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REVIEW

Analgesic conotoxins: block and G protein-coupled receptor modulation of N-type ($Ca_V2.2$) calcium channels

David J Adams, Brid Callaghan and Géza Berecki

Health Innovations Research Institute, RMIT University, Melbourne, Victoria, Australia

Correspondence
Professor David J. Adams, Health Innovations Research Institute, RMIT University, Melbourne, Vic. 3083, Australia. E-mail: david.adams@rmit.edu.au

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Conotoxins (conopeptides) are small disulfide bonded peptides from the venom of marine cone snails. These peptides target a wide variety of membrane receptors, ion channels and transporters, and have enormous potential for a range of pharmaceutical applications. Structurally related ω-conotoxins bind directly to and selectively inhibit neuronal (N)-type voltage-gated calcium channels (VGCCs) of nociceptive primary afferent neurones. Among these, ω-conotoxin MVIIA (Prialt) is approved by the Food and Drug Administration (FDA) as an alternative intrathecal analgesic for the management of chronic intractable pain, particularly in patients refractory to opioids. A series of newly discovered ω-conotoxins from *Conus catus*, including CVID–F, are potent and selective antagonists of N-type VGCCs. In spinal cord slices, these peptides reversibly inhibit excitatory synaptic transmission between primary afferents and dorsal horn superficial lamina neurones, and in the rat partial sciatic nerve ligation model of neuropathic pain, significantly reduce allodynic behaviour. Another family of conotoxins, the α-conotoxins, are competitive antagonists of mammalian nicotinic acetylcholine receptors (nAChRs). α-Conotoxins Vc1.1 and RgIA possess two disulfide bonds and are currently in development as a treatment for neuropathic pain. It was initially proposed that the primary target of these peptides is the α9α10 neuronal nAChR. Surprisingly, however, α-conotoxins Vc1.1, RgIA and PeIA more potently inhibit N-type VGCC currents via a $GABA_B$ GPCR mechanism in rat sensory neurones. This inhibition is largely voltage-independent and involves complex intracellular signalling. Understanding the molecular mechanisms of conotoxin action will lead to new ways to regulate VGCC block and modulation in normal and diseased states of the nervous system.

Abbreviations

AuIB, α-conotoxin AuIB; $Ca_v2.1$, P/Q-type calcium channel; $Ca_v2.2$, N-type calcium channel; CVID, ω-conotoxin CVID; CVIE, ω-conotoxin CVIE; CVIF, ω-conotoxin CVIF; DRG, dorsal root ganglion; epsc, excitatory postsynaptic current; GABA, γ-aminobutyric acid; GEF, guanine nucleotide exchange factors; GIRK, G protein-activated inwardly rectifying potassium channel; GPCR, G protein-coupled receptor; GVIA, ω-conotoxin GVIA; MVIIA, ω-conotoxin MVIIA; nAChR, nicotinic acetylcholine receptor; PeIA, α-conotoxin PeIA; RgIA, α-conotoxin RgIA; Vc1.1, α-conotoxin Vc1.1; VGCC, voltage-gated calcium channel

In recent years, progress in understanding conotoxin genetics and biodiversity and exploring sequence homology within the conotoxin superfamilies has moved rapidly, facilitating the discovery of a series of biologically active conopeptides. Several laboratories have made substantial progress in the identification, separation, synthesis and structural characterization of these peptides (reviewed in Nielsen *et al.*, 2000; Terlau and Olivera, 2004; Schroeder *et al.*, 2005; Jakubowski *et al.*, 2006; Olivera, 2006; Bulaj and Olivera, 2008; Bingham *et al.*, 2010). A number of conopeptides selectively target ion channels, membrane receptors and transporters associated with pain pathways. Among these, N-type voltage-gated

calcium channels (VGCCs) play a major role in pain information processing in sensory neurones (Campbell and Meyer, 2006; Zamponi *et al.*, 2009) and represent a validated target for treating chronic and neuropathic pain (Snutch, 2005; Pexton *et al.*, 2011). The N-type VGCC belongs to the group of high voltage-activated calcium channels and is composed of the pore-forming α_{1B} subunit (also known as $Ca_V2.2$, which in humans is encoded by the *CACNA1B* gene) and ancillary cytoplasmic β subunits (CACNB), membrane associated $\alpha_2\delta$ subunits (CACNA2D) and possibly γ subunits (CACNG) (nomenclature conforms to Alexander *et al.*, 2011). In nociceptive primary afferent neurones, various isoforms of each of these subunits exist, either expressed from similar genes or the result of alternative splicing (for reviews, see Lipscombe *et al.*, 2002; Catterall *et al.*, 2005; Yasuda and Adams, 2007; Catterall, 2011). This review focuses on the effects of ω- and α-conopeptides on N-type VGCC function and evaluates their potential in pain research.

ω-Conotoxins: primary structure and isolation

Typically, ω-conotoxins are composed of 24–30 amino acids, belong to the superfamily of disulfide-rich conopeptides and exhibit a characteristic Cys-residue scaffolding pattern (C-C-CC-C-C) and a protective C-terminal amide, which confers resistance to carboxylase activity (Table 1). Most of the variability in length among ω-conopeptides is in loops 3 and 4. The Cys residues are cross-linked by stabilizing disulfide bridges, resulting in a typical four-loop structure (Terlau and Olivera, 2004). The disulfide-coupled folding and the roles of unique molecular chaperones and enzymes governing post-translational modifications within a single conotoxin sequence are poorly understood. Despite the mechanistic differences in conditions between *in vivo* and *in vitro* oxidative folding, correctly folded and biologically active ω-conotoxins can be obtained in a simple oxidative environment (Figure 1) (Bulaj and Olivera, 2008; Bingham *et al.*, 2010).

The best characterized ω-conotoxins are the N-type VGCC-selective GVIA (Kerr and Yoshikami, 1984) and MVIIA (Olivera *et al.*, 1987), isolated from fish-hunting cone snails. More recently, several novel ω-conotoxins, CVIA-F (Lewis *et al.*, 2000; Adams *et al.*, 2003; Berecki *et al.*, 2010), were identified from the piscivorous *Conus catus* venom using a PCR-based strategy (see Hillyard *et al.*, 1992). This approach had the advantage of supplying large amounts of *synthetic* compounds for structure-function analyses, restricting the need for crude material obtained directly from the cone snail's venom duct. To date, CVID, CVIE and CVIF are the most selective ω-conotoxins for N-type VGCCs (Lewis *et al.*, 2000; Berecki *et al.*, 2010).

N-type VGCC determinants of ω-conotoxin binding

ω-Conotoxins contribute to the rapid prey-immobilizing 'motor cabal' action of the cone snail venom (Terlau *et al.*, 1996), which require multiple neurotoxins coordinately targeting presynaptic VGCCs and postsynaptic nAChRs and voltage-gated Na^+ channels that underlie neuromuscular transmission (Terlau and Olivera, 2004). Calcium influx through VGCCs is necessary for a large number of intracellular events, including the control of neurotransmitter release. The use of ω-conotoxins has been pivotal in both the biochemical and the physiological characterization of VGCCs, primarily due to these agents having very high affinity for VGCC binding sites.

The most commonly used assay to determine the potencies of conotoxins at the N-type VGCC has been radioligand binding (see Nielsen *et al.*, 2000). In such studies, ω-conotoxin affinity for depolarized cells or depolarized membrane fragments is very different from the characteristics of toxin binding to cells exhibiting physiological resting potentials (Table 2). As discussed in more detail below, the most likely reason for this difference is that characteristics of block and/or recovery from block change in accordance with the degree of voltage-dependent inactivation of N-type VGCCs.

N-type VGCCs are relatively heterogeneous in terms of their biophysical properties due to the differences in subunit composition (Yasuda and Adams, 2007), to alternative splicing of the α_{1B} subunit (Lipscombe *et al.*, 2002) or modulation of the α_{1B} subunit by cytosolic proteins, such as G-proteins (Dolphin, 1998). Because of this structural and functional heterogeneity, VGCC sensitivity to various ω-conotoxins can show considerable variation (Table 2). The influence of β and $\alpha_2\delta$ auxiliary subunits on the ability of ω-conotoxins GVIA, MVIIA, CVID and CVID analogues to block N-type VGCCs was examined using peripheral and central forms of rat $Ca_V2.2$ heterologously expressed in *Xenopus* oocytes (Mould *et al.*, 2004). While the β_3 subunit had no significant effect on potency, the $\alpha_2\delta$ subunit dramatically (~100-fold) reduced ω-conotoxin affinity at both the central and the peripheral α_{1B} isoforms (Mould *et al.*, 2004; Berecki *et al.*, 2010). This effect of $\alpha_2\delta$ was not affected by oocyte deglycosylation, which rules out an electrostatic shielding or repulsion effect of the heavily glycosylated extracellular α_2 domain to the conotoxin binding site. Given that the $\alpha_2\delta$ subunit is up-regulated in certain pain states (Luo *et al.*, 2001; Newton *et al.*, 2001), the anti-nociceptive properties of ω-conotoxins might be affected by $\alpha_2\delta$ subunit expression levels.

In rat parasympathetic ganglia *in situ*, CVID inhibits evoked neurotransmitter release from preganglionic cholinergic nerve terminals, whereas MVIIA or GVIA produce no effect (Adams *et al.*, 2003). This is most likely due to the presence of a tissue-specific α_{1B} splice variant or auxiliary subunits of the N-type VGCC. Relatively small differences in the sequence of the N-type VGCC α_{1B} subunit can influence ω-conotoxin-channel interaction: Gly(1326) may form a barrier, which controls the access of peptide toxins to their blocking site within the outer vestibule of the channel pore, as well as stabilizing the toxin-channel interaction (Feng *et al.*, 2001b). In the *Xenopus* expression system, ω-conotoxins CVID and MVIIA have similar potencies to block central ($\alpha_{1B\text{-}d}$) and peripheral ($\alpha_{1B\text{-}b}$) splice variants of the rat N-type VGCCs when co-expressed with rat β_3 (Lewis *et al.*, 2000). Intracellular domains of α_{1B} also affect ω-conotoxin affinity. The human N-type VGCC brain variants Ca_v 2.2

Table 1

Amino acid sequence of selected ω-conotoxins from the venom of *Conus catus, geographus* and *magus*

Toxin	Sequence																											
	Loop 1								Loop 2								Loop 3				Loop 4							
MVIIA	**C**	K	G	K	G	A	K	**C**	S	R	L	M	Y	D	**C**	**C**	T	G	S	**C**	R	–	–	S	G	K	**C**	–	– NH_2
GVIA	**C**	K	S	O	G	S	S	**C**	S	O	T	S	Y	N	**C**	**C**	R	–	S	**C**	N	O	Y	T	K	R	**C**	Y	– NH_2
CVIA	**C**	K	S	T	G	A	S	**C**	R	R	T	S	Y	D	**C**	**C**	T	G	S	**C**	R	–	–	S	G	R	**C**	–	– NH_2
CVIB	**C**	K	G	K	G	A	S	**C**	R	K	T	M	Y	D	**C**	**C**	R	G	S	**C**	R	–	–	S	G	R	**C**	–	– NH_2
CVIC	**C**	K	G	K	G	Q	S	**C**	S	K	L	M	Y	D	**C**	**C**	T	G	S	**C**	S	R	–	R	G	K	**C**	–	– NH_2
CVID	**C**	K	S	K	G	A	K	**C**	S	K	L	M	Y	D	**C**	**C**	S	G	S	**C**	S	G	T	V	G	R	**C**	–	– NH_2
CVIE	**C**	K	G	K	G	A	S	**C**	R	R	T	S	Y	D	**C**	**C**	T	G	S	**C**	R	–	–	S	G	R	**C**	–	– NH_2
CVIF	**C**	K	G	K	G	A	S	**C**	R	R	T	S	Y	D	**C**	**C**	T	G	S	**C**	R	–	–	L	G	R	**C**	–	– NH_2

Note the variation in the numbers of amino acids between non-adjacent cysteines and the three conserved residues Gly^5, Tyr^{13} and Ser^{19}. Conserved cysteine residues are in bold. Shown are the positions of the four loops and the disulfide scaffold in ω-conotoxins.

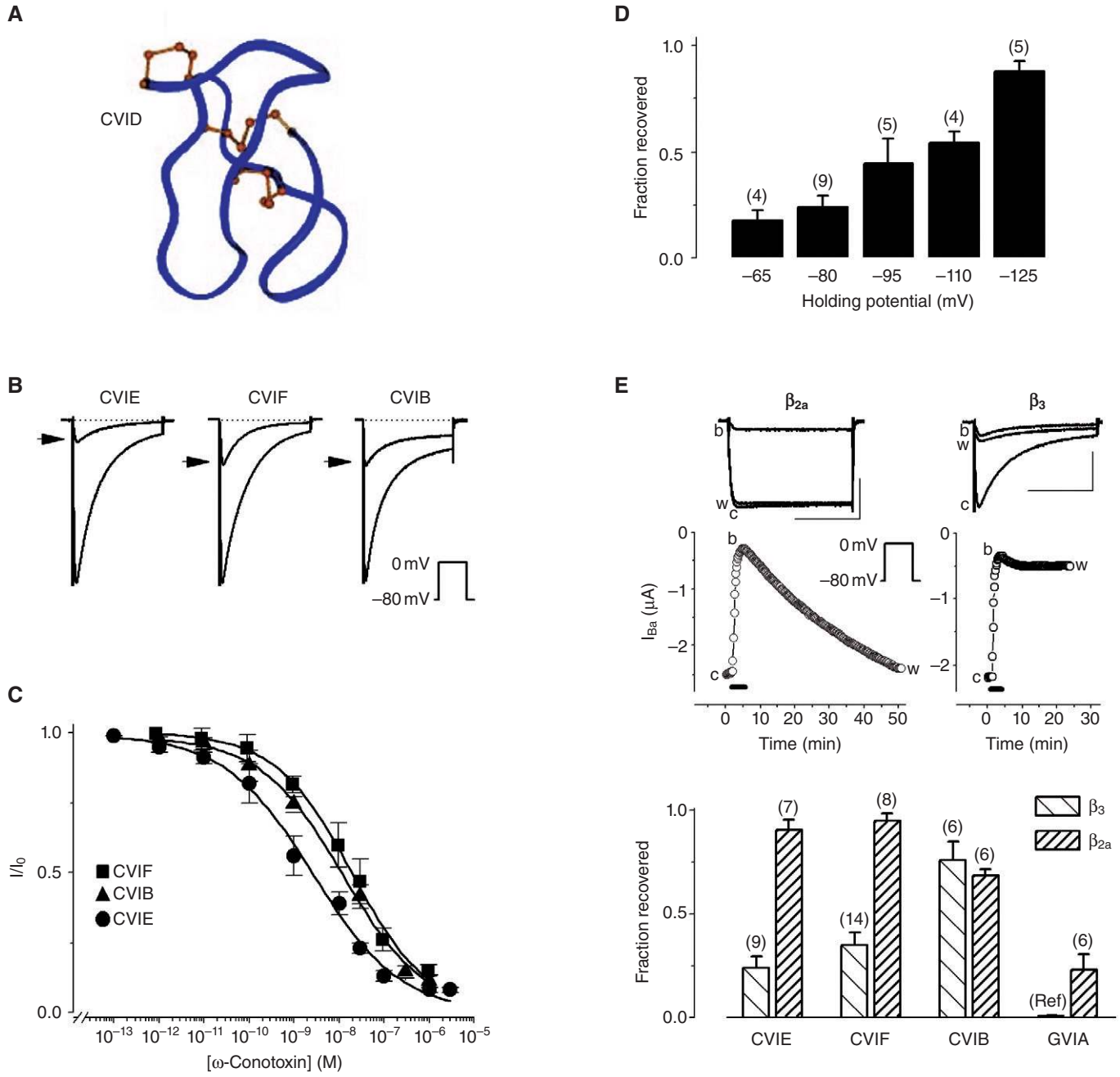

Figure 1

Block of recombinant N-type ($Ca_v2.2$) VGCCs by ω-conotoxins in *Xenopus* oocytes. (A) Three-dimensional structure of ω-conotoxin CVID highlighting the β-bridge and sheets (*red arrows*), the turns (*blue arrows*) and the location of disulfide bridges (*ball*-and-*stick*) [adapted from Lewis *et al.* (2000)]. (B) Representative normalized Ba^{2+} current traces (I_{Ba}) obtained before and after (arrowhead) application of 100 nM ω-conotoxin CVIE, CVIF or CVIB. Inset: voltage protocol. (C) Concentration–response curves for the normalized peak I_{Ba}. (D) Recovery from block by ω-conotoxin CVIE (100 nM) is voltage-dependent. I_{Ba} traces were evoked by 0.1 Hz, 200 ms step depolarization to 0 mV from the indicated holding potential. (E) Recovery from CVIE block depends on the β subunit: channels with the 'non-inactivating' β_{2a} auxiliary subunit show full recovery, whereas channels with β_3 exhibit weak recovery. Scale bars represent 1 μA and 100 ms. Bottom: reversibility of block after bath application of 100 nM ω-conotoxin CVIE, CVIF, CVIB or GVIA seen with $\alpha_{1B\text{-}b}/\alpha_2\delta1/\beta_{2a}$ or $\alpha_{1B\text{-}b}/\alpha_2\delta1/\beta_3$ VGCCs. Data marked by 'Ref' are from Mould *et al.* (2004) and represent recovery from block by 1 nM GVIA. Data in B, C, D and E are modified with permission from Berecki *et al.* (2010).

Table 2

ω-Conotoxins that inhibit mammalian N-type VGCCs

Toxin and species	*Selectivity (VGCC type)	IC_{50} for N-type VGCCs (cloned/native)	Displacement of ^{125}I-GVIA, IC_{50}	Mechanism of VGCC block	Reversibility of N-type VGCC block (cloned/native)
ω-GVIA *Conus geographus*	N[1]	nd/72 nM[2] (DRG)	38 pM[3] and §100 pM[13]	Pore block[4] (complete)	Irreversible in the presence of $\alpha_{1B}/\alpha_2\delta/\beta_{1b}$ subunits[5]/irreversible in spinal cord slice preparation[6]
ω-MVIIA *Conus magus*	N[7]	5 nM[8] (*X*)/200 nM[9] (hc)	55 pM[3]	Pore block[5]	Poorly reversible in the presence of $\alpha_{1B\text{-}b}/\alpha_2\delta1/\beta_3$ subunits; irreversible in the presence of $\alpha_{1B}/\alpha_2\delta/\beta_{1b}$ subunits[5]/irreversible in spinal cord slice preparation[6]
ω-MVIIC *C. magus*	P/Q and N[10]	nd/3–18 nM[11] (SCG)	7 nM[3] and §4 nM[13]	Pore block[4] (complete)	nd/reversible in SCG neurone[11]
ω-MVIID *C. magus*	N[12] and P/Q	nd/nd	§100 nM[13]	nd	nd/reversible in BC cells[13]
ω-RVIA *Conus radiatus*	N[14]	nd/nd	§§8.4–10.4 nM[15]	nd	nd/nd
ω-TVIA *Conus tulipa*	N[16]	nd/nd	251 pM[17]	nd	nd/nd
ω-SVIB *Conus striatus*	N[18] and P/Q	nd/nd	3.2 nM[19]	nd	nd/nd
ω-CnVIIA *Conus consors*	N[20]	nd/nd	2.7–3.7 pM[20]	nd	nd/nd
ω-CVIA *Conus catus*	N[3]	nd/nd	560 pM[3]	nd but likely pore block	nd/nd
ω-CVIB *C. catus*	N[3] and P/Q	33 nM (*X*)[2]/23 nM[2] (DRG)	7.7 nM[3]	nd but likely pore block	Reversible in the presence of $\alpha_{1B\text{-}b}/\alpha_2\delta1/\beta_3$[21] and $\alpha_{1B\text{-}b}/\alpha_2\delta1/\beta_{2a}$[2] subunits/reversible in spinal cord slice preparation[6]
ω-CVIC *C. catus*	N[3] and P/Q	nd/nd	7.6 nM[3]	nd but likely pore block	nd/nd
ω-CVID *C. catus*	N[3]	21.8 nM[8] (*X*)/9 nM[2] (DRG)	70 pM[3]	nd but likely pore block	Reversible in the absence of $\alpha_2\delta1$ subunit[8]; weakly reversible in the presence of $\alpha_2\delta1$ subunit[8]/irreversible in spinal cord slice preparation[6]
ω-SO3 *C. striatus*	N[22]	nd/160 nM[23] (hc)	nd	nd but likely pore block	nd/almost fully reversible in hippocampal neurones[23]
ω-CVIE *C. catus*	N[21]	2.6 nM (*X*)[21]/nd	19 pM[21]	nd but likely pore block	Poorly reversible in the presence of $\alpha_{1B\text{-}b}/\alpha_2\delta1/\beta_3$ subunits[21]; reversible in the presence of $\alpha_{1B\text{-}b}/\alpha_2\delta1/\beta_{2a}$ subunits[21]/poorly reversible in DRG neurone[21]; reversible in spinal cord slice preparation[21]
ω-CVIF *C. catus*	N[21]	19.9 nM[21] (*X*)/nd	25 pM[21]	nd but likely pore block	Poorly reversible in the presence of $\alpha_{1B\text{-}b}/\alpha_2\delta1/\beta_3$ subunits[21]; reversible in the presence of $\alpha_{1B\text{-}b}/\alpha_2\delta1/\beta_{2a}$ subunits[21]/poorly reversible in DRG neurone[21]; reversible in spinal cord slice preparation[21]
ω-FVIA *Conus fulmen*	N[24]	11.5 nM[24] (HEK293 cell)/nd	nd	nd	Reversible in HEK293 cells stably expressing $\alpha_{1B}/\alpha_2\delta/\beta_{1b}$ subunits[24]/nd

*Selectivity determined from relative potencies to displace ^{125}I-GVIA binding to rat brain membrane.
§Displacement of ^{125}I-GVIA binding to bovine adrenal medullary membrane.
§§Displacement of ^{125}I- GVIA binding in Swiss Webster mouse cortex.
Superscript numbers between parentheses refer to references as follows: (l) Olivera *et al.*, 1984; (2) Motin *et al.*, 2007; (3) Lewis *et al.*, 2000; (4) McDonough *et al.*, 2002; (5) Feng *et al.*, 2003; (6) Motin and Adams, 2008; (7) Olivera *et al.*, 1987; (8) Mould *et al.*, 2004; (9) Wen *et al.*, 2005; (10) Hillyard *et al.*, 1992; (11) McDonough *et al.*, 1996; (12) Monje *et al.*, 1993; (13) Gandia *et al.*, 1997; (14) Olivera *et al.*, 1985; (15) Abbott and Litzinger, 1994; (16) Miljanich and Ramachandran, 1995; (17) Miljanich *et al.*, 1993; (18) Ramilo *et al.*, 1992; (19) Nielsen *et al.*, 2000; (20) Favreau *et al.*, 2001; (21) Berecki *et al.*, 2010; (22) Lu *et al.*, 1999; (23) Wen *et al.*, 2005; (24) Lee *et al.*, 2010.
BC cell, bovine chromaffin cell; DRG, dorsal root ganglion neurone; hc, hippocampal neurone; HEK239 cell, human embryonic kidney cell line; GPCR, G protein-coupled receptor pathway; nACh, nicotinic acetylcholine receptor; nd, not determined (no published information); N, N-type VGCC; P/Q, P/Q-type VGCC; PTX, *pertussis* toxin; SCG, rat superior cervical ganglion neurone; *X*, *Xenopus* oocyte.

Delta 1 and Delta 2 lack the synaptic protein interaction site and are less sensitive to both GVIA and MVIIA (Kaneko *et al.*, 2002).

The membrane potential can severely affect the severity of ω-conotoxin block and the kinetics of its onset and removal (Nowycky *et al.*, 1985; Neely *et al.*, 1993; Stocker *et al.*, 1997; Feng *et al.*, 2003). Block by ω-conotoxins GVIA, MVIIA and SNX-331, a derivative of MVIIC, is reversible by strong hyperpolarization. This effect of the membrane potential on reversibility of block could be accounted for by a modulated receptor model, in which toxin dissociation from the inactivated state is slower than from the resting state (state-dependent block) (Stocker *et al.*, 1997). Recovery from CVIE and CVIF block also increases with membrane hyperpolarization, indicating that these molecules have a higher affinity for channels in the inactivated state (Figure 1) (Berecki *et al.*, 2010). Contrary to this, hyperpolarization does not significantly enhance the extent of recovery from CVID block (Mould *et al.*, 2004), whereas CVIB block and recovery also appear to be largely voltage-independent (Motin *et al.*, 2007; Berecki *et al.*, 2010).

Several other factors and mechanisms, including N-type VGCC heterogeneity, Ca^{2+}–calmodulin binding (Liang *et al.*, 2003), palmitoylation (Hurley *et al.*, 2000) and steady-state N-type VGCC inactivation, affect the extent of recovery from block by different ω-conotoxins. Auxiliary subunits $\alpha_2\delta$ and β exert a pronounced regulation of steady-state inactivation characteristics of N-type VGCCs (Scott *et al.*, 1996; Yasuda *et al.*, 2004). Slowly inactivating N-type VGCCs with β_{2a} subunits almost completely recover from CVIE or CVIF block, whereas rapidly inactivating VGCCs containing the β_3 subunits exhibit only weak recovery (Figure 1E) (Berecki *et al.*, 2010). Co-expression of $\alpha_2\delta$ with α_{1B} and β_3 subunits reduces recovery from block for CVID but not for MVIIA (Mould *et al.*, 2004). Reversibility of block may be correlated with the effectiveness of these compounds to reverse different painful conditions *in vivo* and could indicate whether the side effects during the administration of these peptides can be controlled (Wright *et al.*, 2000).

The site and mechanism of block of N-type VGCCs by ω-conotoxins

Although the blocking mechanism by GVIA, MVIIA and MVIIC has been studied in detail, the precise site of interaction of ω-conotoxins with the N-type VGCC is yet to be fully elucidated. At the single channel level, N-type VGCCs exhibit decreased open-state probability in the presence of GVIA, and the Ca^{2+} binding site (or sites), located in the pore of the channel, allosterically regulates the GVIA binding site (Witcher *et al.*, 1993). Amino acid residues within the S5–H5 region of domain III (located in the vestibule of the α_{1B} subunit of the N-type VGCC) are critical for binding ω-conotoxin GVIA, suggesting that this toxin acts via physical occlusion of the pore (Ellinor *et al.*, 1994). The results of secondary structure prediction methods show a helix–coil–helix structure for this pore region, highly reminiscent of the classical EF hand, but with a weaker Ca^{2+} binding site than that found in calmodulin and other calcium-binding proteins (Doughty *et al.*, 1998). There is sound electrophysiological evidence for the existence of this ion-permeation pathway-lining, weak Ca^{2+} binding – EF hand homology motif, at which both Ca^{2+} and Ba^{2+} can compete but only Ca^{2+} can produce an effect on channel gating (Zamponi and Snutch, 1996). An increase in external Ba^{2+} reduces both the potency and the rate of MVIIC block (Boland *et al.*, 1994; McDonough *et al.*, 1996), and conversely, a decrease in external Ba^{2+} accelerates the off-rate of GVIA (Liang and Elmslie, 2002). These findings support the idea that toxin molecules compete with Ba^{2+} bound at the locus of selectivity at the EF hand homology motif on the a_{1B} subunit, immediately adjacent to the ω-conotoxin GVIA binding site.

There are at least two distinct toxin binding sites on the N-type channel and three on the P/Q-type ($Ca_v2.1$) channel. MVIIC binding produces complete N- and P/Q-type channel block, but this is prevented by previous partial channel block by ω-agatoxin-IIIA from the venom of the funnel-web spider *Agelenopsis aperta*, suggesting that MVIIC binds closer to the external mouth of the pore than ω-agatoxin-IIIA. On N-type channels, results are consistent with blockade of the channel pore by GVIA, MVIIA and MVIIC (McDonough *et al.*, 2002; Feng *et al.*, 2003). Although GVIA appears to be an open channel blocker, Yarotskyy and Elmslie (2009) showed that the N-type VGCC gating charge movement is also affected to some extent during the block, and this gating modulation alone inhibited the N-type VGCC current by 50% in a mathematical model.

As previously mentioned, single residues of the N-type VGCC can have a significant impact on toxin-channel interaction (Feng *et al.*, 2001a). Stocker *et al.* (1997) suggested that, during toxin blockade, preferential interaction of the toxin with the inactivated channel must be accompanied by an externally detectable conformational change near the extracellular pore mouth. It therefore appears that ω-conopeptides exert their biological actions by occluding the ion-conducting pore of VGCCs, with a 1:1 stoichiometry for GVIA (McDonough *et al.*, 2002; Feng *et al.*, 2003). Because CVIA-F are structurally related to GVIA and MVIIA, it is likely that common molecular determinants underlie the block of N-type VGCCs by these ω-conopeptides.

ω-Conotoxin residues affecting the interaction with N-type VGCCs

C. catus ω-conotoxins are small, hydrophilic and stable, and are therefore ideally suited for NMR spectroscopy (Daly and Craik, 2009). CVIA-F adopt a common fold, including the anti-parallel triple-stranded β-sheet previously reported in MVIIA, MVIIC and GVIA (Figure 1) (Nielsen *et al.*, 2000). Detailed NMR studies of CVID have revealed the unique orientation of loop 4, which may account for the improved selectivity for the N-type VGCC over MVIIA and GVIA, and the presence of two hydrogen bonds (from the NH protons of Lys^{10} and Leu^{11}) that enhance the stability of loop 2 (Lewis *et al.*, 2000; Nielsen *et al.*, 2000). Compared with CVID, CVIB lacks the two stabilizing hydrogen bonds between loops 2 and 4 and, like other ω-conotoxins lacking this structural stabilizing feature, it has a disordered loop 2 (Motin *et al.*, 2007).

A series of studies determined the most important ω-conotoxin residues for interaction with VGCCs. The N-terminal amino group (Lampe *et al.*, 1993) and Tyr13 (Kim *et al.*, 1995) are essential for the binding to a number of residues, which partially overlap with the putative EF hand domain on the channel (Ellinor *et al.*, 1994; Feng *et al.*, 2001b; 2003). Homology models of a series of ω-conotoxins exhibit similar structural fold at positions 10, 11 and 13 (Berecki *et al.*, 2010) (see Table 1). The amino acid in position 10 has a secondary effect on binding and affects the extent of ω-conotoxin reversibility (Mould *et al.*, 2004); it is therefore likely that the pharmacophore of ω-conotoxins might be affected by amino acid substitution in this position. There is also evidence that some ω-conopeptides exert allosteric effects on channel structure when they bind (Stocker *et al.*, 1997). The distribution of electrostatic surface properties of ω-conotoxins suggests that the ω-conotoxin–N-type VGCC interactions are dominated by ionic/electrostatic forces (Berecki *et al.*, 2010). The difference in net charge between ω-conotoxins, in addition to differences in structure, can give us clues to explain the difference in potency, selectivity and reversibility among these related peptides.

ω-Conotoxins as neurophysiological tools and potential drug leads for the treatment of pain

The N-type VGCC is revealed as an exciting new target for pain treatment, following the approval of ω-conotoxin MVIIA (Ziconotide or Prialt) by the US Food and Drug Administration and European Medicines Agency for the management of severe chronic pain associated with cancer, acquired immune deficiency syndrome (AIDS) and neuropathies – refractory to other current pain medications. However, due to the wide distribution of N-type VGCCs and the peptidergic nature of MVIIA, current therapy with Prialt is limited to intrathecal delivery and has many side effects (Park and Luo, 2010). In the ascending pain pathways, the pathophysiological roles of N-type VGCCs have been demonstrated by using gene knockout mice or ω-conopeptides (Snutch, 2005; McGivern, 2006). CVIB and CVID-F have been efficiently used to evaluate the contribution of the N-type VGCC component to whole-cell currents in dorsal root ganglia (DRG) neurones (Motin *et al.*, 2007; Berecki *et al.*, 2010) and to characterize excitatory synaptic transmission between primary afferents and dorsal horn superficial lamina neurones. CVID completely and irreversibly inhibits excitatory postsynaptic current (epsc), whereas the epsc amplitude-reducing effect of CVIB and CVIE-F is reversible (Motin and Adams, 2008; Berecki *et al.*, 2010).

During intrathecal application, ω-conopeptides bind to N-type VGCCs of the nociceptive A-δ and C fibres, the cell bodies of which reside in the DRG and project to secondary neurones of the dorsal horn in the spinal cord (Zamponi *et al.*, 2009). N-type VGCC blocker ω-conopeptides are potent inhibitors of nociceptive signalling and therefore represent potential drug leads for the treatment of pain states. Clearly, there is a need for new conotoxins, which may have potential for the treatment of neuropathic pain, depression and various other neurological conditions, as well as exhibiting a more favourable ratio of anti-nociception to side effect profile than MVIIA. CVID-F exhibits the highest N-type over P/Q-type selectivity and could therefore serve as lead structures for novel analgesics. CVID (AM-336, Leconotide) has been tested as a therapeutic agent and has completed phase II clinical trial. Compared with MVIIA, CVID has an improved specificity for N-type VGCCs, better *in vitro* reversibility, and a more rapid onset and offset of action (Mould *et al.*, 2004); in animal models, intrathecally applied CVID shows improved efficacy (Wright *et al.*, 2000) and fewer cardiovascular side effects (Smith *et al.*, 2002). Similar to CVID, ω-conotoxins CVIE or CVIF completely and reversibly relieve mechanical allodynia, as demonstrated in a nerve injury model of neuropathic pain (Berecki *et al.*, 2010). However, further experiments are required to clarify the anti-nociception to side effect profiles of CVIE-F. Currently, the methods for administering ω-conopeptides as therapeutic agents are limited to intrathecal delivery. Peptides, in general, are unable to cross the blood–brain barrier due to their inherently large size and hydrophilic nature. Therefore, alternative strategies are needed, which would convert active peptides into non-peptidic small molecules and/or would limit their systemic degradation. Schroeder *et al.* (2004) identified cyclic pentapeptides, which contain residues of loop 2 of CVID and mimic the binding of CVID at the N-type VGCC. A better understanding of conopeptide-VGCC interaction would help in the development of small molecules that selectively and tissue-specifically target N-type VGCCs. Future experiments with structural analogues could result in compounds with stabilized structure, which would permit oral administration. However, development of ω-conotoxin mimetics (through the mimicry of N-type VGCC binding domains) is currently very challenging (Baell *et al.*, 2004). Recently, the cell-specific inhibition of P/Q- and N-type VGCCs *in vivo* was demonstrated with a tethered-toxin approach, resulting in cell-specific and cell-autonomous silencing of neurotransmission (Auer *et al.*, 2010). This approach could serve as a viable alternative therapy to intrathecal ω-conopeptide delivery.

α-Conotoxins: isolation, structures, targets

α-Conotoxins have been isolated either by fractionation and purification from cone snail crude venom duct contents or via PCR screens of cDNA sequences derived from *Conus* venom ducts of fish-, worm- and mollusc-hunting species of *Conus* (Olivera *et al.*, 1985; Janes, 2005; Kaas *et al.*, 2008). The first α-conotoxins were discovered more than 30 years ago (Spence *et al.*, 1977; Gray *et al.*, 1981) and are currently classified according to their gene superfamily membership and cysteine framework connectivity. The number of amino acids between the C2 and C3 and the C3 and C4 residues further divides the α-conotoxins into five subfamilies: α3/5, α4/3, α4/4, α4/6 and α4/7 (Azam and McIntosh, 2009). The venom repertoire of cone snails undergoes ontogenetic changes, suggestive of alternative functions of peptide toxins during development (Safavi-Hemami *et al.*, 2011). To date, at least 39 mature α-conotoxin sequences of the type I framework are included in the ConoServer database (Kaas *et al.*, 2010).

Table 3

Amino acid sequences of α-conotoxins that inhibit N-type ($Ca_v2.2$) calcium channels via $GABA_B$ receptor activation

Toxin	Sequence and disulfide connectivity																	IC_{50} (nM)	References
			Loop 1							Loop 2									
Vc1.1	G	**C**	**C**	S	D	P	R	**C**	N	Y	D	H	P	E	I	**C**	$-NH_2$	1.7	Callaghan *et al.*, 2008
Vc1.2	G	**C**	**C**	S	N	P	A	**C**	M	V	N	N	P	Q	I	**C**	$-NH_2$	47.5	Safavi-Hemami *et al.*, 2011
RgIA	G	**C**	**C**	S	D	P	R	**C**	R	Y	R	–	–	–	–	**C**	R	7.3	Callaghan and Adams, 2010
PeIA	G	**C**	**C**	S	H	P	A	**C**	S	V	N	H	P	E	L	**C**	$-NH_2$	1.1	Daly *et al.*, 2011
AuIB	G	**C**	**C**	S	Y	P	P	**C**	F	A	T	N	P	D	–	**C**	$-NH_2$	1.5	Klimis *et al.*, 2011

IC_{50} values were determined for N-type VGCCs in rat DRG neurons. Conserved cysteine residues are in bold; the positions of the two loops and the disulfide scaffold are shown.

Members of this family typically range in size from 12 to 16 amino acids, exhibit a cysteine framework with two disulfide bonds in C1–C3 and C2–C4 connectivity, and often have an amidated C-terminus (Table 3).

In contrast to the ω-conotoxins that are usually classified as selective antagonists of VGCCs, α-conotoxins are pharmacologically classified as competitive antagonists of muscle and neuronal nAChRs (Adams *et al.*, 1999; Janes, 2005; Bingham *et al.*, 2010). However, a series of recent publications clearly demonstrates that a subset of α-conotoxins indirectly inhibit neuronal N-type VGCCs by acting effectively as agonists of the $GABA_B$ GPCR (Figure 2) (Callaghan *et al.*, 2008; Daly *et al.*, 2011; Klimis *et al.*, 2011; Safavi-Hemami *et al.*, 2011). There are also examples of conotoxins that target other GPCRs, including the α_1-adrenoreceptor (Sharpe *et al.*, 2001; 2003) and the neurotensin receptor (Craig *et al.*, 1999). $GABA_B$ receptors are heterodimers composed of a $GABA_{B1}$ subunit and the $GABA_{B2}$ subunit (Figure 3) (Bettler *et al.*, 2004), and their modulation by agonists is well established as a mechanism for producing pain relief (Goudet *et al.*, 2009). Activation of presynaptic $GABA_B$ receptors leads to inhibition of N-type calcium channels, which, in turn, results in diminished release of GABA (autoreceptors) and other neurotransmitters (heteroreceptors), whereas postsynaptic $GABA_B$ receptor activation is associated with G-protein regulated inward-rectifying potassium (GIRK) channel activation, resulting in hyperpolarization. In the human peripheral and central nervous system, $GABA_{B1}$ has two major site variants (isoforms), $GABA_{B1a}$ and $GABA_{B1b}$, which result from alternative transcription initiation and primarily differ by the presence of a pair of sushi repeats (domains) in $GABA_{B1a}$ (Kaupmann *et al.*, 1997), thought to interact with proteins involved in cell–cell adhesion (Bettler *et al.*, 2004). The $GABA_{B1}$ subunit contains an endoplasmic reticulum (ER) retention domain that is concealed when it forms a dimer with $GABA_{B2}$, allowing for surface expression (Margeta-Mitrovic *et al.*, 2000), whereas $GABA_{B2}$ regulates internalization of the receptor (Hannan *et al.*, 2011). Recently, the $GABA_B$ heterodimeric receptor has been shown to spontaneously form dimers of heterodimers, or larger complexes in heterologous systems via the interaction of $GABA_{B1}$ subunits, which leads to negative functional cooperativity between $GABA_B$ heterodimers (Comps-Agrar *et al.*, 2011; Kniazeff *et al.*, 2011).

GPCR stimulation is associated with GDP replacement with GTP and dissociation of the heterotrimeric G protein into Gα-GTP and Gβγ dimers, which then interact with a variety of different effectors (for reviews, see Pierce *et al.*, 2002; Oldham and Hamm, 2006; 2008). Regulators of G-protein signalling proteins accelerate the Gα subunit GTPase activity, thus increasing the rate of G-protein inactivation (McCudden *et al.*, 2005). The activated GPCRs are also phosphorylated by G protein-coupled receptor kinases, resulting in recruitment of β-arrestins to terminate G protein-dependent signalling (Shukla *et al.*, 2010).

N-type VGCC determinants of modulation by GPCRs

The N-type VGCC can be depressed by several neurotransmitters and this modulation constitutes a major mechanism of presynaptic inhibition (Bean, 1989). Two distinct pathways are generally identified in this signalling, between which the voltage-dependent inhibition is the most common form, involving Gβγ binding directly to the I-II linker of the VGCC α1 subunit of the N-type VGCC (Bean, 1989; Hille, 1994; Tedford *et al.*, 2010) (Figure 3). The free Gβγ heterodimers do not only signal exclusively to VGCCs but also modulate downstream effectors such as phospholipase C and adenylyl cyclase. The Gα subunits trigger various intracellular pathways, which may converge on VGCCs to either up-regulate or inhibit their activities (Bernheim *et al.*, 1991; Beech *et al.*, 1992; Delmas *et al.*, 1998; Kammermeier *et al.*, 2000). Because VGCC modulation via second messenger pathways cannot be reversed by strong membrane depolarizations, it is referred to as voltage-independent inhibition. This pathway involves diffusible second messengers and occurs over a slower time

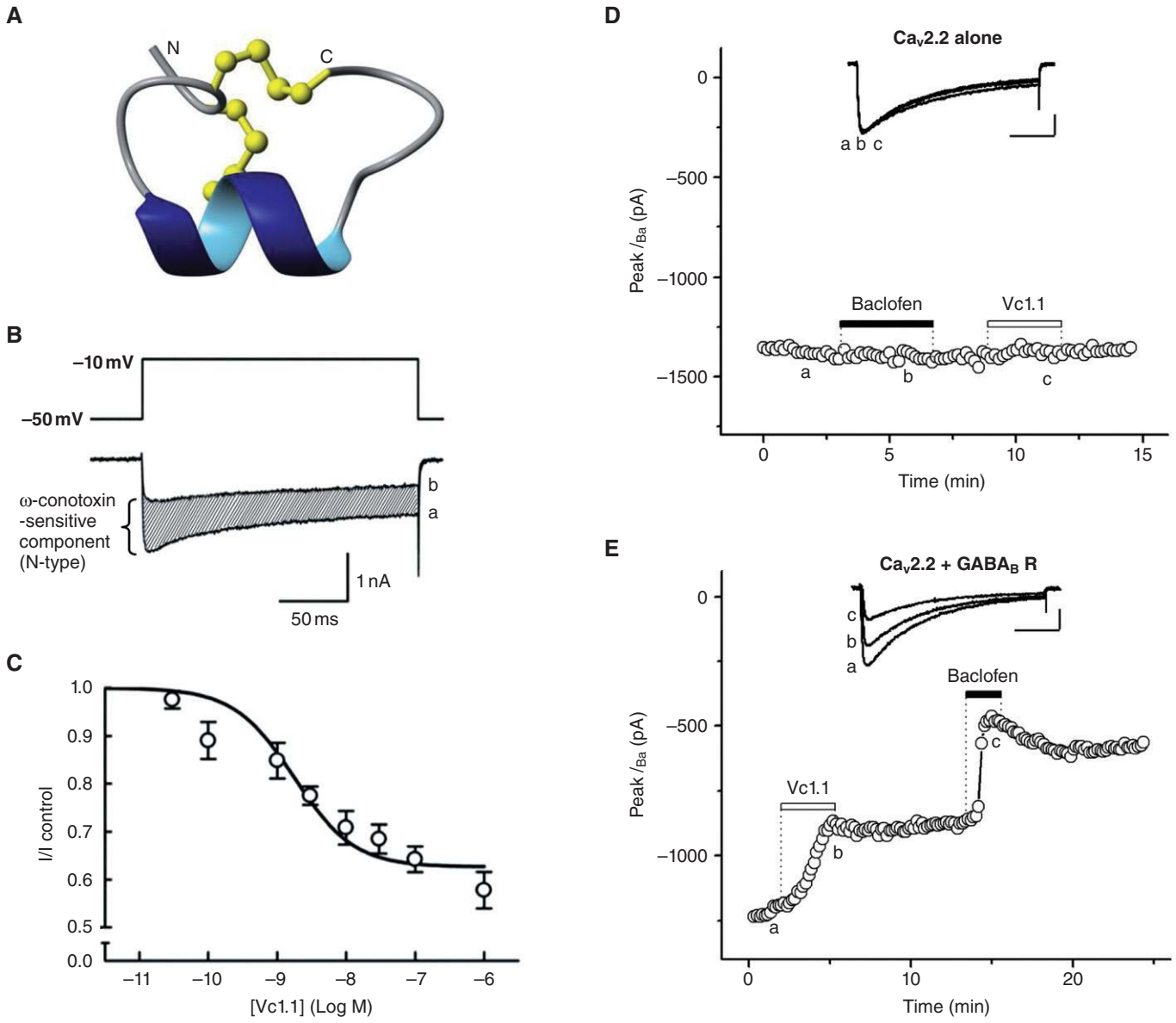

Figure 2

α-Conotoxins inhibit N-type ($Ca_V2.2$) VGCCs via the $GABA_B$ receptor. (A) Three-dimensional structure of α-conotoxin Vc1.1. Disulfide bonds are shown in *ball-and-stick* representation and the N and C termini are marked adapted from Clark *et al.* (2006). (B) Superimposed traces of depolarization-activated whole-cell I_{Ba} recorded using 2 mM Ba^{2+} as the charge carrier, elicited in the absence (a) and presence (b) of 100 nM Vc1.1. Inset: voltage protocol. (C) Concentration–response curve for inhibition of I_{Ba} in DRG neurones by Vc1.1; IC_{50} = 1.7 nM. B and C are adapted from Callaghan *et al.* (2008), with permission. (D) The $GABA_B$ receptor mediates Vc1.1 inhibition of N-type ($Ca_V2.2$) VGCC currents in transiently transfected HEK-293 cells. Time course of peak I_{Ba} evoked with 0.1 Hz, 250 ms depolarizing test pulses from –80 to 10 mV and recorded using 20 mM Ba^{2+} as the charge carrier; upward deflections represent current inhibition. Baclofen (50 μM) or Vc1.1 (100 nM) does not affect I_{Ba} in cells co-transfected only with cDNAs of $\alpha_{1\text{-}B}$, $\alpha_2\delta_1$ and β_1 VGCC subunits (n = 3). Inset: representative I_{Ba} traces are shown at the times indicated by lowercase letters. In cells co-transfected with ($Ca_V2.2$) VGCC subunits and $GABA_{B1,B2}$ subunits, Vc1.1 and baclofen (n = 4) (E) inhibit I_{Ba}. Scale bars in D and E represent 500 pA and 50 ms (D and E; G. Berecki and D.J. Adams, unpubl. obs.).

course than voltage-dependent inhibition (Luebke and Dunlap, 1994). The voltage-dependent and voltage-independent modulation of neuronal Ca_v2 channels has been reviewed recently (Tedford and Zamponi, 2006; Brown and Sihra, 2008; Weiss, 2009; Currie, 2010).

As previously mentioned, N-type VGCCs are rather heterogeneous regarding their biophysical properties due to the differences in subunit composition. Alternative splicing markedly expands the functional potential of VGCCs (Gray *et al.*, 2007). In pain pathways, exon 37a not only tailors $Ca_V2.2$ channels towards specific roles in pain (Altier *et al.*, 2007) but also alters $Ca_V2.2$ channel modulation by GPCRs, acting as a molecular switch linking GPCRs to voltage-independent inhibition of the N-type VGCC (Lipscombe and

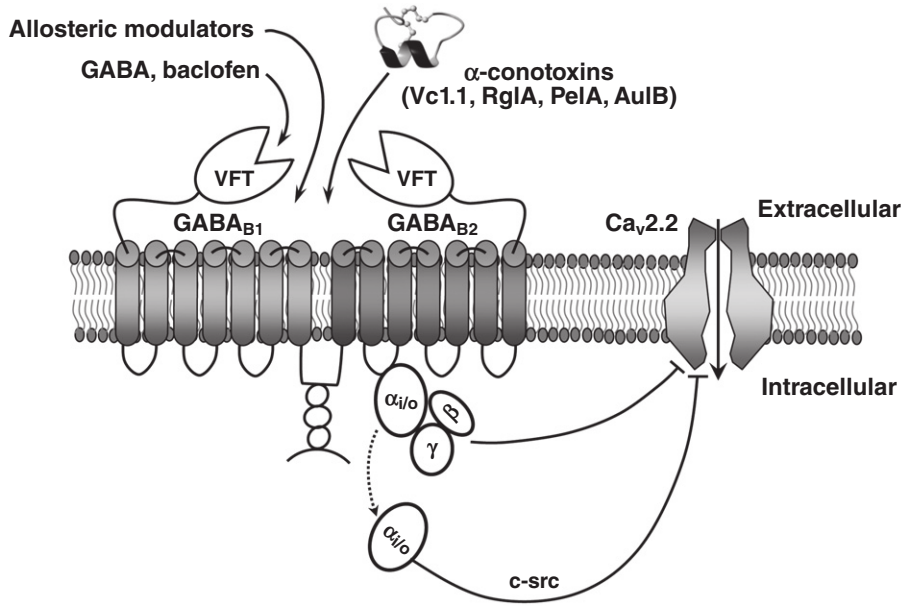

Figure 3

GABA$_B$ receptor activation by analgesic α-conotoxins. The highly conserved structural scaffold of the α-conotoxins consists of two disulfide bonds that stabilize a central helical region. GABA$_B$ receptor is a heterodimer with two almost identical subunits that are both required for a functional receptor. The GABA$_{B1}$ subunit is involved in ligand binding and the GABA$_{B2}$ subunit interacts with the G-protein. The natural ligand of the receptor, GABA, binds to a cleft within the large N-terminal 'Venus fly-trap (VFT)' domain of the GABA$_{B1}$ subunit, triggering a conformational change in the receptor that facilitates interaction with the G-protein by the GABA$_{B2}$ subunit. GPCR activation leads to dissociation of Gα from Gβγ subunits. A single GPCR can couple to either one or more families of Gα proteins, which activate several downstream effectors (Tedford and Zamponi, 2006). Upon ligand binding, Gβγ subunits function as a dimer to interact with many signalling molecules and with ion channels. The schematically shown pore-forming α$_{1\text{-}B}$ subunit of the N-type (Ca$_V$2.2) VGCC consists of IV homologous domains linked by cytoplasmic loops (referred to as I-II, II-III and III-IV linkers) and cytoplasmic N- and C-terminal regions.

Raingo, 2007). The 37a splice isoform, which is preferentially expressed in nociceptive neurones (Bell *et al.*, 2004), exhibits both voltage-dependent and voltage-independent inhibition, whereas the 37b isoform exhibits only voltage-dependent inhibition due to the absence of a consensus site for tyrosine kinase phosphorylation in position 1747 (Raingo *et al.*, 2007). In nociceptors of a murine model lacking exon 37a in Ca$_V$2.2, spinal morphine analgesia is diminished *in vivo* due to a decreased activity-independent G protein inhibition of N-type VGCCs (Andrade *et al.*, 2010).

A novel mechanism of N-type VGCC modulation by α-conotoxins

We examined α-conotoxin Vc1.1 (α4/7), a synthetic peptide, against other known targets involved in pain pathways as it had previously been shown that conotoxins may target more than one class of channel or receptor family. For example, pI14a, a conotoxin from the vermivorous cone *Conus planorbis*, was reported to inhibit both voltage-gated (Kv1.6) and ligand-gated (α3β4, α1β1εδ nAChR) ion channels (Imperial *et al.*, 2006). We found that Vc1.1 and RgIA (α4/3) inhibit N-type VGCC currents in mammalian DRG neurones, whereas vc1a, the native post-translationally modified peptide of Vc1.1, was inactive. The inhibition was indirect and was abolished in the presence of GDPβS, *Pertussis* toxin (PTX), the selective peptide inhibitor of pp60c-src tyrosine kinase and GABA$_B$ receptor antagonists (CGP55845, CGP54626 and phaclofen), whereas selective nAChR, muscarinic AChR, GABA$_A$, α$_1$- and α$_2$-adrenergic, μ-opioid antagonists had no effect on N-type VGCC currents (Callaghan *et al.*, 2008). Inhibition of N-type VGCC currents depended on the frequency and duration of the depolarizing pulse in the presence of Vc1.1, indicating use-dependence of block. The onset of the Vc1.1 effect was slower than that of baclofen and was not reversed by prepulse depolarizations or by toxin washout. It was suggested that this novel type of VGCC inhibition is not a conventional Gβγ-mediated voltage-dependent mechanism but is largely voltage-independent, with the involvement of a PTX-sensitive Gα$_{i/o}$ subfamily and of a c-src kinase (Callaghan *et al.*, 2008). Taken together, these results indicate that analgesic Vc1.1 and Rg1A modulate native N-type Ca$_V$2.2 channel currents via a novel mechanism, acting effectively as agonists via G protein-coupled GABA$_B$ receptors.

In rat DRG neurones, the presence of GABA$_B$ receptor subunits GABA$_{B1}$ and GABA$_{B2}$ has been confirmed (Towers *et al.*, 2000) and the GABA$_B$ receptor-mediated inhibition of Ca$_V$2.2 channels by baclofen has been reported previously (Dolphin and Scott, 1987; Tatebayashi and Ogata, 1992). Neither baclofen nor Vc1.1 mobilized intracellular calcium release in rat DRG neurones, consistent with a Gα$_o$-mediated modulation of N-type VGCCs (Callaghan *et al.*, 2008). In α9

nAChR knockout mice, the inhibition of N-type VGCC currents by Vc1.1 and RgIA was found to be intact, indicating that the α9-containing nAChRs do not mediate the actions of these α-conotoxins (Callaghan and Adams, 2010). Furthermore, we have recently shown that AuIB (α4/6), a selective antagonist of α3β4 nAChRs, inhibits N-type VGCCs via a $GABA_B$ receptor-mediated pathway but does not antagonize α9α10 nAChRs (Klimis *et al.*, 2011). PeIA (α4/7), another conotoxin selective for α9α10 nAChRs, inhibits N-type VGCC currents in DRG neurones (Daly *et al.*, 2011). Vc1.2 (α4/7), a recently identified toxin isolated from embryonic *Conus victoriae*, also inhibits N-type VGCCs; however, it exhibits distinct nAChR selectivity to the adult toxin (Safavi-Hemami *et al.*, 2011). siRNA knockdown of functional $GABA_B$ receptor expression significantly reduces the inhibition of N-type VGCC current in response to baclofen, Vc1.1, Rg1A and AuIB in isolated rat DRG neurones (H. Cuny and D.J. Adams, unpubl. obs.). Vc1.1 and RgIA were unable to displace binding of the selective $GABA_B$ receptor antagonist [^{3}H]CGP54626 to either human $GABA_B$ (1b,2) receptors transiently expressed in HEK293T cells (McIntosh *et al.*, 2009) or rat DRG neurones (S. Nevin and D.J. Adams, unpubl. obs.). These results suggest that Vc1.1 binds to a different ligand binding site than [^{3}H]CGP54626, the competitive antagonist for the GABA binding site on the $GABA_{B1}$ subunit (see Figure 3). The sequences and IC_{50} values of α-conotoxins that inhibit N-type calcium channels are shown in Table 3. Generally, α-conopeptides suffer from the disadvantage of short biological half-lives and poor activity when administered orally. Remarkably, synthetic cyclization of Vc1.1 produces an orally active compound with improved *in vivo* stability, higher potency for $GABA_B$-mediated inhibition of N-type calcium channels and lower potency at α9α10 nAChRs (Clark *et al.*, 2010).

Vc1.1 had no direct effect on recombinant $Ca_V2.1$ (P/Q-type), $Ca_V2.3$ (R-type) and $Ca_V1.2$ (L-type) VGCCs expressed in *Xenopus* oocytes (Callaghan *et al.*, 2008). However, in approximately two-thirds of oocytes expressing recombinant $Ca_V2.2$ channels, application of baclofen inhibited Ba^{2+} currents, consistent with the previously reported presence of endogenous $GABA_B$ receptors in batches of *Xenopus* oocytes (Yang *et al.*, 2001). In these oocytes, Vc1.1 and Rg1A also inhibited Ba^{2+} currents through recombinant $Ca_V2.2$ channels (see supplemental data in Callaghan *et al.*, 2008). McIntosh *et al.* (2009) reported that α-conotoxin Vc1.1 does not modulate GIRK (1,4) channels heterologously expressed with $GABA_B$ receptors in *Xenopus* oocytes. Recently, $GABA_B$ receptor-mediated modulation of transiently or stably expressed $Ca_V2.2$ channels in HEK-293 cells, co-transfected with cDNAs of cloned human $GABA_{B1}$ and $GABA_{B2}$ receptor subunits, was successfully reconstituted (Figure 2D,E) (G. Berecki and D.J. Adams, unpubl. obs.). This approach is particularly useful for investigating $Ca_v2.2$ channel modulation via $GABA_B$ receptors without significant interference of endogenous ion channels.

Anti-allodynic effects of α-conotoxins

Several α-conotoxins with different selectivity for nAChR subtypes have been shown to be effective analgesics in rat models of neuropathic pain (Sandall *et al.*, 2003; Satkunanathan *et al.*, 2005; McIntosh *et al.*, 2009; Klimis *et al.*, 2011). Intramuscular injection of Vc1.1 reversed surgically induced hyperalgesia in rats following chronic constriction injury or partial nerve ligation, with the relief persisting for 1 week post-treatment (Satkunanathan *et al.*, 2005). RgIA also exhibited acute and persistent anti-nociceptive effects in rats following partial nerve ligation (Vincler *et al.*, 2006). Both conopeptides most selectively target α9α10 nAChRs over other nAChR subtypes (Vincler *et al.*, 2006; Nevin *et al.*, 2007), leading to the proposal that antagonism of this receptor is a molecular target for the treatment of neuropathic pain (Vincler *et al.*, 2006). Conversely, the native peptide of Vc1.1, vc1a, has been found to be equipotent at α9α10 nAChRs but inactive in rat neuropathic pain assays (Livett *et al.*, 2006; Nevin *et al.*, 2007), suggesting that an alternative mechanism may underlie the analgesic effects observed with Vc1.1 and RgIA. Recently, Klimis *et al.* (2011) provided evidence that α9α10 nAChRs are not significantly involved in the pain-relieving actions of α-conotoxins, and that Vc1.1 and AuIB exhibit distinct selectivity profiles for N-type VGCCs and nAChRs. The intramuscular administration of Vc1.1 and AuIB produced a long lasting reversal of allodynia in partial nerve ligation rats. The anti-allodynic effect of Vc1.1 was almost completely antagonized by the selective $GABA_B$ inhibitor SCH 50911 *in vivo* (Klimis *et al.*, 2011). It was concluded that the activation of $GABA_B$ receptors by α-conotoxins and subsequent inhibition of N-type VGCCs are the most likely mechanisms mediating these effects, despite their modulation, at lower efficacy, of the α9α10 nAChR.

N-type VGCC inhibition by selective antagonists or via GPCRs produces analgesia in animals and humans (McGivern, 2006; Zamponi *et al.*, 2009). The precise mechanism within the nociceptive pathway responsible for the reversal of allodynia by α-conotoxins is not yet fully understood. *In vitro* patch-clamp electrophysiological studies in rat spinal cord slices could not identify any modulation of primary afferent synaptic transmission by α-conotoxins Vc1.1 or RgIA (L. Motin and D.J. Adams, unpubl. obs.). This might be due to differences in the signalling mechanisms in DRG cell bodies and their central nerve terminals and/or due to the presence of a $Ca_v2.2$ channel splice variant(s) in the presynaptic nerve terminal (Altier *et al.*, 2007; Raingo *et al.*, 2007; Andrade *et al.*, 2010; Gardezi *et al.*, 2010) that is not affected by α-conotoxins.

Conclusion and future directions

Understanding the precise mechanism of action of conotoxins is critical to fully exploit the potential of these peptides to target the N-type VGCCs. Future research will be required to determine the precise binding site(s) for the ω-conotoxins on Cav2.2 and to elucidate the potential of ω-conotoxin CVID-F analogues as pain therapeutics. Similarly, the precise binding site on $GABA_B$ receptor subunits for α-conotoxins has to be determined. Specific radiolabelling of the α-conopeptides could help to confirm specific binding to and distribution of $GABA_B$ receptors. A more complete understanding of the structure–activity relationships of known and chemically

modified α-conotoxins that target $GABA_B$ receptors will require further mutational studies (e.g. alanine scanning). This will help in the design of more potent and specific analogues for the $GABA_B$ receptor versus nAChR subtypes, including peptide modification to improve stability and oral availability. Further studies aimed at determining the precise signalling pathway and the mechanism of VGCC inhibition by various α-conotoxins via GPCRs will be invaluable for the development of more potent and selective analogues of the $GABA_B$ receptor.

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Conflict of interest

None.

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