thus, the acute analgesic action of some α-Ctxs may be mediated by activity at $GABA_B$ receptors, although

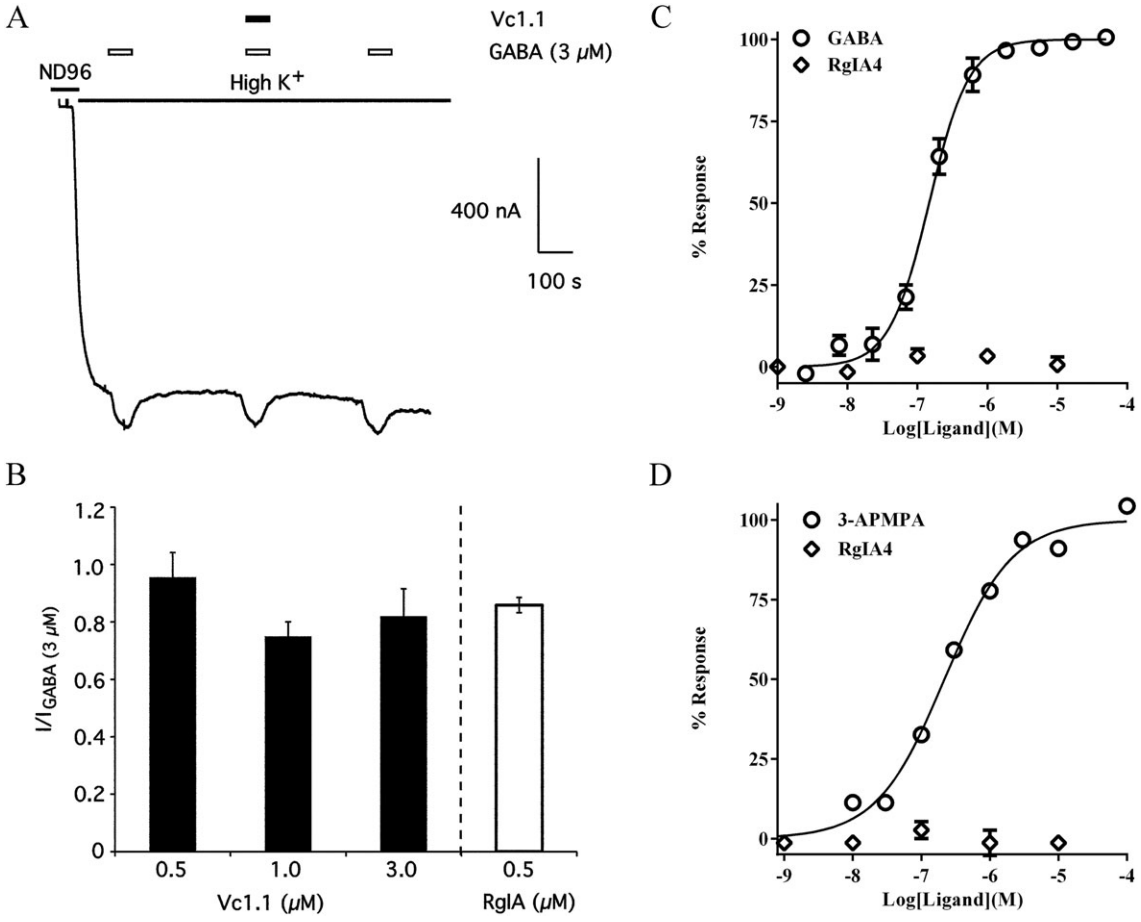

Figure 2

Analgesic α-Ctxs Vc1.1, RgIA and RgIA4 fail to activate heterologously expressed $GABA_B$ receptors. α-Ctxs have not shown agonist activity in a variety of functional assays designed to measure $GABA_B$ receptor activity. (A) Vc1.1 failed to activate human $GABA_B$ receptors functionally coupled to K_{ir} (GIRK) channels expressed in *Xenopus* oocytes. The oocytes were stimulated with 45 mM K^+ and then pulsed with GABA or GABA + Vc1.1 to stimulate $GABA_B$ receptor activation of K_{ir} channels. Note that GABA, but not Vc1.1, increased K^+ currents. Similar results were obtained with RgIA in the same assay. (B) Histogram showing the lack of effect of Vc1.1 and RgIA on K_{ir} channel activation. (figures in A and B were used with permission of McIntosh *et al.*, 2009). (C) G_i-coupled cAMP modulation was assessed in CHO cells expressing $GABA_{B1a/B2}$ receptors with $G_{i/o}$ coupling. GABA produced an increase in cAMP response in a concentration-dependent manner, whereas RgIA4 had no effect at any of the concentrations tested. (D) Cellular dielectric spectroscopy was used to assay changes in electrical impedance using CHO cells expressing human $GABA_{B1a/B2}$ receptors during stimulation with the $GABA_B$ receptor agonist 3-aminopropyl(methyl)phosphonic acid (3-AMPA). 3-AMPA produced changes in cellular impedance in a concentration-dependent manner, whereas RgIA4 had no effect. (Figures in C and D were used with the permission of Romero *et al.*, 2017).

the lack of reproducibility across laboratories and/or systems renders this interpretation unclear. Multiple pharmacological families of compounds that inhibit α9α10 nAChRs have been shown to favourably alter the expression and/or maintenance of chronic pain in a variety of experimental paradigms. Evidence from α9 subunit null mice also supports a role for α9α10 nAChRs in attenuating the expression of neuropathic pain (Mohammadi and Christie, 2014; Romero *et al.*, 2017).

Non-α-conotoxin antagonists of α9α10 nAChRs are analgesic

Studies demonstrating that Vc1.1 and RgIA produce analgesia in rodent models of neuropathic pain provided initial evidence for α9α10 nAChRs as an important target in the treatment of neuropathic pain syndromes. Since the initial studies with α-Ctxs, small molecules and other selective antagonists of α9α10 nAChRs (tetrakis-, tris- and bis-azaaromatic quaternary ammonium salts) have been discovered and tested for their ability to produce effects similar to those observed with α-Ctxs (Holtman *et al.*, 2011; Zheng *et al.*, 2011; Wala *et al.*, 2012). One such molecule, ZZ-204G has been shown to be effective at decreasing nociceptive behaviour and reducing mechanical hyperalgesia (Holtman *et al.*, 2011). Another small molecule ZZ1-61C is effective in reducing symptoms of peripheral neuropathy induced by the chemotherapeutic agent vincristine in rodents (Wala *et al.*, 2012). More recently, a novel conopeptide belonging to the O1-Ctx superfamily was discovered by a PCR screen of the venom duct from *Conus generalis* (Luo *et al.*, 2015). A well-known member of this superfamily is the VGCC

Table 1

Therapeutic effects of small molecule antagonists of α9α10 nAChRs, α-Ctxs, and α-Ctx analogues in neuropathic pain

Ligand	Pain model	Therapeutic effects
RgIA	CCI	Reduced mechanical allodynia and mechanical hyperalgesia; disease-modifying effects[a,b]
	Oxaliplatin-induced neuropathy	Reduced mechanical allodynia and mechanical hyperalgesia; reduced cold allodynia; disease-modifying effects[c]
RgIA4	Oxaliplatin-induced neuropathy	Reduced mechanical hyperalgesia; reduced cold allodynia; prolonged effects[d]
Vc1.1	CCI	Reduced mechanical allodynia and mechanical hyperalgesia; disease-modifying effects[b,e]
	PSNL	Reduced mechanical allodynia and mechanical hyperalgesia[e,f,h]
	Streptozotocin-induced neuropathy	Reduced mechanical allodynia[i,j]
	CVH	Reduced excitability of nociceptors[k]
vc1a	CCI	No effect on mechanical allodynia[h]; accelerated recovery of sensory nerves[i]
cVc1.1	CCI	Reduced mechanical allodynia[l]
[Ser3]Vc1.1(1–8)	VMR	Reduced visceromotor response[m]
AuIB	PSNL	Reduced mechanical allodynia[f,h]
αO-GeXIVA	CCI	Reduced mechanical allodynia[n,o]
ZZ-204G	CCI	Reduced mechanical hyperalgesia[p]
	Formalin test	Reduced inflammatory pain[p]
	Tail flick	No effect[p]
ZZ1-61c	Vincristine-induced neuropathy	Reduced mechanical allodynia and mechanical hyperalgesia[q]

CVP, chronic visceral hypersensitivity; VMR, visceromotor response.
Mechanical allodynia measured with the Von Frey test; mechanical hyperalgesia measured with the paw pressure test.

[a]Di Cesare Mannelli *et al.*, 2014.
[b]Vincler *et al.*, 2006.
[c]Pacini *et al.*, 2016.
[d]Romero *et al.*, 2017.
[e]Satkunanathan *et al.*, 2005.
[f]Napier *et al.*, 2012.
[g]Klimis *et al.*, 2011.
[h]Nevin *et al.*, 2007.
[i]Livett *et al.*, 2008.
[j]McIntosh *et al.*, 2009.
[k]Castro *et al.*, 2017.
[l]Clark *et al.*, 2010.
[m]Carstens *et al.*, 2016a.
[n]Luo *et al.*, 2015.
[o]Li *et al.*, 2016.
[p]Holtman *et al.*, 2011.
[q]Wala *et al.*, 2012.

antagonist ω-Ctx MVIIA (FDA approved as ziconotide). However, in contrast to the potent inhibition of VGCCs by MVIIA, GeXIVA lacks inhibitory activity on rat DRG neuron-expressed VGCCs. Instead, GeXIVA was observed to be a potent inhibitor of rat α9α10 nAChRs expressed in *Xenopus* oocytes. A closer examination of the activity of GeXIVA on α9α10 nAChRs revealed that the peptide binds to a site distinct from that of RgIA. Nevertheless, when tested in the rat CCI model of neuropathic pain, GeXIVA significantly reduced signs of mechanical hyperalgesia induced by CCI injury (Luo *et al.*, 2015; Li *et al.*, 2016). Taken together, evidence obtained from studies using α-Ctxs, small molecule compounds and GeXIVA as receptor antagonists are consistent with an important role for α9α10 nAChRs in neuropathic pain. Table 1 summarizes the effects of these compounds in the various models of neuropathic pain.

Summary and conclusions

In summary, three separate structural families of molecules that share the common feature of being selective antagonists of α9α10 nAChRs (α-Ctxs, an O1-superfamily of Ctxs and quaternary ammonium salts) have been identified. Members from each of these families have been shown to not only reverse pain but also prevent pain. As disease modification is not a common feature of analgesics, these effects support a role for α9* nAChRs. While we cannot rule out the possibility that each of these molecules also acts on some unidentified, yet therapeutic, target, the parsimonious explanation is that their common characteristic, inhibition of α9* nAChRs, is mechanistically important. Evidence from α9 subunit null mice further supports a role for α9* nAChRs; however, since these are germline deletions, rather than conditional KO mice, developmental compensation may affect both pain phenotype expression and response to compounds, particularly if complex neuroimmuno-modulatory processes are involved. In contrast, multiple compelling studies indicate that one of these families, the α-Ctxs, have $GABA_B$ receptor agonist activity and are analgesic. One member, AuIB, lacks activity at the α9α10 nAChR yet is analgesic, calling into question the necessity of activity at α9α10 nAChRs. Furthermore, analogues of α-Ctxs that have α9α10 nAChR activity

but lack $GABA_B$ receptor activity have diminished or lack analgesic activity. In the midst of these otherwise convincing data, the very activity of these compounds on $GABA_B$ receptors is inconsistent or absent across assays and laboratories somewhat diminishing the confidence in this explanation. In addition, the therapeutic profile of the one FDA-approved $GABA_B$ receptor agonists, baclofen, does not closely resemble that of the α-Ctxs with $GABA_B$ receptor activity. While there is clearly much yet to learn, as a group, these α9α10 antagonists not only produce analgesia, but perhaps more importantly, may also alter the course of disease progression, thereby averting sequela normally associated with nerve injury. These findings raise the possibility of developing therapeutics that may be given prophylactically to protect against the development of nerve injury-induced neuropathic pain.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015a,b,c).

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Conflict of interest

Conotoxins, including some of those referenced in this paper, and quaternary ammonium salts have been patented by the University of Utah, Hainan University or the University of Kentucky, or with J.M.M. listed as an inventor.

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