

REVIEW

Analgesic conotoxins: block and G protein-coupled receptor modulation of N-type ($\text{Ca}_v2.2$) 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

Keywords

ω -conotoxin; α -conotoxin; voltage-gated calcium channel; GABA_B receptor; G protein-coupled receptor; dorsal root ganglion; *Xenopus* oocyte; HEK293 cell; pain pathway

Received

5 September 2011

Revised

24 October 2011

Accepted

3 November 2011

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 Rg1A 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 $\alpha_9\alpha_{10}$ neuronal nAChR. Surprisingly, however, α -conotoxins Vc1.1, Rg1A and Pe1A 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

Au1B, α -conotoxin Au1B; $\text{Ca}_v2.1$, P/Q-type calcium channel; $\text{Ca}_v2.2$, 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; Pe1A, α -conotoxin Pe1A; Rg1A, α -conotoxin Rg1A; 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 character-

ization 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 (α_{1B-d}) and peripheral (α_{1B-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 $Ca_v2.2$

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 Gly⁵, Tyr¹³ and Ser¹⁹. Conserved cysteine residues are in bold. Shown are the positions of the four loops and the disulfide scaffold in ω -conotoxins.

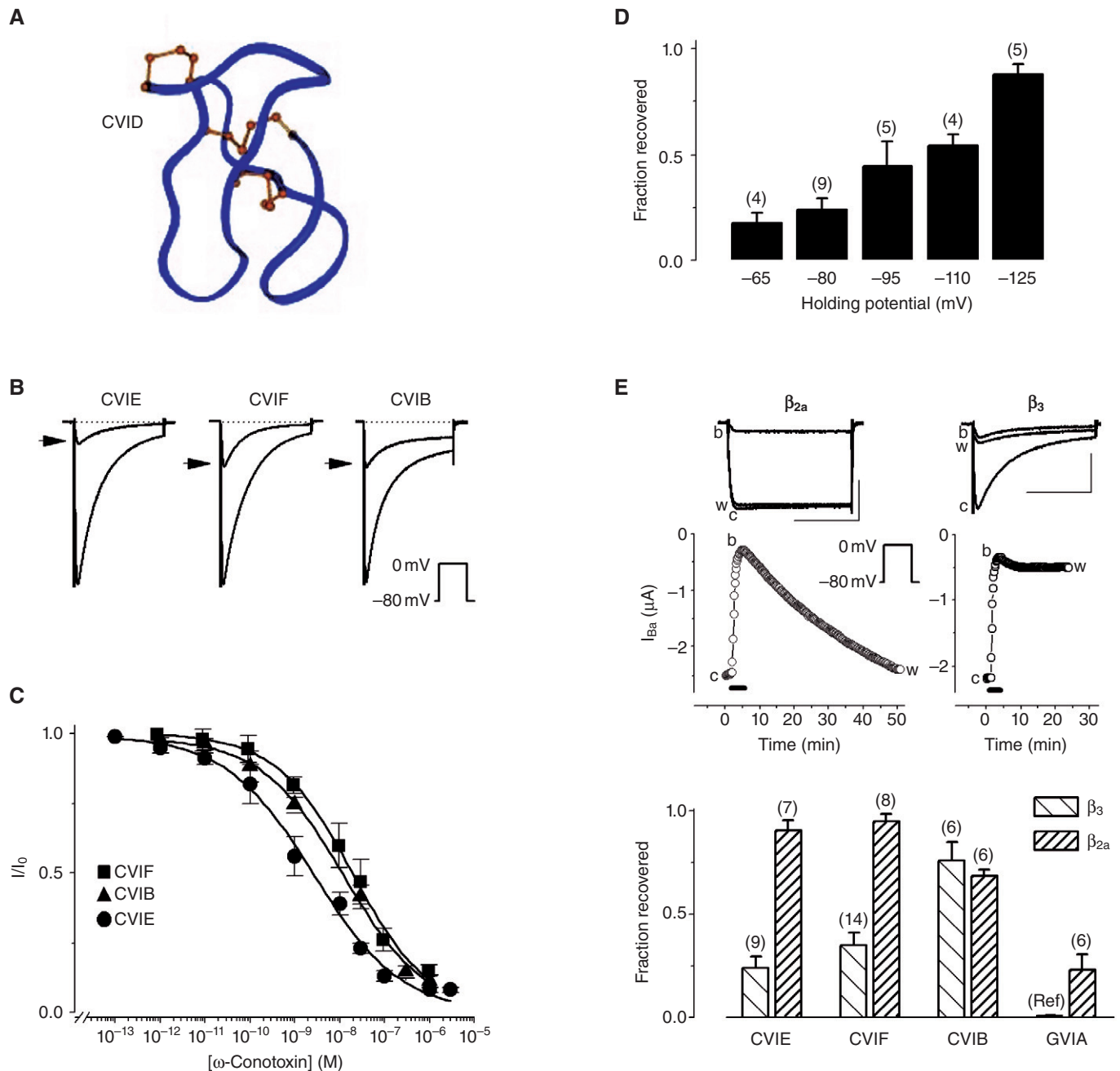


Figure 1

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 $\alpha_{1B-b}/\alpha_{2\delta 1}/\beta_{2a}$ or $\alpha_{1B-b}/\alpha_{2\delta 1}/\beta_3$ 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- ω VIA, IC ₅₀	Mechanism of VGCC block	Reversibility of N-type VGCC block (cloned/native)
ω -GVIA <i>Conus geographus</i>	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 <i>Conus magus</i>	N ⁷	5 nM ⁸ (X)/200 nM ⁹ (hc)	55 pM ³	Pore block ⁵	Poorly reversible in the presence of $\alpha_{1B-b}/\alpha_{2\delta 1}/\beta_3$ subunits; irreversible in the presence of $\alpha_{1B}/\alpha_{2\delta}/\beta_{1b}$ subunits ⁵ /irreversible in spinal cord slice preparation ⁶
ω -MWIIC <i>C. magus</i>	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 -MWIID <i>C. magus</i>	N ¹² and P/Q	nd/nd	[§] 100 nM ¹³	nd	nd/reversible in BC cells ¹³
ω -RVIA <i>Conus radiatus</i>	N ¹⁴	nd/nd	^{§§} 8.4–10.4 nM ¹⁵	nd	nd/nd
ω -TVIA <i>Conus tulipa</i>	N ¹⁶	nd/nd	251 pM ¹⁷	nd	nd/nd
ω -SVIB <i>Conus striatus</i>	N ¹⁸ and P/Q	nd/nd	3.2 nM ¹⁹	nd	nd/nd
ω -CnVIIA <i>Conus consors</i>	N ²⁰	nd/nd	2.7–3.7 pM ²⁰	nd	nd/nd
ω -CVIA <i>Conus catus</i>	N ³	nd/nd	560 pM ³	nd but likely pore block	nd/nd
ω -CVIB <i>C. catus</i>	N ³ and P/Q	33 nM (X)/23 nM ² (DRG)	7.7 nM ³	nd but likely pore block	Reversible in the presence of $\alpha_{1B-b}/\alpha_{2\delta 1}/\beta_3^{21}$ and $\alpha_{1B-b}/\alpha_{2\delta 1}/\beta_{2a}^{22}$ subunits/reversible in spinal cord slice preparation ⁶
ω -CVIC <i>C. catus</i>	N ³ and P/Q	nd/nd	7.6 nM ³	nd but likely pore block	nd/nd
ω -CVID <i>C. catus</i>	N ³	21.8 nM ⁸ (X)/9 nM ² (DRG)	70 pM ³	nd but likely pore block	Reversible in the absence of $\alpha_{2\delta 1}$ subunit ⁸ ; weakly reversible in the presence of $\alpha_{2\delta 1}$ subunit ⁸ /irreversible in spinal cord slice preparation ⁶
ω -S03 <i>C. striatus</i>	N ²²	nd/160 nM ²³ (hc)	nd	nd but likely pore block	nd/almost fully reversible in hippocampal neurones ²³
ω -CVIE <i>C. catus</i>	N ²¹	2.6 nM (X) ²¹ /nd	19 pM ²¹	nd but likely pore block	Poorly reversible in the presence of $\alpha_{1B-b}/\alpha_{2\delta 1}/\beta_3$ subunits ²¹ ; reversible in the presence of $\alpha_{1B-b}/\alpha_{2\delta 1}/\beta_{2a}$ subunits ²¹ /poorly reversible in DRG neurone ²¹ ; reversible in spinal cord slice preparation ²¹
ω -CVIF <i>C. catus</i>	N ²¹	19.9 nM ²¹ (X)/nd	25 pM ²¹	nd but likely pore block	Poorly reversible in the presence of $\alpha_{1B-b}/\alpha_{2\delta 1}/\beta_3$ subunits ²¹ ; reversible in the presence of $\alpha_{1B-b}/\alpha_{2\delta 1}/\beta_{2a}$ subunits ²¹ /poorly reversible in DRG neurone ²¹ ; reversible in spinal cord slice preparation ²¹
ω -FVIA <i>Conus fulmen</i>	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 ¹²⁵I- ω VIA binding to rat brain membrane.

[§]Displacement of ¹²⁵I- ω VIA binding to bovine adrenal medullary membrane.

^{§§}Displacement of ¹²⁵I- ω VIA binding in Swiss Webster mouse cortex.

Superscript numbers between parentheses refer to references as follows: (1) 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; HEK293 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 α_{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 ($\text{Ca}_v2.1$) 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, Yarotsky 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 Lys¹⁰ and Leu¹¹) 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 knock-out 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: α 3/5, α 4/3, α 4/4, α 4/6 and α 4/7 (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 ($\text{Ca}_v2.2$) 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 <i>et al.</i> , 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 <i>et al.</i> , 2011
RglA	G	C	C	S	D	P	R	C	R	Y	R	–	–	–	–	C	R	7.3	Callaghan and Adams, 2010
PelA	G	C	C	S	H	P	A	C	S	V	N	H	P	E	L	C	– NH ₂	1.1	Daly <i>et al.</i> , 2011
AulB	G	C	C	S	Y	P	P	C	F	A	T	N	P	D	–	C	– NH ₂	1.5	Klimis <i>et al.</i> , 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 $\text{G}\alpha$ -GTP and $\text{G}\beta\gamma$ 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 $\text{G}\alpha$ 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 $\text{G}\beta\gamma$ binding directly to the I-II linker of the VGCC α_1 subunit of the N-type VGCC (Bean, 1989; Hille, 1994; Tedford *et al.*, 2010) (Figure 3). The free $\text{G}\beta\gamma$ heterodimers do not only signal exclusively to VGCCs but also modulate downstream effectors such as phospholipase C and adenylyl cyclase. The $\text{G}\alpha$ 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

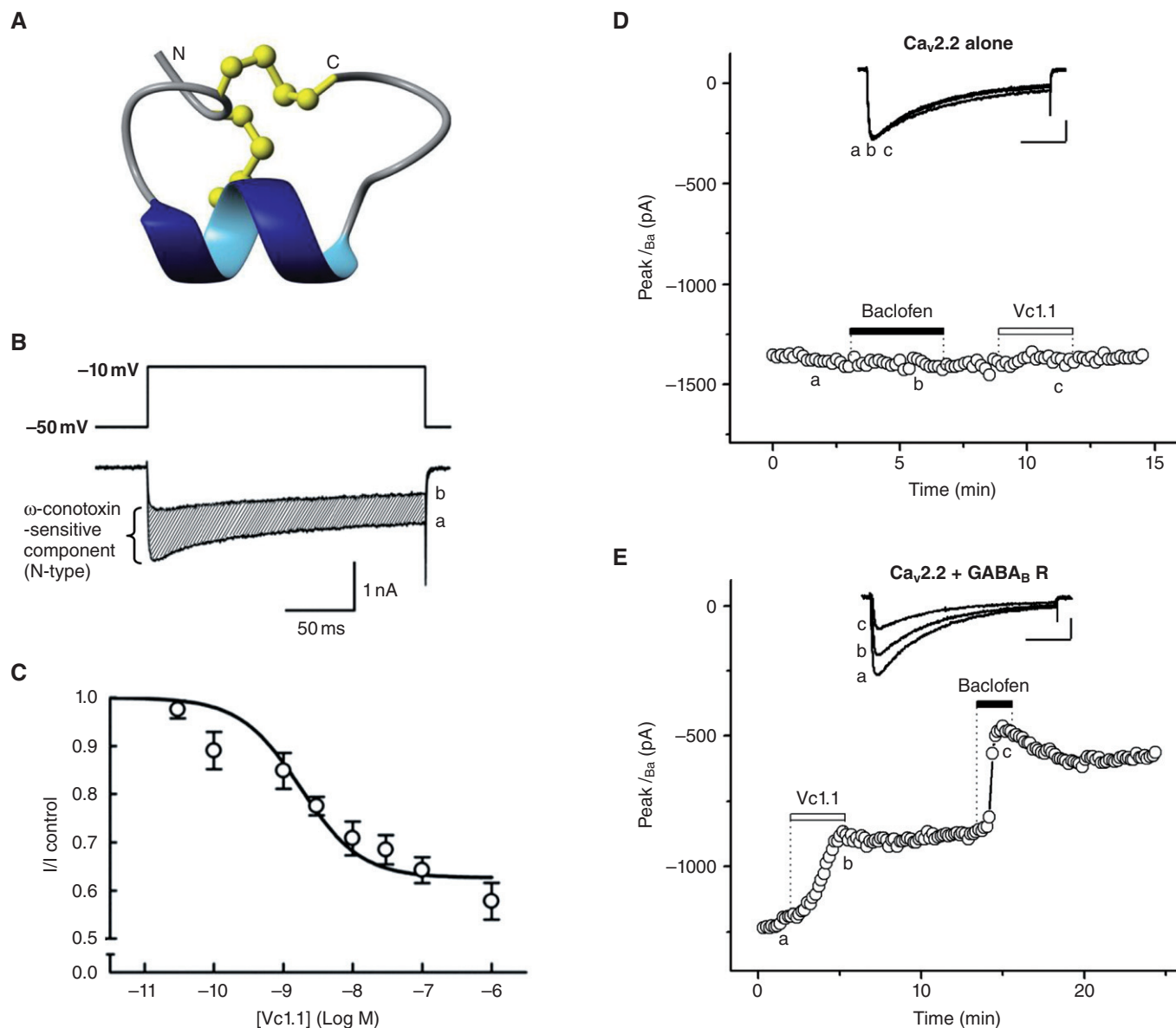


Figure 2

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 μ M) or Vc1.1 (100 nM) does not affect I_{Ba} in cells co-transfected only with cDNAs of α_{1-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 $GABA_{B1,2}$ 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

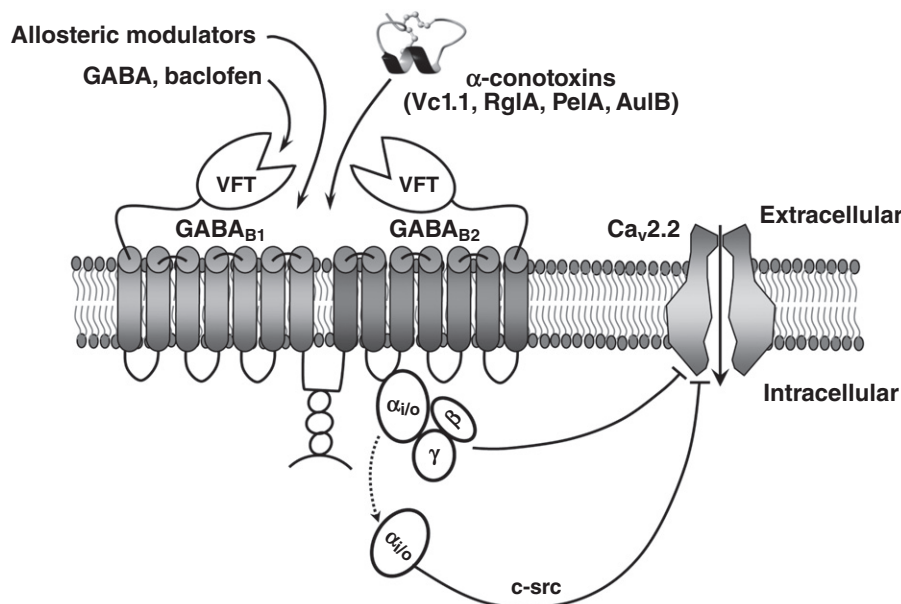


Figure 3

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 G α from G $\beta\gamma$ subunits. A single GPCR can couple to either one or more families of G α proteins, which activate several downstream effectors (Tedford and Zamponi, 2006). Upon ligand binding, G $\beta\gamma$ subunits function as a dimer to interact with many signalling molecules and with ion channels. The schematically shown pore-forming α_{1-B} subunit of the N-type (Ca_v2.2) 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_v2.2, 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 (α 4/7), 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, p114a, a conotoxin from the vermivorous cone *Conus planorbis*, was reported to inhibit both voltage-gated (Kv1.6) and ligand-gated (α 3 β 4, α 1 β 1 ϵ δ nAChR) ion channels (Imperial *et al.*, 2006). We found that Vc1.1 and RglA (α 4/3) 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 GDP β S, *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 G $\beta\gamma$ -mediated voltage-dependent mechanism but is largely voltage-independent, with the involvement of a PTX-sensitive G $\alpha_{i/o}$ subfamily and of a c-src kinase (Callaghan *et al.*, 2008). Taken together, these results indicate that analgesic Vc1.1 and RglA modulate native N-type Ca_v2.2 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_v2.2 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 G α_o -mediated modulation of N-type VGCCs (Callaghan *et al.*, 2008). In α 9

nAChR knockout mice, the inhibition of N-type VGCC currents by Vc1.1 and Rg1A was found to be intact, indicating that the $\alpha 9$ -containing nAChRs do not mediate the actions of these α -conotoxins (Callaghan and Adams, 2010). Furthermore, we have recently shown that AuIB ($\alpha 4/6$), a selective antagonist of $\alpha 3\beta 4$ nAChRs, inhibits N-type VGCCs via a GABA_B receptor-mediated pathway but does not antagonize $\alpha 9\alpha 10$ nAChRs (Klimis *et al.*, 2011). PeIA ($\alpha 4/7$), another conotoxin selective for $\alpha 9\alpha 10$ nAChRs, inhibits N-type VGCC currents in DRG neurones (Daly *et al.*, 2011). Vc1.2 ($\alpha 4/7$), 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 Rg1A were unable to displace binding of the selective GABA_B receptor antagonist [³H]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 [³H]CGP54626, the competitive antagonist for the GABA binding site on the GABA_{B1} subunit (see Figure 3). The sequences and IC₅₀ 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 $\alpha 9\alpha 10$ 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 Ba²⁺ 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 Ba²⁺ 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; Satkunathan *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). Rg1A also exhibited acute and persistent anti-nociceptive effects in rats following partial nerve ligation (Vincler *et al.*, 2006). Both conopeptides most selectively target $\alpha 9\alpha 10$ 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 $\alpha 9\alpha 10$ 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 Rg1A. Recently, Klimis *et al.* (2011) provided evidence that $\alpha 9\alpha 10$ 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 $\alpha 9\alpha 10$ 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 Rg1A (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 α -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.

Acknowledgements

Our research is supported by grants from the National Health & Medical Research Council and the Australian Research Council (ARC) to DJA. DJA is an ARC Australian Professorial Fellow.

Conflict of interest

None.

References

- Abbott JR, Litzinger MJ (1994). Different ω -conotoxins mark the development of Swiss Webster mouse cortex suggesting N-type voltage sensitive calcium channel subtypes. *Int J Dev Neurosci* 12: 43–47.
- Adams DJ, Alewood PF, Craik DJ, Drinkwater RD, Lewis RJ (1999). Conotoxins and their potential pharmaceutical applications. *Drug Dev Res* 46: 219–234.
- Adams DJ, Smith AB, Schroeder CI, Yasuda T, Lewis RJ (2003). ω -Conotoxin CVIE inhibits a pharmacologically distinct voltage-sensitive calcium channel associated with transmitter release from preganglionic nerve terminals. *J Biol Chem* 278: 4057–4062.
- Alexander SPH, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th Edition. *Br J Pharmacol* 164 (Suppl. 1): S1–S324.
- Altier C, Dale CS, Kisilevsky AE, Chapman K, Castiglioni AJ, Matthews EA *et al.* (2007). Differential role of N-type calcium channel splice isoforms in pain. *J Neurosci* 27: 6363–6373.
- Andrade A, Denome S, Jiang YO, Marangoudakis S, Lipscombe D (2010). Opioid inhibition of N-type Ca²⁺ channels and spinal analgesia couple to alternative splicing. *Nat Neurosci* 13: 1249–1256.
- Auer S, Stürzebecher AS, Jüttner R, Santos-Torres J, Hanack C, Frahm S *et al.* (2010). Silencing neurotransmission with membrane-tethered toxins. *Nat Methods* 7: 229–236.
- Azam L, McIntosh JM (2009). Alpha-conotoxins as pharmacological probes of nicotinic acetylcholine receptors. *Acta Pharmacol Sin* 30: 771–783.
- Baell JB, Duggan PJ, Lok YP (2004). ω -Conotoxins and approaches to their non-peptide mimetics. *Aust J Chem* 57: 179–185.
- Bean BP (1989). Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature* 340: 153–156.
- Beech DJ, Bernheim L, Hille B (1992). Pertussis toxin and voltage dependence distinguish multiple pathways modulating calcium channels of rat sympathetic neurons. *Neuron* 8: 97–106.
- Bell TJ, Thaler C, Castiglioni AJ, Helton TD, Lipscombe D (2004). Cell-specific alternative splicing increases calcium channel current density in the pain pathway. *Neuron* 41: 127–138.
- Berecki G, Motin L, Haythornthwaite A, Vink S, Bansal P, Drinkwater R *et al.* (2010). Analgesic ω -conotoxins CVIE and CVIF selectively and voltage-dependently block recombinant and native N-type calcium channels. *Mol Pharmacol* 77: 139–148.
- Bernheim L, Beech DJ, Hille B (1991). A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons. *Neuron* 6: 859–867.
- Bettler B, Kaupmann K, Mosbacher J, Gassmann M (2004). Molecular structure and physiological functions of GABA_B receptors. *Physiol Rev* 84: 835–867.
- Bingham JP, Mitsunaga E, Bergeron ZL (2010). Drugs from slugs – past, present and future perspectives of ω -conotoxin research. *Chem Biol Interact* 183: 1–18.
- Boland LM, Morrill JA, Bean BP (1994). ω -Conotoxin block of N-type calcium channels in frog and rat sympathetic neurons. *J Neurosci* 14: 5011–5027.
- Brown DA, Sihra TS (2008). Presynaptic signaling by heterotrimeric G-proteins. *Handb Exp Pharmacol* 184: 207–260.
- Bulaj G, Olivera BM (2008). Folding of conotoxins: formation of the native disulfide bridges during chemical synthesis and biosynthesis of *Conus* peptides. *Antioxid Redox Signal* 10: 141–155.
- Callaghan B, Adams DJ (2010). Analgesic α -conotoxins Vc1.1 and Rg1A inhibit N-type calcium channels in sensory neurons of alpha9 nicotinic receptor knockout mice. *Channels (Austin)* 4: 51–54.
- Callaghan B, Haythornthwaite A, Berecki G, Clark RJ, Craik DJ, Adams DJ (2008). Analgesic α -conotoxins Vc1.1 and Rg1A inhibit N-type calcium channels in rat sensory neurons via GABA_B receptor activation. *J Neurosci* 28: 10943–10951.
- Campbell JN, Meyer RA (2006). Mechanisms of neuropathic pain. *Neuron* 52: 77–92.
- Catterall WA (2011). Voltage-gated calcium channels. *Cold Spring Harb Perspect Biol* 3: a003947.
- Catterall WA, Perez-Reyes E, Snutch TP, Striessnig J (2005). Nomenclature and structure-function relationships of voltage-gated calcium channels. *Pharmacol Rev* 57: 411–425.
- Clark RJ, Fischer H, Nevin ST, Adams DJ, Craik DJ (2006). The synthesis, structural characterization, and receptor specificity of the α -conotoxin Vc1.1. *J Biol Chem* 281: 23254–23263.
- Clark RJ, Jensen J, Nevin ST, Callaghan BP, Adams DJ, Craik DJ (2010). The engineering of an orally active conotoxin for the treatment of neuropathic pain. *Angew Chem Int Ed Engl* 49: 6545–6548.
- Comps-Agrar L, Kniazeff J, Nørskov-Lauritsen L, Maurel D, Gassmann M, Gregor N *et al.* (2011). The oligomeric state sets GABA_B receptor signalling efficacy. *EMBO J* 30: 2336–2349.
- Craig AG, Norberg T, Griffin D, Hoeger C, Akhtar M, Schmidt K *et al.* (1999). Contulakin-G, an O-glycosylated invertebrate neurotensin. *J Biol Chem* 274: 13752–13759.

- Currie KP (2010). G protein modulation of Ca_v2 voltage-gated calcium channels. *Channels (Austin)* 4: 497–509.
- Daly NL, Craik DJ (2009). Structural studies of conotoxins. *IUBMB Life* 61: 144–150.
- Daly NL, Callaghan B, Clark RJ, Nevin ST, Adams DJ, Craik DJ (2011). Structure and activity of alpha-conotoxin PeIA at nicotinic acetylcholine receptor subtypes and GABA_B receptor-coupled N-type calcium channels. *J Biol Chem* 286: 10233–10237.
- Delmas P, Brown DA, Dayrell M, Abogadie FC, Caulfield MP, Buckley NJ (1998). On the role of endogenous G-protein $\beta\gamma$ subunits in N-type Ca^{2+} current inhibition by neurotransmitters in rat sympathetic neurones. *J Physiol* 506: 319–329.
- Dolphin AC (1998). Mechanisms of modulation of voltage-dependent calcium channels by G proteins. *J Physiol* 506: 3–11.
- Dolphin AC, Scott RH (1987). Calcium channel currents and their inhibition by (-)-baclofen in rat sensory neurones: modulation by guanine nucleotides. *J Physiol* 386: 1–17.
- Doughty SW, Blaney FE, Orlek BS, Richards WG (1998). A molecular mechanism for toxin block in N-type calcium channels. *Protein Eng* 11: 95–99.
- Ellinor PT, Zhang J-F, Horne WA, Tsien RW (1994). Structural determinants of the blockade of N-type calcium channels by a peptide neurotoxin. *Nature* 372: 272–275.
- Favreau P, Gilles N, Lamthanh H, Bournaud R, Shimahara T, Bouet F *et al.* (2001). A new ω -conotoxin that targets N-type voltage-sensitive calcium channels with unusual specificity. *Biochemistry* 40: 14567–14575.
- Feng ZP, Hamid J, Doering C, Jarvis SE, Boley GM, Bourinet E *et al.* (2001a). Amino acid residues outside of the pore region contribute to N-type calcium channel permeation. *J Biol Chem* 276: 5726–5730.
- Feng ZP, Hamid J, Doering C, Boley GM, Snutch TP, Zamponi GW (2001b). Residue Gly1326 of the N-type calcium channel α_1B subunit controls reversibility of ω -conotoxin GVIA and MVIIA block. *J Biol Chem* 276: 15728–15735.
- Feng ZP, Doering CJ, Winkfein RJ, Beedle AM, Spafford JD, Zamponi GW (2003). Determinants of inhibition of transiently expressed voltage-gated calcium channels by ω -conotoxins GVIA and MVIIA. *J Biol Chem* 278: 20171–20178.
- Gandia L, Lara B, Imperial JS, Villarroya M, Albillos A, Maroto R *et al.* (1997). Analogies and differences between ω -conotoxins MVIIIC and MVIIID: binding sites and functions in bovine chromaffin cells. *Pflügers Arch* 435: 55–64.
- Gardezi SR, Taylor P, Stanley EF (2010). Long C terminal splice variant $\text{Ca}_v2.2$ identified in presynaptic membrane by mass spectrometric analysis. *Channels (Austin)* 4: 58–62.
- Goudet C, Magnaghi V, Landry M, Nagy F, Gereau RW, Pin JP (2009). Metabotropic receptors for glutamate and GABA in pain. *Brain Res Brain Res Rev* 60: 43–56.
- Gray AC, Raingo J, Lipscombe D (2007). Neuronal calcium channels: splicing for optimal performance. *Cell Calcium* 42: 409–417.
- Gray WR, Luque A, Olivera BM, Barrett J, Cruz LJ (1981). Peptide toxins from *Conus geographus* venom. *J Biol Chem* 256: 4734–4740.
- Hannan S, Wilkins ME, Dehghani-Tafti E, Thomas P, Baddeley SM, Smart TG (2011). γ -Aminobutyric acid type B (GABA_B) receptor internalization is regulated by the R2 subunit. *J Biol Chem* 286: 24324–24335.
- Hille B (1994). Modulation of ion-channel function by G-protein-coupled receptors. *Trends Neurosci* 17: 531–536.
- Hillyard DR, Monje VD, Mintz IM, Bean BP, Nadasdi L, Ramachandran J *et al.* (1992). A new *Conus* peptide ligand for mammalian presynaptic Ca^{2+} channels. *Neuron* 9: 69–77.
- Hurley JH, Cahill AL, Currie KP, Fox AP (2000). The role of dynamic palmitoylation in Ca^{2+} channel inactivation. *Proc Natl Acad Sci U S A* 97: 9293–9298.
- Imperial JS, Bansal PS, Alewood PF, Daly NL, Craik DJ, Sporning A *et al.* (2006). A novel conotoxin inhibitor of Kv1.6 channel and nAChR subtypes defines a new superfamily of conotoxins. *Biochemistry* 45: 8331–8340.
- Jakubowski JA, Kelley WP, Sweedler JV (2006). Screening for post-translational modifications in conotoxins using liquid chromatography/mass spectrometry: an important component of conotoxin discovery. *Toxicon* 47: 688–699.
- Janes RW (2005). α -Conotoxins as selective probes for nicotinic acetylcholine receptor subclasses. *Curr Opin Pharmacol* 5: 280–292.
- Kaas Q, Westermann JC, Halai R, Wang CK, Craik DJ (2008). ConoServer, a database for conopeptide sequences and structures. *Bioinformatics* 24: 445–446.
- Kaas Q, Westermann JC, Craik DJ (2010). Conopeptide characterization and classifications: an analysis using ConoServer. *Toxicon* 55: 1491–1509.
- Kammermeier PJ, Ruiz-Velasco V, Ikeda SR (2000). A voltage-independent calcium current inhibitory pathway activated by muscarinic agonists in rat sympathetic neurons requires both $\text{G}\alpha_q/11$ and $\text{G}\beta\gamma$. *J Neurosci* 20: 5623–5629.
- Kaneko S, Cooper CB, Nishioka N, Yamasaki H, Suzuki A, Jarvis SE *et al.* (2002). Identification and characterization of novel human $\text{Ca}_v2.2$ (α_{1B}) calcium channel variants lacking the synaptic protein interaction site. *J Neurosci* 22: 82–92.
- Kaupmann K, Hugel K, Heid J, Flor PJ, Bischoff S, Mickel SJ *et al.* (1997). Expression cloning of GABA_B receptors uncovers similarity to metabotropic glutamate receptors. *Nature* 386: 239–246.
- Kerr LM, Yoshikami D (1984). A venom peptide with a novel presynaptic blocking action. *Nature* 308: 282–284.
- Kim JI, Takahashi M, Ohtake A, Wakamiya A, Sato K (1995). Tyr13 is essential for the activity of ω -conotoxin MVIIA and GVIA, specific N-type calcium channel blockers. *Biochem Biophys Res Commun* 206: 449–454.
- Klimis H, Adams DJ, Callaghan B, Nevin S, Alewood PF, Vaughan CW *et al.* (2011). A novel mechanism of inhibition of high-voltage activated calcium channels by α -conotoxins contributes to relief of nerve injury-induced neuropathic pain. *Pain* 152: 259–266.
- Kniazeff J, Prézeau L, Rondard P, Pin JP, Goudet C (2011). Dimers and beyond: the functional puzzles of class C GPCRs. *Pharmacol Ther* 130: 9–25.
- Lampe RA, Lo MMS, Keith RA, Horn MB, McLane MW, Herman JL *et al.* (1993). Effects of site-specific acetylation on omega-conotoxin GVIA binding and function. *Biochemistry* 32: 3255–3260.
- Lee S, Kim Y, Back SK, Choi HW, Lee JY, Jung HH *et al.* (2010). Analgesic effect of highly reversible ω -conotoxin FVIA on N type Ca^{2+} channels. *Mol Pain* 6: 97.
- Lewis RJ, Nielsen KJ, Craik DJ, Loughnan ML, Adams DA, Sharpe IA *et al.* (2000). Novel ω -conotoxins from *Conus catus* discriminate among neuronal calcium channel subtypes. *J Biol Chem* 275: 35335–35344.

- Liang H, Elmslie KS (2002). Rapid and reversible block of N-type calcium channels (Cav 2.2) by ω -conotoxin GVIA in the absence of divalent cations. *J Neurosci* 22: 8884–8890.
- Liang H, DeMaria CD, Erickson MG, Mori MX, Alseikhan BA, Yue DT (2003). Unified mechanisms of Ca^{2+} regulation across the Ca^{2+} channel family. *Neuron* 39: 951–960.
- Lipscombe D, Raingo J (2007). Alternative splicing matters: N-type calcium channels in nociceptors. *Channels (Austin)* 1: 225–227.
- Lipscombe D, Pan JQ, Gray AC (2002). Functional diversity in neuronal voltage-gated calcium channels by alternative splicing of $\text{Ca}_v\alpha 1$. *Mol Neurobiol* 26: 21–44.
- Livett BG, Sandall DW, Keays D, Down J, Gayler KR, Satkunathan N *et al.* (2006). Therapeutic applications of conotoxins that target the neuronal nicotinic acetylcholine receptor. *Toxicon* 48: 810–829.
- Lu BS, Yu F, Zhao D, Huang PT, Huang CF (1999). Conopeptides from *Conus striatus* and *Conus textile* by cDNA cloning. *Peptides* 20: 1139–1144.
- Luebke JI, Dunlap K (1994). Sensory neuron N-type calcium currents are inhibited by both voltage-dependent and -independent mechanisms. *Pflugers Arch* 428: 499–507.
- Luo ZD, Chaplan SR, Higuera ES, Sorkin LS, Stauderman KA, Williams ME *et al.* (2001). Upregulation of dorsal root ganglion $\alpha_2\delta$ calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. *J Neurosci* 21: 1868–1875.
- Margeta-Mitrovic M, Jan YN, Jan LY (2000). A trafficking checkpoint controls GABA_A receptor heterodimerization. *Neuron* 27: 97–106.
- McCudden CR, Hains MD, Kimple RJ, Siderovski DP, Willard FS (2005). G-protein signaling: back to the future. *Cell Mol Life Sci* 62: 551–577.
- McDonough SI, Swartz KJ, Mintz IM, Boland LM, Bean BP (1996). Inhibition of calcium channels in rat central and peripheral neurons by ω -conotoxin MVIIC. *J Neurosci* 16: 2612–2623.
- McDonough SI, Boland LM, Mintz IM, Bean BP (2002). Interactions among toxins that inhibit N-type and P-type calcium channels. *J Gen Physiol* 119: 313–328.
- McGivern JG (2006). Targeting N-type and T-type calcium channels for the treatment of pain. *Drug Discov Today* 11: 245–253.
- McIntosh JM, Absalom N, Chebib M, Elgoyhen AB, Vincler M (2009). Alpha9 nicotinic acetylcholine receptors and the treatment of pain. *Biochem Pharmacol* 78: 693–702.
- Miljanich GP, Ramachandran J (1995). Antagonists of neuronal calcium channels: structure, function, and therapeutic implications. *Annu Rev Pharmacol Toxicol* 35: 707–734.
- Miljanich GP, Bowersox SS, Fox JA, Valentino KL, Bitner RS, Yamashiro DH (1993). Compositions for delayed treatment of ischaemia-related neuronal damage. Publication no. WO 93/10145; International application no. PCT/US92 09766; International Patent Classification, C07K 7/10, A61K37/02.
- Monje VD, Haack JA, Naisbitt SR, Miljanich G, Ramachandran J, Nadasdi L *et al.* (1993). A new *Conus* peptide ligand for Ca channel subtypes. *Neuropharmacology* 32: 1141–1149.
- Motin L, Adams DJ (2008). ω -Conotoxin inhibition of excitatory synaptic transmission evoked by dorsal root stimulation in rat superficial dorsal horn. *Neuropharmacology* 55: 860–864.
- Motin L, Yasuda T, Schroeder C, Lewis R, Adams D (2007). ω -Conotoxin CVIB differentially inhibits native and recombinant N- and P/Q-type calcium channels. *Eur J Neurosci* 25: 435–444.
- Mould J, Yasuda T, Schroeder CI, Beedle AM, Doering CJ, Zamponi GW *et al.* (2004). The $\alpha_2\delta$ auxiliary subunit reduces affinity of ω -conotoxins for recombinant N-type ($\text{Ca}_v2.2$) calcium channels. *J Biol Chem* 279: 34705–34714.
- Neely A, Wei X, Olcese R, Birnbaumer L, Stefani E (1993). Potentiation by the beta subunit of the ratio of the ionic current to the charge movement in the cardiac calcium channel. *Science* 262: 575–578.
- Nevin ST, Clark RJ, Klimis H, Christie MJ, Craik DJ, Adams DJ (2007). Are $\alpha 9\alpha 10$ nicotinic acetylcholine receptors a pain target for α -conotoxins? *Mol Pharmacol* 72: 1406–1410.
- Newton RA, Bingham S, Case PC, Sanger G, Lawson SN (2001). Dorsal root ganglion neurons show increased expression of the calcium channel $\alpha_2\delta$ -1 subunit following partial sciatic nerve injury. *Brain Res Mol Brain Res* 95: 1–8.
- Nielsen KJ, Schroeder T, Lewis R (2000). Structure-activity relationships of ω -conotoxins at N-type voltage-sensitive calcium channels. *J Mol Recognit* 13: 55–70.
- Nowicky MC, Fox AP, Tsien RW (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* 316: 440–443.
- Oldham WM, Hamm HE (2006). Structural basis of function in heterotrimeric G proteins. *Q Rev Biophys* 39: 117–166.
- Oldham WM, Hamm HE (2008). Heterotrimeric G protein activation by G-protein-coupled receptors. *Nat Rev Mol Cell Biol* 9: 60–71.
- Olivera BM (2006). *Conus* peptides: biodiversity-based discovery and exogenomics. *J Biol Chem* 281: 31173–31177.
- Olivera BM, McIntosh JM, Cruz LJ, Luque FA, Gray WR (1984). Purification and sequence of a presynaptic peptide toxin from *Conus geographus* venom. *Biochemistry* 23: 5087–5090.
- Olivera BM, Gray WR, Zeikus R, McIntosh JM, Varga J, Rivier J *et al.* (1985). Peptide neurotoxins from fish-hunting cone snails. *Science* 230: 1338–1343.
- Olivera BM, Cruz LJ, de Santos V, LeCheminant GW, Griffin D, Zeikus R *et al.* (1987). Neuronal calcium channel antagonists. Discrimination between calcium channel subtypes using ω -conotoxin from *Conus magus* venom. *Biochemistry* 26: 2086–2090.
- Park J, Luo ZD (2010). Calcium channel functions in pain processing. *Channels (Austin)* 4: 510–517.
- Pexton T, Moeller-Bertram T, Schilling JM, Wallace MS (2011). Targeting voltage-gated calcium channels for the treatment of neuropathic pain: a review of drug development. *Expert Opin Investig Drugs* 20: 1277–1284.
- Pierce KL, Premont RT, Lefkowitz RJ (2002). Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 3: 639–650.
- Raingo J, Castiglioni AJ, Lipscombe D (2007). Alternative splicing controls G protein-dependent inhibition of N-type calcium channels in nociceptors. *Nat Neurosci* 10: 285–292.
- Ramilo CA, Zafaralla GC, Nadasdi L, Hammerland LG, Yoshikami D, Gray WR *et al.* (1992). Novel α - and ω -conotoxins from *Conus striatus* venom. *Biochemistry* 31: 9919–9926.

- Safavi-Hemami H, Siero WA, Kuang Z, Williamson NA, Karas JA, Page LR *et al.* (2011). Embryonic toxin expression in the cone snail *Conus victoriae*: primed to kill or divergent function? *J Biol Chem* 286: 22546–22557.
- Sandall DW, Satkunathan N, Keays DA, Polidano MA, Liping X, Pham V *et al.* (2003). A novel α -conotoxin identified by gene sequencing is active in suppressing the vascular response to selective stimulation of sensory nerves *in vivo*. *Biochemistry* 42: 6904–6911.
- Satkunathan N, Livett B, Gayler K, Sandall D, Down J, Khalil Z (2005). α -Conotoxin Vc1.1 alleviates neuropathic pain and accelerates functional recovery of injured neurones. *Brain Res* 1059: 149–158.
- Schroeder CI, Smythe ML, Lewis RJ (2004). Development of small molecules that mimic the binding of ω -conotoxins at the N-type voltage-gated calcium channel. *Mol Divers* 8: 127–134.
- Schroeder CI, Lewis RJ, Adams DJ (2005). Block of voltage-gated calcium channels by peptide toxins. In: Zamponi GW (ed.). *Voltage-Gated Calcium Channels*. Eureka.com and Kluwer Academic/Plenum Publishers: Georgetown, pp. 294–308.
- Scott VE, De Waard M, Liu H, Gurnett CA, Venzke DP, Lennon VA *et al.* (1996). β subunit heterogeneity in N-type Ca^{2+} channels. *J Biol Chem* 271: 3207–3212.
- Sharpe IA, Gehrmann J, Loughnan ML, Thomas L, Adams DA, Atkins A *et al.* (2001). Two new classes of conopeptides inhibit the α_1 -adrenoceptor and noradrenaline transporter. *Nat Neurosci* 4: 902–907.
- Sharpe IA, Thomas L, Loughnan M, Motin L, Palant E, Croker DE *et al.* (2003). Allosteric α_1 -adrenoceptor antagonism by the conopeptide p-TIA. *J Biol Chem* 278: 34451–34457.
- Shukla AK, Kim J, Ahn S, Xiao K, Shenoy SK, Liedtke W *et al.* (2010). Arresting a TRP channel β -arrestin 1 mediates ubiquitination and functional downregulation of TRPV4. *J Biol Chem* 285: 30115–30125.
- Smith MT, Cabot PJ, Ross FB, Robertson AD, Lewis RJ (2002). The novel N-type calcium channel blocker, AM336, produces potent dose-dependent antinociception after intrathecal dosing in rats and inhibits substance P release in rat spinal cord slices. *Pain* 96: 119–127.
- Snutch TP (2005). Targeting chronic and neuropathic pain: the N-type calcium channel comes of age. *NeuroRx* 2: 662–670.
- Spence I, Gillessen D, Gregson RP, Quinn RJ (1977). Characterization of the neurotoxic constituents of *Conus geographus* (L.) venom. *Life Sci* 21: 1759–1769.
- Stocker JW, Nadasdi L, Aldrich RW, Tsien RW (1997). Preferential interaction of ω -conotoxins with inactivated N-type Ca^{2+} channels. *J Neurosci* 17: 3002–3013.
- Tatebayashi H, Ogata N (1992). Kinetic analysis of the GABA_B-mediated inhibition of the high-threshold Ca^{2+} current in cultured rat sensory neurones. *J Physiol* 447: 391–407.
- Tedford HW, Zamponi GW (2006). Direct G protein modulation of Ca_v2 calcium channels. *Pharmacol Rev* 58: 837–862.
- Tedford HW, Kisilevsky AE, Vieira LB, Varela D, Chen L, Zamponi GW (2010). Scanning mutagenesis of the I-II loop of the $\text{Ca}_v2.2$ calcium channel identifies residues arginine 376 and valine 416 as molecular determinants of voltage dependent G protein inhibition. *Mol Brain* 3: 6.
- Terlau H, Olivera BM (2004). *Conus* venoms: a rich source of novel ion channel-targeted peptides. *Physiol Rev* 84: 41–68.
- Terlau H, Shon K, Grilley M, Stocker M, Stuhmer W, Olivera BM (1996). Strategy for rapid immobilization of prey by a fish-hunting cone snail. *Nature* 381: 148–151.
- Towers S, Princiville A, Billinton A, Edmunds M, Bettler B, Urban L *et al.* (2000). GABA_B receptor protein and mRNA distribution in rat spinal cord and dorsal root ganglia. *Eur J Neurosci* 12: 3201–3210.
- Vinclair M, Wittenauer S, Parker R, Ellison M, Olivera BM, McIntosh JM (2006). Molecular mechanism for analgesia involving specific antagonism of $\alpha 9\alpha 10$ nicotinic acetylcholine receptors. *Proc Natl Acad Sci U S A* 103: 17880–17884.
- Weiss N (2009). Regulation of N-type calcium channels by G-proteins: multiple pathways to control calcium entry into neurons. *Channels (Austin)* 3: 219–220.
- Wen L, Yang S, Qiao HF, Liu ZW, Zhou WX, Zhang YX *et al.* (2005). SO-3, a new O-superfamily conopeptide derived from *Conus striatus*, selectively inhibits N-type calcium currents in cultured hippocampal neurons. *Br J Pharmacol* 145: 728–739.
- Witcher DR, DeWaard M, Campbell KP (1993). Characterization of the purified N-type Ca^{2+} channel and the cation sensitivity of ω -conotoxin GVIA binding. *Neuropharmacology* 32: 1127–1139.
- Wright CE, Robertson AD, Whorlow SL, Angus JA (2000). Cardiovascular and autonomic effects of ω -conotoxins MVIIA and CVID in conscious rabbits and isolated tissue assays. *Br J Pharmacol* 131: 1325–1336.
- Yang Q, Li ZW, Wei JB (2001). Current responses mediated by endogenous GABA_B and GABA_C receptors in *Xenopus* oocytes (in Chinese). *Sheng Li Xue Bao* 53: 311–315.
- Yarotsky V, Elmslie KS (2009). ω -Conotoxin GVIA alters gating charge movement of N-type ($\text{Ca}_v2.2$) calcium channels. *J Neurophysiol* 101: 332–340.
- Yasuda T, Adams DJ (2007). Voltage-gated calcium channels in nociception. In: Martinac B (ed.). *Sensing with Ion Channels*. Springer: Heidelberg, pp. 267–298.
- Yasuda T, Chen L, Barr W, McRory JE, Lewis RJ, Adams DJ *et al.* (2004). Auxiliary subunit regulation of high-voltage activated calcium channels expressed in mammalian cells. *Eur J Neurosci* 20: 1–13.
- Zamponi GW, Snutch TP (1996). Evidence for a specific site for modulation of calcium channel activation by external calcium ions. *Pflügers Arch* 431: 470–472.
- Zamponi GW, Lewis RJ, Todorovic SM, Arneric SP, Snutch TP (2009). Role of voltage-gated calcium channels in ascending pain pathways. *Brain Res Brain Res Rev* 60: 84–89.