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REVIEW

Targeting voltage-gated calcium channels: developments in peptide and small-molecule inhibitors for the treatment of neuropathic pain

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Chronic pain affects approximately 20% of people worldwide and places a large economic and social burden on society. Despite the availability of a range of analgesics, this condition is inadequately treated, with complete alleviation of symptoms rarely occurring. In the past 30 years, the voltage-gated calcium channels (VGCCs) have been recognized as potential targets for analgesic development. Although the majority of the research has been focused on $Ca_v2.2$ in particular, other VGCC subtypes such as $Ca_v3.2$ have recently come to the forefront of analgesic research. Venom peptides from marine cone snails have been proven to be a valuable tool in neuroscience, playing a major role in the identification and characterization of VGCC subtypes and producing the first conotoxin-based drug on the market, the ω-conotoxin, ziconotide. This peptide potently and selectively inhibits $Ca_v2.2$, resulting in analgesia in chronic pain states. However, this drug is only available via intrathecal administration, and adverse effects and a narrow therapeutic window have limited its use in the clinic. Other $Ca_v2.2$ inhibitors are currently in development and offer the promise of an improved route of administration and safety profile. This review assesses the potential of targeting VGCCs for analgesic development, with a main focus on conotoxins that block $Ca_v2.2$ and the developments made to transform them into therapeutics.

Abbreviations
VGCC, voltage-gated calcium channel

Introduction

Over the past 30 years, peptide toxins from cone snails have been recognized as potential therapeutic candidates due to their exquisite selectivity and high potency at a range of different ion channels and receptors. To compensate for their slow speed, cone snails have evolved a large repertoire of toxins in their venom to rapidly and irreversibly interrupt the nervous system function, thus immobilizing their unsuspecting prey. Recent MS-based studies have suggested that an individual cone snail venom comprises greater than 1000 pharmacologically active components (Davis *et al.*, 2009). To date, only a small portion of these toxins have been characterized, leaving a plethora of toxins that are yet to be investigated.

The majority of the biologically active *Conus* venom components are small, disulfide-rich peptides typically composed of 10–30 residues (Figure 1) (Bingham *et al.*, 2010). These are largely targeted at voltage- and ligand-gated ion channels in the peripheral nervous system and CNS (Becker and Terlau, 2008). Due to the high structural diversity of these peptides, several conotoxin superfamilies have been defined. The peptides are initially synthesized as pre-propeptide precursors, containing a highly conserved signal sequence, a pro-region

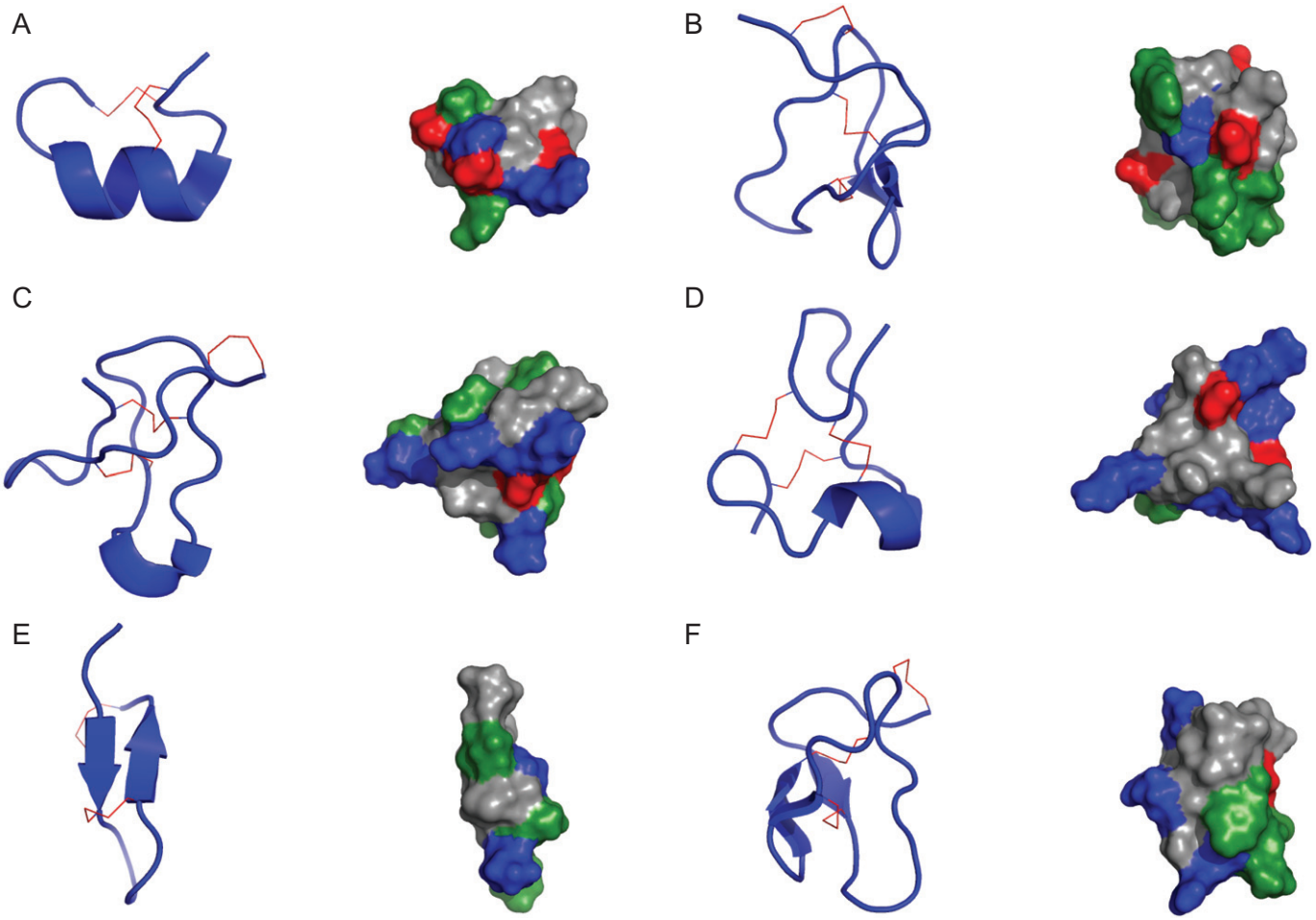

Figure 1

Structures of representative conotoxins from the main pharmacological families. Disulfide bonds are shown in red. Surface profiles highlight the positive (blue), negative (red) and hydrophobic (green) residues. (A) α-Vc1.1 (PDB code 2H8S), (B) δ-TxVIA (PDB code 1FU3), (C) κ-PVIIA (PDB code 1AV3), (D) μ-GIIIA (PDB code 1TCG), (E) χ-MrIA (PDB code 2EW4) and (F) ω-MVIIA (PDB code 1MVI).

and the variable mature toxin at the C-terminus (Terlau and Olivera, 2004; Bingham *et al.*, 2010). The superfamilies contain a homologous signal peptide sequence and a characteristic arrangement of cysteine residues (Terlau and Olivera, 2004; Bingham *et al.*, 2010). The peptides within each superfamily target a range of ion channels and receptors; thus, the superfamilies have been further divided into pharmacological families (Table 1). Conotoxins have been shown to have potential applications in a variety of pathological conditions, although the majority of research has focused on analgesic development (Terlau and Olivera, 2004).

Chronic pain

Chronic pain affects approximately 20% of people worldwide and places a large economic and social burden on society (Gureje *et al.*, 1998; Blyth *et al.*, 2001; Breivik *et al.*, 2006; Bouhassira *et al.*, 2008). Depending on the origin of the pain syndrome, it can be classified as either inflammatory or neuropathic pain, with complex syndromes commonly involving aspects of both. While inflammatory pain is the result of tissue injury or an invading foreign substance, neuropathic pain develops specifically due to nervous system damage or dysfunction (Baron, 2006). Neuropathic pain can often be a secondary product of a range of prior conditions, including infection, trauma, autoimmune diseases, metabolic disease, vascular disease and cancer (Campbell and Meyer, 2006). Due to the complex central and peripheral mechanisms involved in neuropathic pain, the range of therapeutic targets is extensive (Figure 2). Consequently, there have been few drugs specifically approved for the treatment of neuropathic pain. Re-evaluation of existing therapies for other indications has resulted in a greater number of therapeutic options for symptomatic pain relief. However, these drugs are frequently required in high doses, have limited efficacy for this indication, are effective in a small subset of patients and are associated with a range of adverse effects. Currently, only 30–40% of patients experience greater than 40–50% of pain relief with existing analgesics (Backonja and Woolf, 2010).

Current treatment options include opioids, non-steroidal anti-inflammatory drugs (NSAIDs), antidepressants, anticon-

Table 1

Conotoxin classification (Terlau and Olivera, 2004; Peng *et al.*, 2008; Loughnan *et al.*, 2009; Bingham *et al.*, 2010)

Superfamily	Cysteine arrangement	Cysteine pattern class designation	Pharmacological family designation	Target receptor/channel
A	CC-C-C	I	α	nACh
	CC-C-C-C-C	IV	αA	nACh
	–	IV	κA	K^+
M	CC-C-C-CC	III	μ	Na^+
	–	III	ψ	nACh
	–	III	κM	K^+
O	C-C-CC-C-C	VI/VII	ω	Ca^{2+}
	–	VII	κ	K^+
	–	VI	δ	Na^+
	–	VI	μO	Na^+
P	C-C-C-C-C-C	IX	–	ND
S	C-C-C-C-C-C-C-C-C-C	VIII	σ	5-HT_3 receptor
T	CC-CC	V	τ	Ca^{2+}
	CC-CPC	X	χ	NE transporter
I	C-C-CC-CC-C-C	XI	κI	K^+
J	C-C-C-C	XIV	κJ	K^+, nACh
V	C-C-CC-C-C-C-C	–	–	ND
D	C-CC-C-CC-C-C-C-C	XX	αD	nACh
L	C-C-C-C	XIV	αL	K^+, nACh
C	C-C	X	αC	nACh
	C-C	–	Contryphans	Ca^{2+}
	–	–	Conopressin	Vasopressin
	No cysteines	–	Conantokins	NMDA
	–	–	Conofarmide	RF amide
	–	–	Contulakins	Neurotensin

ND, not determined.

vulsants, skeletal muscle relaxants and topical agents (Turk *et al.*, 2011). Although opioids are the most commonly used therapeutic class for neuropathic pain treatment, their efficacy is highly debated. Opioids are commonly used for chronic neuropathic pain, although these drugs are plagued with problems such as user dependence, common side effects including constipation, nausea and somnolence, and the development of tolerance and opioid-induced hyperalgesia (Turk *et al.*, 2011). Currently, the European Federation of Neurological Societies Task Force recommends opioids as the second- or third-line treatment for neuropathic pain due to the risk of user dependence and the lack of studies assessing the long-term safety of opioid use (Attal *et al.*, 2010). The NSAIDs are mainly used for the treatment of acute pain, but have shown efficacy in osteoarthritis, rheumatoid arthritis and back pain (Turk *et al.*, 2011). These drugs have limited utility for neuropathic pain syndromes. The recommended first-line treatments for neuropathic pain include the tricyclic antidepressants and the calcium current-blocking gabapentinoids (Attal *et al.*, 2010). The efficacy of tricyclic antidepressants has mainly been investigated in patients with peripheral neuropathic pain (Max *et al.*, 1987; Verdu *et al.*, 2008). This drug class is non-selective, thus potentially produces several adverse effects, which are particularly pronounced at high doses (Verdu *et al.*, 2008; Attal *et al.*, 2010). Of main concern are the cardiovascular adverse effects (Attal *et al.*, 2010). For this reason, treatment with tricyclic antidepressants is not recommended for the elderly or patients with cardiovascular risk factors (Verdu *et al.*, 2008). Some of the later generation tricyclic antidepressants such as the serotonin and noradrenaline reuptake inhibitors, venlafaxine and duloxetine, have been shown to be more widely accepted (Dobecki *et al.*, 2006; Jann and Slade, 2007). The gabapentinoid drugs, gabapentin and pregabalin, reduce the number of voltage-gated calcium channel (VGCC) complexes at the plasma membrane through the inhibition of α2-δ auxiliary subunit recycling (Heblich *et al.*, 2008; Tran-Van-Minh and Dolphin, 2010). This mechanism of action explains the discrepancies in the effect on calcium current and neurotransmitter release that have been observed between acute and chronic administration (Fehrenbacher *et al.*, 2003; Brown and Randall, 2005; Heblich *et al.*, 2008). These compounds

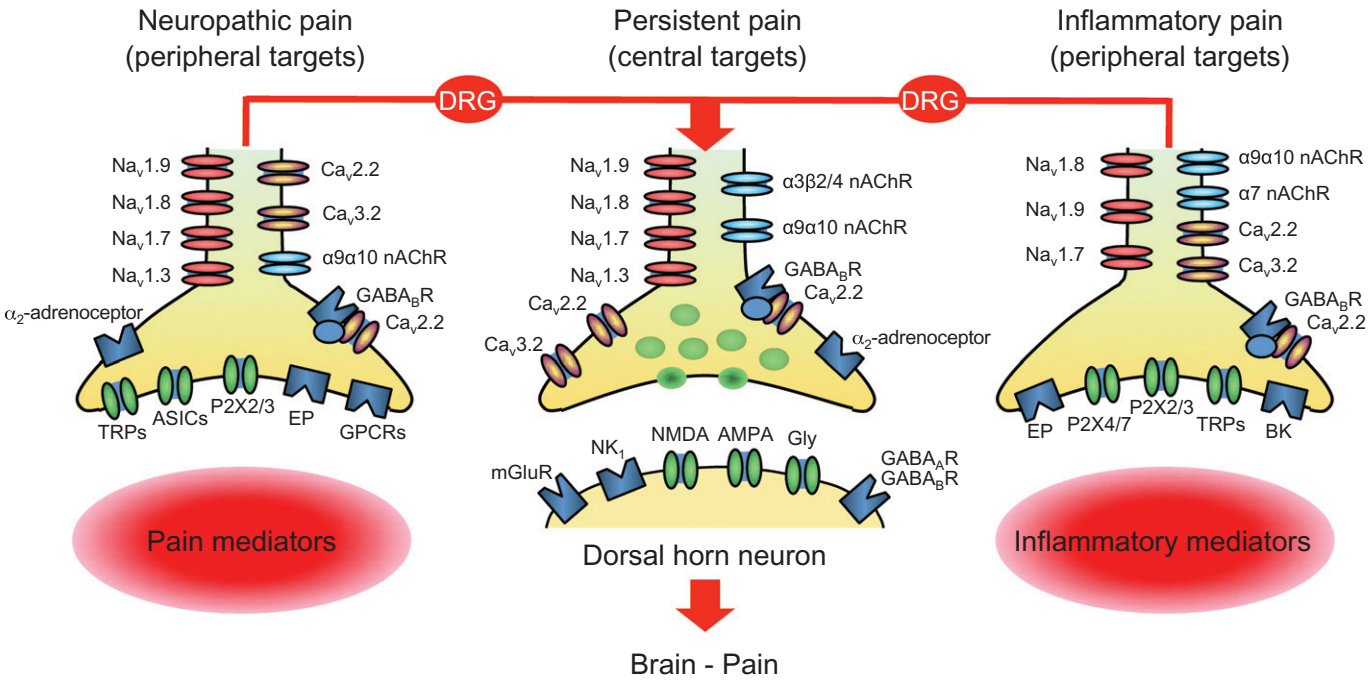

Figure 2

Potential peripheral and central neuropathic and inflammatory pain targets. The development of neuropathic and inflammatory pain is a complex process involving a wide range of ion channels and receptors, thus providing a large number of potential analgesic targets.; ASICs, acid sensing ion channels; BK, bradykinin 1 and 2 receptors; DRG, dorsal root ganglion; EP, PG receptor 1; Gly, glycine receptor; mGluR, metabotropic glutamate receptor; NK_1, neurokinin 1 receptor; TRPs, transient receptor potential channels. Figure modified from Lewis and Garcia (2003).

are used for the treatment of neuropathic conditions such as diabetic painful neuropathy (Lesser *et al.*, 2004; Sandercock *et al.*, 2009) and postherpetic neuralgia (Rowbotham *et al.*, 1998; Dworkin *et al.*, 2003; Irving *et al.*, 2009), despite being originally designed and used as anticonvulsants for epilepsy treatment (Sivenius *et al.*, 1991; French *et al.*, 2003). These compounds suffer from fewer adverse effects as compared with the opioids and tricyclic antidepressants, such that gabapentin is currently the leading drug for neuropathic pain treatment. Less commonly used analgesics include skeletal muscle relaxants and topical agents such as lidocaine and capsaicin creams and patches. Treatment with the current therapeutics for chronic neuropathic pain rarely results in complete alleviation of the symptoms, thus highlighting a need for new therapy options.

In 1984, the discovery of a novel peptide in *Conus geographus* venom led to the emergence of a new direction in analgesic development (Olivera *et al.*, 1984). After much research, this peptide, GVIA, was found to be anti-hyperalgesic upon inhibition of a VGCC subtype, $Ca_v2.2$, which underlies N-type calcium currents. At this time, the molecular biology of VGCCs was relatively unknown, but this discovery turned them into a prominent analgesic target.

Voltage-gated calcium channels

Calcium entry into cells through the excitation of VGCCs is involved in electrical excitability, repetitive firing patterns, excitation–contraction coupling and gene expression (Snutch, 2005). VGCC-mediated calcium entry is the initial trigger for the release of neurotransmitters at presynaptic nerve terminals (Snutch, 2005). Due to their essential role in calcium signalling, VGCCs are important targets for the treatment of pain, stroke, epilepsy, migraine and hypertension (Table 2).

VGCCs are multiple subunit complexes located in both excitable and non-excitable cells. VGCCs are made up of a large pore-forming transmembrane subunit α1, an intracellular β subunit, a disulfide-linked α2-δ transmembrane subunit and a γ subunit (McEnery *et al.*, 1991; Witcher *et al.*, 1993b). Four homologous domains, each consisting of six transmembrane regions, form the α1 subunit (Catterall, 2000). This subunit contains the binding sites for most channel blockers, including that of the ω-conotoxins. The ω-conotoxin binding site lies in the third domain between the fifth and sixth transmembrane region (Ellinor *et al.*, 1994; Feng *et al.*, 2001). The β subunit exists entirely in the cytoplasm and assists in the trafficking of the α1 subunit to the plasma membrane and regulates the gating properties of the channel (Bichet *et al.*, 2000). α2-δ subunits are expressed in a wide range of tissues, including skeletal muscle, heart and brain (Arikkath and Campbell, 2003). The α2-δ subunit has been shown to increase the expression and membrane targeting of the α1 subunit (Felix *et al.*, 1997; Dolphin *et al.*, 1999), increase current amplitude (Gurnett *et al.*, 1996), cause rapid activation and inactivation kinetics (Felix *et al.*, 1997), and produce a hyperpolarizing shift in the voltage dependence of activation (Felix *et al.*, 1997). Unlike β and α2-δ subunits, γ subunits are not thought to be involved in α1 trafficking to the plasma

Table 2

Therapeutic significance of VGCCs (Triggle, 2007; Mohan and Gandhi, 2008; Bingham *et al.*, 2010)

Systematic name	Current	Expression	Therapeutic significance
$Ca_v1.1$	L	Skeletal muscle	–
$Ca_v1.2$	L	Cardiac muscle, endocrine cells and neurons	Cardiovascular disorders
$Ca_v1.3$	L	Endocrine cells and neurons	Parkinson's disease, cardiac arrhythmia
$Ca_v1.4$	L	Retina	
$Ca_v2.1$	P Q	Dendrites and nerve terminals	Epilepsy, migraine
$Ca_v2.2$	N	Dendrites and nerve terminals	Pain
$Ca_v2.3$	R	Cell bodies, dendrites and nerve terminals	Diabetes
$Ca_v3.1$	T	Cardiac muscle, skeletal muscle and neurons	Cardiac arrhythmia, epilepsy, hypertension, sleep disorders
$Ca_v3.2$	T	Cardiac muscle and neurons	Pain, epilepsy
$Ca_v3.3$	T	Neurons	Sleep disorders, epilepsy

membrane. The γ subunit is primarily involved in the regulation of the biophysical properties of the channel, but this role remains controversial (Arikkath and Campbell, 2003; Ferron *et al.*, 2008; Dolphin, 2009).

Initially, the majority of VGCC research focused on finding inhibitors of $Ca_v1.2$ channels due to their localization in the CVS and role in BP regulation (nomenclature conforms to Alexander *et al.*, 2011). Currently, these channels are inhibited by the orally available, small-molecule dihydropyridines with which a peptide drug would find it difficult to compete in the pharmaceutical market. Subsequently, interest has been shifted to a variety of VGCC subtypes, as increased research has revealed their numerous roles in disease states. The T-type VGCCs ($Ca_v3.1$–3.3) have recently been implicated in the regulation of different pain states. This has been reviewed by Todorovic and Jevtovic-Todorovic (2011). These channels are distributed in both neuronal and non-neuronal cells and are highly involved in cellular excitability (Kim *et al.*, 2003). Unlike $Ca_v3.1$, which was found to be pro-nociceptive when silenced (Kim *et al.*, 2003), $Ca_v3.2$ knockdown was found to have an analgesic effect in mechanical, thermal and chemical pain states (Bourinet *et al.*, 2005; Choi *et al.*, 2007). The lack of selective inhibitors has hindered the understanding of the roles of the Ca_v3 channels in disease states; thus, the development of $Ca_v3.2$ antagonists for pain treatment has only recently gained attention.

Conversely, the ω-conotoxins have played a major role in the realization that $Ca_v2.2$ blockers have an analgesic effect, thus shifting the focus of VGCC research towards $Ca_v2.2$. Of the several VGCCs expressed in the CNS, $Ca_v2.1$ and $Ca_v2.2$ are dominant in presynaptic nerve terminals and are mainly associated with neurotransmitter release (French and Zamponi, 2005). $Ca_v2.2$ channels are highly concentrated in both dorsal root ganglia cell bodies and synaptic terminals in the spinal cord dorsal horn and are involved in the sensation of thermal, mechanical and inflammatory painful stimuli (Snutch, 2005).

VGCCs, particularly $Ca_v2.2$, play an essential role in the perception of pain. In the ascending pain pathway, a painful peripheral stimulus triggers the activation of peripheral nociceptors, which transmits action potentials along the primary afferent nerve fibres into $Ca_v2.2$-containing sensory neurons within the peripheral nervous system (Winquist *et al.*, 2005; Park and Luo, 2010). The generated nociceptive signal is propagated to spinothalamic tract neurons in laminae I and II of the spinal cord dorsal horn (Snutch, 2005; Zamponi *et al.*, 2009b; Park and Luo, 2010). $Ca_v2.2$-mediated Ca^{2+} influx initiates the release of neurotransmitters such as substance P and glutamate, which act postsynaptically on spinothalamic tract neurons (Snutch, 2005; Winquist *et al.*, 2005; Zamponi *et al.*, 2009b; Park and Luo, 2010). Pain is sensed when these neurons relay the signal to the thalamus (Zamponi *et al.*, 2009b). VGCCs have been shown to be involved in both ascending and descending pain pathways (Zamponi *et al.*, 2009b; Park and Luo, 2010). Thus, these channels are key targets for the treatment of chronic pain.

Further validation of this target has come from animal models of neuropathic pain. Dorsal horn expression of the $Ca_v2.2$ α_{1B} subunit has been shown to be up-regulated in parallel with the development of mechanical and thermal allodynia in a rat chronic sciatic nerve constriction injury model of neuropathic pain (Cizkova *et al.*, 2002; Winquist *et al.*, 2005). $Ca_v2.2$ knockout mice display reduced sensitivity to inflammatory and neuropathic pain, but remain behaviourally normal (Saegusa *et al.*, 2001). Additionally, $Ca_v2.2$ activity is modulated by GPCR activation, many of which are confirmed targets for analgesics, including receptors for opioids, cannabinoids, neuropeptide Y and substance P (Snutch, 2005). Thus, $Ca_v2.2$ selective conotoxins would be effective therapeutic candidates for chronic and neuropathic pain treatment.

The $Ca_v2.2$ channels have significant functional diversity, which is dependent on the cell population in which they are expressed. This diversity can be a result of the association with various auxiliary subunits and also from alternative splicing of the $Ca_v2.2$ RNA (Altier *et al.*, 2007). There are several alternative splice sites within the $Ca_v2.2$ gene, with the majority of the research being performed on the exon 37a/37b splice site. Recent research has shown that $Ca_v2.2$ e37a is involved in the transmission of nociception in both

inflammatory and neuropathic pain models (Altier *et al.*, 2007). This isoform is also predominantly expressed in nociceptive neurons, indicating that neuronal targeting can be achieved with a $Ca_v2.2$ e37a selective peptide (Bell *et al.*, 2004). The main hurdle to overcome is the intracellular location of the alternatively spliced exon. $Ca_v2.2$ e37a involves the replacement of exon 37b with the mutually exclusive exon 37a in the intracellular, C-terminal region of the receptor (Bell *et al.*, 2004). As the ω-conotoxin binding site lies in the extracellular region of $Ca_v2.2$, selective targeting of the e37a isoform is difficult. Nonetheless, reports have indicated that current inhibition caused by ω-conotoxins MVIIA and GVIA was decreased in an intracellular Δ1 $Ca_v2.2$ splice variant, suggesting that selective splice variant targeting can be achieved (Kaneko *et al.*, 2002). More research is required on the structural features of the splice variants to further explore this new direction in therapeutic development.

VGCC-targeting conotoxins

While there are many small-molecule, inorganic ion and peptide blockers that target VGCCs, few have the selectivity and potency of the peptides from the venom of marine cone snails. The conotoxins that target VGCCs come from several different structural and pharmacological families. These include the ω-conotoxins from the O superfamily, the τ-conotoxins from the T superfamily and the contryphans. This review will mainly focus on the ω-conotoxins, as they are the largest and most therapeutically significant VGCC-targeting family.

ω-conotoxins

The principal pharmacological family that targets VGCCs are the ω-conotoxins (Table 3). The ω-conotoxins are the most widely used *Conus* peptide family in neuroscience and inhibit synaptic transmission due to their inhibition of $Ca_v2.1$ and $Ca_v2.2$ (Terlau and Olivera, 2004). These peptides have been intensively studied for the inhibition of neurotransmitter release and for the treatment of inflammatory and chronic neuropathic conditions (Lewis *et al.*, 2000). $Ca_v2.2$-selective inhibitors are highly sought after as alternative analgesics for the management of intractable chronic pain (Malmberg and Yaksh, 1995; Snutch, 2005). The ω-conotoxins act on $Ca_v2.2$ channels, which are highly expressed on the presynaptic terminals of primary afferent neurons that terminate in the dorsal horn of the spinal cord (Schmidtko *et al.*, 2010). Upon blockage of $Ca_v2.2$ in the dorsal horn by ω-conotoxins, neurotransmitter release is inhibited and the sensation of painful stimuli is prevented (Schmidtko *et al.*, 2010). The site and mechanism of $Ca_v2.2$ block by ω-conotoxins has recently been reviewed by Adams *et al.* (2012).

The ω-conotoxins are typically basic peptides between 24 and 31 residues in length. The six cysteine residues form a cystine knot motif when fully folded, which imparts chemical, thermal and mechanical stability to the peptide (Pallaghy *et al.*, 1994; Daly and Craik, 2009). This motif involves the formation of a ring structure by two disulfide bonds and the peptide backbone, which is pierced by the third disulfide bond (Pallaghy *et al.*, 1994). The cystine knot motif is present in many toxins from other venomous animals, such as spiders and scorpions, and is being extensively used as a scaffold for peptide-based drug design (Craik *et al.*, 2001; Reiss *et al.*, 2006; Wang *et al.*, 2009; Kolmar, 2010; Silverman *et al.*, 2011).

Most of the known ω-conotoxins have been identified from either piscivorous and, more recently, molluscivorous cone snails. This does not necessarily indicate that vermivorous cone snails are lacking ω-conotoxins, as the majority of the research has focused on the fish and mollusc hunters. The most intensively studied ω-conotoxins are GVIA from *C. geographus*, MVIIA and MVIIC from *Conus magus* and CVID from *Conus catus*. The first ω-conotoxin to be characterized was GVIA from *C. geographus*, which has been extensively studied over the last 30 years (Olivera *et al.*, 1984). The main benefit of GVIA discovery has been the integral role that this peptide has played in the identification, localization and characterization of VGCCs, particularly $Ca_v2.2$ (Cruz and Olivera, 1986; Kerr *et al.*, 1988; Jones *et al.*, 1989; Robitaille *et al.*, 1990; McEnery *et al.*, 1991; Witcher *et al.*, 1993a,b; Filloux *et al.*, 1994). This 27-residue peptide has greater potency at $Ca_v2.2$ than both MVIIA and CVID, although the irreversibility of the block has limited its utility as a therapeutic (Scott *et al.*, 2002).

MVIIA is a 25-residue peptide from *C. magus* venom that potently and reversibly blocks VGCCs (Olivera *et al.*, 1987). This peptide has greater selectivity for $Ca_v2.2$ over $Ca_v2.1$, such that it became the first conotoxin-based therapeutic (Kristipati *et al.*, 1994). In 2004, a synthetic version of MVIIA (ziconotide) was approved for the treatment of long-term neuropathic pain in the USA and Europe. Ziconotide is discussed in more detail in the Therapeutic implications section.

MVIIC was also isolated from *C. magus*, yet, despite sharing over 70% homology with MVIIA, is more selective for $Ca_v2.1$ over $Ca_v2.2$ (Hillyard *et al.*, 1992). Due to the different selectivity profile, this peptide has been used in combination with GVIA, MVIIA and other VGCC blockers to distinguish VGCC subtypes in various tissues (Hillyard *et al.*, 1992; Gaur *et al.*, 1994; Kristipati *et al.*, 1994; Woppmann *et al.*, 1994; Sugiura *et al.*, 1995; Foehring and Armstrong, 1996). MVIIC is not therapeutically significant for neuropathic pain treatment due to its subtype selectivity profile and toxicity issues.

In 2000, four novel ω-conotoxins (CVIA-D) were isolated from *C. catus* venom (Lewis *et al.*, 2000). CVIA and CVID were selective for $Ca_v2.2$, whereas CVIB and CVIC antagonized both $Ca_v2.1$ and $Ca_v2.2$ (Lewis *et al.*, 2000). CVID was the most potent peptide at $Ca_v2.2$ and remains the most selective ω-conotoxin for $Ca_v2.2$ that has been found to date (Lewis *et al.*, 2000). Based on the $Ca_v2.2$ selectivity, much research has been performed on CVID to transform it into a therapeutic. CVID is further discussed in the Therapeutic implications section. Recently, two more ω-conotoxins, CVIE and CVIF, were identified from *C. catus* venom glands (Berecki *et al.*, 2010) and were found to be potent, selective and reversible $Ca_v2.2$ inhibitors (Berecki *et al.*, 2010).

The rationale for using ω-conotoxin $Ca_v2.2$ antagonists for neuropathic pain treatment is further supported by the observation that the pore-forming α_{1B} subunit of $Ca_v2.2$ is up-regulated after peripheral nerve injury (Cizkova *et al.*, 2002). However, many other subunits that modulate ω-conotoxin binding are also up-regulated in pain states. The

Table 3

ω-Conotoxin sequences and their corresponding VGCC subtype selectivity

Species	Name	Sequence	VGCC target	Reference/s
Conus geographus	GVIA	---CKSOGSSC---SOTSYNCC-RSCNOYTKRCY*	$Ca_v2.2$	Olivera *et al.* (1984); Rivier *et al.* (1987); Kim *et al.* (1995); Lew *et al.* (1997); Nielsen *et al.* (2000); Schroeder and Lewis (2006)
	GVIB	---CKSOGSSC---SOTSYNCC-RSCNOYTKRCYG*	ND	Olivera *et al.* (1985)
	GVIC	---CKSOGSSC---SOTSYNCC-RSCNOYTKRC*	ND	Olivera *et al.* (1985)
	GVIIA	---CKSOGTOC---SRGMRDCC-TSCLLYSNKCRRY*	ND	Olivera *et al.* (1985)
	GVIIB	---CKSOGTOC---SRGMRDCC-TSCLSYSNKCRRY*	ND	Olivera *et al.* (1985)
Conus magus	MVIIA	---CKGKGAKC---SRLMYDCCTGSC--RSGKC*	$Ca_v2.2$	Olivera *et al.* (1985; 1987); Kim *et al.* (1995); Nadasdi *et al.* (1995); Nielsen *et al.* (1999a,b; 2000); Schroeder and Lewis (2006)
	MVIIB	---CKGKGASC---HRTSYDCCTGSC--NRGKC*	$Ca_v2.1$	Olivera *et al.* (1987)
	MVIIC	---CKGKGAPC---RKTMYDCCSGSC-GRRGKC*	$Ca_v2.1$ and $Ca_v2.2$	Hillyard *et al.* (1992); Nielsen *et al.* (1999a,b); Sato *et al.* (2000a)
	MVIID	---CQGRGASC---RKTMYNCCSGSC--NRGRC*	$Ca_v2.1$ and $Ca_v2.2$	Monje *et al.* (1993)
Conus catus	CVIA	---CKSTGASC---RRTSYDCCTGSC--RSGRC*	$Ca_v2.2$	Lewis *et al.* (2000)
	CVIB	---CKGKGASC---RKTMYDCCRGSC--RSGRC*	$Ca_v2.1$ and $Ca_v2.2$	Lewis *et al.* (2000)
	CVIC	---CKGKGQSC---SKLMYDCCTGSC-SRRGKC*	$Ca_v2.1$ and $Ca_v2.2$	Lewis *et al.* (2000)
	CVID	---CKSKGAKC---SKLMYDCCSGSCSGTVGRC*	$Ca_v2.2$	Lewis *et al.* (2000); Nielsen *et al.* (2000); Schroeder and Lewis (2006)
	CVIE	---CKGKGASC---RRTSYDCCTGSC--RSGRC*	$Ca_v2.2$	Berecki *et al.* (2010)
	CVIF	---CKGKGASC---RRTSYDCCTGSC--RLGRC*	$Ca_v2.2$	Berecki *et al.* (2010)
Conus striatus	SVIA	---CRSSGSOC----GVTSICC-GRC--YRGRCT*	Poor specificity to mammalian VGCCs Rapid paralysis and death in fish	Ramilo *et al.* (1992)
	SVIB	---CKLKGQSC---RKTSYDCCSGSC-GRSGKC*	$Ca_v2.1$ and $Ca_v2.2$	Ramilo *et al.* (1992)
	SO3	---CKAAGKPC---SRIAYNCCTGSC--RSGKC*	$Ca_v2.2$	Wen *et al.* (2005; 2006)
Conus tulipa	TVIA	---CLSOGSSC---SOTSYNCC-RSCNOYSRKCR*	$Ca_v2.2$	Wang *et al.* (1998)
Conus radiatus	RVIA	---CKPOGSOC---RVSSYNCC-SSCKSYNKKC*	$Ca_v2.2$	Miljanich *et al.* (1991)
Conus consors	CnVIIA	---CKGKGAOC---TRLMYDCCHGSCSSSKGRC*	$Ca_v2.2$	Favreau *et al.* (2001)
Conus pennaceus	PnVIA	--GCLEVDYFCGIPFANNGLCCSGNC---VFVCTPQ*	Blocks molluscan HVA VGCCs	Kits *et al.* (1996)
	PnVIB	DDDCEPPGNFC-GMIKIGPPCCSGWC---FFACA*	Blocks molluscan HVA VGCCs	Kits *et al.* (1996)
Conus textile	TxVII	---CKQADEPC---DVFSLDCCTGIC---LGVCMW*	Ca_v1	Fainzilber *et al.* (1996); Kobayashi *et al.* (2000)
Conus fulmen	FVIA	---CKGTGKSC---SRIAYNCCTGSC--RSGKC*	$Ca_v2.2$	Lee *et al.* (2010)

*C-terminal amidation.
ND, not determined.

most significant for ω-conotoxin development is the up-regulation of the α2-δ subunit. The presence of this subunit has been shown to decrease MVIIA and CVID affinity by approximately 100-fold (Mould *et al.*, 2004), whereas the affinity of the recently identified CVIE and CVIF is only reduced by 20-fold (Berecki *et al.*, 2010). The α2-δ subunit has been shown to be up-regulated in the spinal cord and dorsal root ganglia after nerve injury (Luo *et al.*, 2001; Newton *et al.*,

2001). To date, ω-conotoxins that are unaffected by the presence of the α2-δ subunit are yet to be identified, although the search continues as it is anticipated that $Ca_v2.2$ antagonists that avoid interaction with this auxiliary channel subunit are more desirable for neuropathic pain treatment.

τ-conotoxins

The τ-conotoxins are members of the T superfamily along with the χ-conotoxins. Unlike the ω-conotoxins, this pharmacological family is structurally and functionally diverse.

TxIX from *Conus textile* has been shown to reduce presynaptic Ca^{2+} influx, although it has not yet been discerned whether this is through interaction with VGCCs or GPCRs (Rigby *et al.*, 1999). This 13-residue peptide is highly post-translationally modified, containing two γ-carboxyglutamate residues, a D-tryptophan, an *O*-glycosylated threonine and a C-terminal hydroxyproline (Rigby *et al.*, 1999). Although TxIX has been shown to reduce Ca^{2+} influx, more research is required to determine the specific molecular target of this pharmacological family.

Contryphans

The contryphans are small peptides between 8 and 11 residues and contain only a single disulfide bond. In contrast to the ω-conotoxins, this family of peptides has been identified in piscivorous, molluscivorous and vermivorous cone snails. The contryphans have been found to have a variety of actions on Ca^{2+} influx. Glacontryphan-M from *Conus marmoreus* inhibits $Ca_v1.2$ (Hansson *et al.*, 2004), contryphan-Am from *Conus amadis* and contryphan-Lo from *Conus loroisii* modulate high voltage-activated calcium channels (Sabareesh *et al.*, 2006), and contryphan-Vn from *Conus ventricosus* modulates voltage-gated and Ca^{2+}-activated K^+ channels (Massilia *et al.*, 2003). Further research is required to identify the precise molecular targets and potencies of these peptides to determine their therapeutic potential.

Structure–activity relationships

Development of structure–activity relationships (SAR) between ω-conotoxins and $Ca_v2.2$ is essential to probe the specific features for $Ca_v2.2$ binding, thus underpinning the design of selective inhibitors. The majority of the SAR analysis between ω-conotoxins and $Ca_v2.2$ has focused on MVIIA, GVIA, CVID and MVIIC. Structural analysis of ω-conotoxins has identified that loops 2 and 4 are important for VGCC subtype selectivity (Nielsen *et al.*, 1999a). In particular, Tyr13 in loop 2 of MVIIA has been found to play an essential role in $Ca_v2.2$ binding (Kim *et al.*, 1995). The substitution of this residue with the corresponding D-amino acid resulted in a fivefold loss of activity (Nielsen *et al.*, 1999b). A high content of basic amino acid residues within the mature peptide sequence also plays an important role in VGCC inhibition (Sato *et al.*, 1993). Initially, Lys2 of GVIA and MVIIA was thought to directly interact with $Ca_v2.2$ upon binding (Lew *et al.*, 1997), although further research has identified that this residue indirectly contributes to binding through the structural stability of loop 2 residues (Schroeder *et al.*, 2006).

The recovery of $Ca_v2.2$ from block has been shown to be membrane-potential-dependent, being weak at physiological membrane potentials (Mould *et al.*, 2004). VGCC recovery from block could impact on the effectiveness of ω-conotoxins for chronic pain treatment *in vivo* and is important in minimizing adverse effects (Wright *et al.*, 2000). *In vivo* studies have identified that CVID, MVIIA and GVIA irreversibly inhibit synaptic transmission in rat dorsal horn neurons, whereas CVIB inhibition is reversible (Motin and Adams, 2008). The mutation of Arg10 to Lys in MVIIA has been shown to improve channel recovery from block at physiological membrane potentials, suggesting that this can be applied to other irreversible blockers to improve the therapeutic potential (Mould *et al.*, 2004). Fortunately, this residue does not significantly contribute to VGCC subtype selectivity, making this a viable approach (Sato *et al.*, 2000b).

The residues responsible for VGCC subtype selectivity have been investigated using chimeric ω-conotoxins and point mutations. A peptide encompassing the N-terminus of MVIIA with the C-terminus of MVIIC had increased affinity for $Ca_v2.2$ as compared with native MVIIC (Sato *et al.*, 2000b). Point mutations made to the MVIIA/MVIIC chimera and native MVIIC found that replacement of Thr11 of MVIIC with Leu (the corresponding MVIIA residue) increased the affinity for $Ca_v2.2$ and decreased the affinity for $Ca_v2.1$ (Sato *et al.*, 2000b). Despite this observation, $Ca_v2.2$-selective GVIA, CVIE and CVIF all contain a Thr in this position, indicating that this residue may contribute to the overall structure of the peptide, rather than directly interacting with the receptor. MVIIC [P7K] had increased affinity for both channels (Sato *et al.*, 2000b). The other varying residues in the N-terminal portion of MVIIA and MVIIC were not found to significantly contribute to subtype selectivity (Sato *et al.*, 2000b). Further research by this group implicated a larger binding surface including Lys4 and Arg22 in determining $Ca_v2.1$ selectivity over other VGCCs (Sato *et al.*, 2000a).

A comparison of ω-conotoxin three-dimensional structures has highlighted the point that the overall fold has been retained even though a different VGCC subtype selectivity is present within this peptide family (Figure 3). Furthermore, MVIIA and MVIIC have very similar surface profiles, including Tyr13, making it difficult to discern critical features for $Ca_v2.1$ selectivity. Specifically, the conserved important residue Tyr13 in MVIIA and MVIIC is bordered by a hydrophobic patch, although this feature is absent in GVIA. This observation further highlights the importance of Tyr13 over the surrounding loop 2 residues for $Ca_v2.2$ selectivity. The recent exclusion of Lys2 from the pharmacophore for $Ca_v2.2$ binding has reduced the required structural features for $Ca_v2.2$ inhibition (Schroeder *et al.*, 2006). Thus, it appears that small-molecule inhibitors could effectively inhibit $Ca_v2.2$ due to the minimized pharmacophore.

In contrast to the $Ca_v2.2$ blockers, the Ca_v1 blocker, TxVII, of similar fold contains very few basic residues (Fainzilber *et al.*, 1996). Large clusters of positively charged residues that are particularly evident in the surface profiles of MVIIA and MVIIC have been replaced with hydrophobic patches in TxVII. Thus, although the overall structure of the ω-conotoxins is similar, the amino acid side chains play a major role in determining the subtype selectivity.

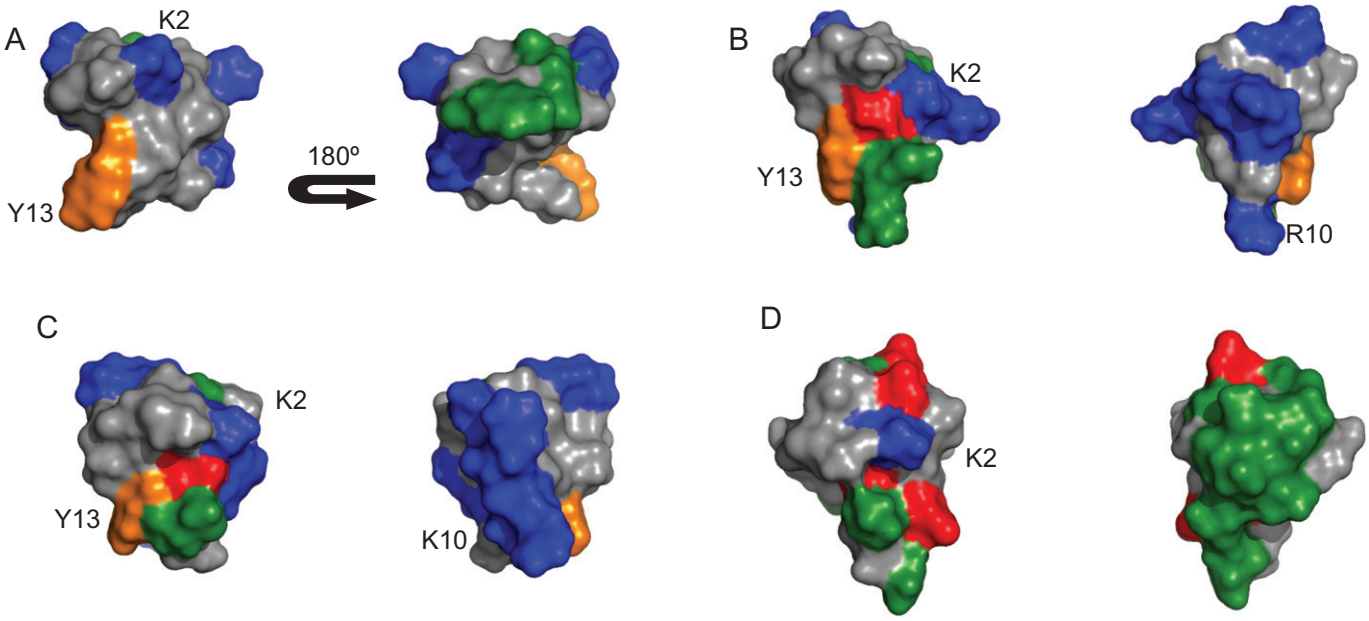

Figure 3

Surface profiles of (A) GVIA (PDB ID: 1TTL), (B) MVIIA (PDB ID: 1MVI), (C) MVIIC (PDB ID: 1CNN) and (D) TxVII (PDB ID: 1F3K), where positively charged residues are blue, negatively charged residues are red and hydrophobic residues are green. The conserved Tyr13 in GVIA, MVIIA and MVIIC is highlighted in orange.

VGCC subunit composition also plays an important role in ω-conotoxin binding and potency. In certain pain states, different subunits are up-regulated. As previously mentioned, the α2-δ auxiliary subunit is up-regulated in neuropathic pain states and decreases the potency of ω-conotoxins (Newton *et al.*, 2001; Mould *et al.*, 2004; Berecki *et al.*, 2010). This effect has been suggested to be due to electrostatic shielding or repulsion around the ω-conotoxin binding site, but this has not been further investigated (Mould *et al.*, 2004). Additionally, the β_3 subunit has increased expression in neuropathic pain states, implicating this subunit as a novel therapeutic target (Li *et al.*, 2011). The presence of this subunit has also been shown to reduce the $Ca_v2.2$ affinity of certain ω-conotoxins (Lewis *et al.*, 2000).

Further structural analysis is required to determine critical residues and structural motifs that confer subtype specificity and avoid the interactions with auxiliary channel subunits and intracellular domains. This will allow the design of ω-conotoxin therapeutics with improved selectivity, potency and, consequently, fewer adverse effects.

An important aspect for the design of peptide drugs is the avoidance of degradation. Some methods of conotoxin degradation include methionine oxidation (Schoneich *et al.*, 1997), asparagine deamidation and the isomerization or cleavage of Asp-Pro peptide bonds (Wakankar and Borchardt, 2006). All ω-conotoxin sequences contain an amidated C-terminus, which, along with being important for $Ca_v2.2$ binding, can reduce the susceptibility to proteolysis by carboxypeptidases. The stability towards proteolytic breakdown can be increased by the substitution of D-amino acids in place of the corresponding L-amino acid, with some native conotoxins incorporating this modification, for example, contryphans (Jimenez *et al.*, 1996). In come cases, this improves the stability, but it can also reduce the potency if the substitution is incorrectly positioned (Nielsen *et al.*, 1999b). D-amino acids have been included in the design of numerous small-molecule $Ca_v2.2$ inhibitors (Pallaghy and Norton, 2000; Schroeder *et al.*, 2004).

The increased understanding into the SAR of ω-conotoxins and $Ca_v2.2$ has produced vital information on ω-conotoxin features that are essential for binding and VGCC selectivity, which may be used to produce designer peptides with optimal properties for therapeutic applications. Further research is required to identify features that may modulate interaction with auxiliary subunits and the reversibility of $Ca_v2.2$ block. These developments can be directly applied to existing peptides or can be used to assist the design and production of minimized structures.

Small-molecule $Ca_v2.2$ inhibitors

While conotoxins are effective $Ca_v2.2$ blockers, the development of minimized structures is also desired to facilitate alternate routes of administration and to produce more cost-effective therapeutics. The $Ca_v2.2$ pharmacophore identified in the SAR studies above has allowed for the development of small-molecule inhibitors. This has been reviewed by Yamamoto and Takahara (2009).

Several approaches have been undertaken, including the positioning of mimetics of Tyr13, Leu11 and Arg10 of MVIIA around a dendritic backbone (Menzler *et al.*, 1998; 2000; Guo *et al.*, 2000), the rational design of type-III mimetics of GVIA based on Lys2, Tyr13 and Arg17 side chains (Baell *et al.*, 2001;

2004; 2006; Duggan *et al.*, 2008; 2009; Andersson *et al.*, 2009), and the grafting of important GVIA residues onto the backbone structure of contryphan-R (Figure 4) (Pallaghy and Norton, 2000). Although the functional groups of the important loop 2 residues were retained, the resulting molecules failed to possess the potency of the native ω-conotoxins.

Schroeder *et al.* (2004) used molecular modelling techniques to produce small-molecule mimetics of loop 2 of CVID. A range of cyclic pentapeptide CVID loop 2 mimetics was produced with some of these peptides being active at $Ca_v2.2$. The CVID loop 2 mimetics ($IC_{50} \geq 20$ μM) were less potent than native CVID, although retained the selectivity for $Ca_v2.2$ over $Ca_v2.1$. This research established that it is possible to produce $Ca_v2.2$-selective small molecules, although further pharmacophore development is required to refine the resulting molecules in order to retain the potency of the native conotoxins.

The development of selective $Ca_v2.2$ inhibitors has not been limited to peptide mimetics. Several non-peptidic small molecules have been in various stages of preclinical and clinical development but have yet to result in a successful therapeutic (Knutsen *et al.*, 2007; Zamponi *et al.*, 2009a).

Recently developed small-molecule $Ca_v2.2$ antagonists have been exploiting state-dependent VGCC block with the aim of reducing the adverse effects that are common with $Ca_v2.2$-selective peptides. The recognition that state-dependent block of VGCCs widens the therapeutic window of antagonists was first implied by Bean (1984) upon the discovery that dihydropyridines preferentially bind to inactivated L-type calcium channels (Snutch, 2005). The rationale behind the $Ca_v2.2$-directed approach is the inhibition of channels that are involved in the sustained firing of neurons in neuropathic pain, while sparing channels that are necessary for normal cardiovascular and CNS function (Snutch, 2005; Winquist *et al.*, 2005). This is achieved by the preferential inhibition of inactivated channels. Winquist *et al.* (2005) have recently reviewed the benefits of use-dependent block of VGCCs in neuropathic pain treatment. This method of inhibition overcomes the poor subtype selectivity that is common with small molecules as compared with peptide inhibitors.

Development of the most advanced small molecule, NMED-160 (MK-6721), was recently ceased after phase II clinical trials due to inadequate pharmaceutical properties. NMED-160 was designed using the scaffolds of the non-selective $Ca_v2.2$ antagonists, flunarizine and lomerizine (Figure 4) (Zamponi *et al.*, 2009a). This small molecule causes a use-dependent block of $Ca_v2.2$, in which channel block increases with a greater frequency of action potentials (Grigoriadis *et al.*, 2009). Further research into the SAR of the diphenylpiperazine class of compounds has been reported (Pajouhesh *et al.*, 2010).

More recently, there have been reports of the development of an *N*-triazole oxindole and diphenyl lactam variants that are state-dependent Ca_v2 inhibitors (Figure 4) (Abbadie *et al.*, 2010; Doherty *et al.*, 2012; Scott *et al.*, 2012; Swensen *et al.*, 2012). The adverse effects experienced with non-selective Ca_v2 inhibitors and $Ca_v2.1$ deletion or mutation were not observed, which is thought to be due to the state-dependent mechanism of inhibition (Abbadie *et al.*, 2010; Doherty *et al.*, 2012; Scott *et al.*, 2012; Swensen *et al.*, 2012). Despite the lack of a successful drug candidate, the novel drug classes that have been developed may assist in the development of future inhibitors.

It is anticipated that the increased development of high-throughput techniques and assays, and easier access to peptide libraries could lead to the discovery of a novel small-molecule $Ca_v2.2$ inhibitor in the near future (Finley *et al.*, 2010).

In vitro and *in vivo* assays

The recent search for $Ca_v2.2$ inhibitors has necessitated the development of a variety of *in vitro* and *in vivo* testing methods to evaluate the therapeutic potential of these compounds. The development of improved testing methods is integral for the efficient production of pain therapeutics without unnecessary *in vivo* testing of unsuitable candidates.

Many cell lines and expression systems have been used to evaluate the potency and selectivity of a variety of peptides and compounds at $Ca_v2.2$. Rat dorsal root ganglion neurons have been essential for the initial screening of potential VGCC inhibitors. Dorsal root ganglion neurons have been shown to express several different calcium channel subtypes and the use of various dyes and inhibitors allows for the identification of the affected channel subtype/s (Kostyuk *et al.*, 1981; Berecki *et al.*, 2010).

Following this initial screening, focused testing is performed through the use of cloned channels. A range of assays has been developed and are routinely used, particularly competition binding experiments involving radiolabelled ω-conotoxins and electrophysiological recording of the Ca^{2+} or Ba^{2+} current using *Xenopus* oocytes or mammalian cells (Cruz and Olivera, 1986; Williams *et al.*, 1992; Lewis *et al.*, 2000; Berecki *et al.*, 2010; Gowd *et al.*, 2010).

Over the several decades of pain research, there have been numerous animal models that have been developed. Prior to the development of peripheral nerve injury models in the 1970s, commonly used animal models for the testing of analgesic compounds only evaluated the behavioural and physiological reaction to thermal and mechanical stimuli. Today, there is a wide range of animal models that have been developed to assess different types of neuropathic pain, including peripheral and central nerve injury, drug- and disease-induced neuropathy and several others (Table 4). Jaggi *et al.* (2011) has comprehensively reviewed the various animal models of neuropathic pain. This range of animal models improves the ability to develop therapeutics that effectively and specifically treat various pain syndromes.

Therapeutic implications

MVIIA (ziconotide)

The first conotoxin-based therapeutic is from the ω-conotoxin pharmacological family. In 2004, the Food and Drug Administration (FDA) approved a synthetic version of ω-conotoxin MVIIA, ziconotide (Prialt), for the management of long-term neuropathic pain. As previously mentioned, this peptide is a selective, reversible $Ca_v2.2$ inhibitor (Kristipati

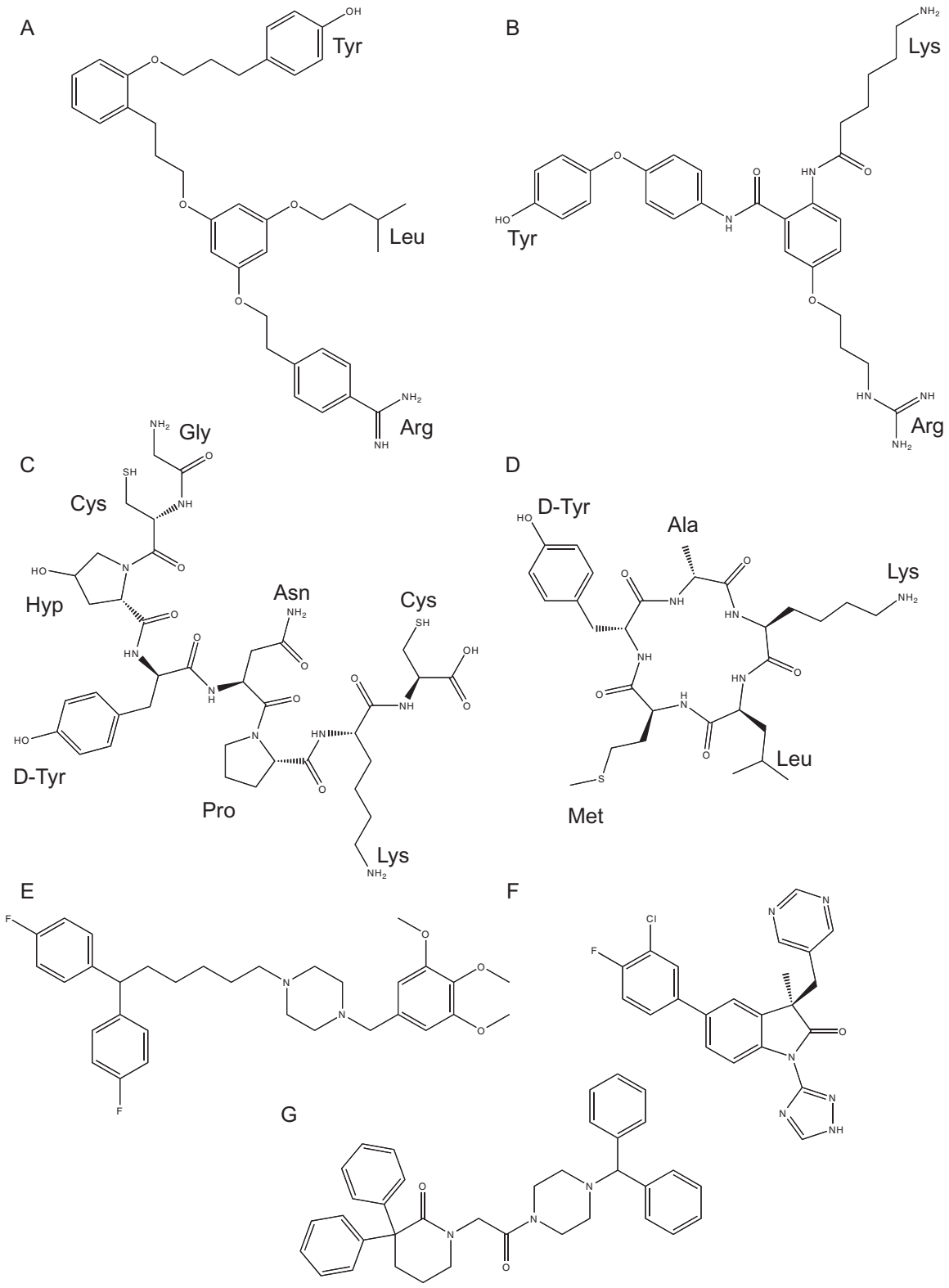

Figure 4

Small-molecule $Ca_v2.2$ inhibitors. (A) Dendritic backbone displaying key MVIIA residues (Menzler *et al.*, 2000). (B) Type-III mimetic of GVIA using the Lys2, Tyr13 and Arg17 side chains (Baell *et al.*, 2001). (C) Grafting of D-Tyr, Asn and Lys from GVIA onto the contryphan-R scaffold (Pallaghy and Norton, 2000). (D) Loop 2 cyclic pentapeptide mimetics of CVID (Schroeder *et al.*, 2004). (E) Example of NMED-160 class of non-peptidic $Ca_v2.2$ inhibitors (Zamponi *et al.*, 2009a). (F) *N*-triazole oxindole, TROX-1 (Abbadie *et al.*, 2010). (G) Diphenyl lactam variant A-1048400 (Scott *et al.*, 2012).

Table 4

VGCC involvement in models of acute, persistent, inflammatory and neuropathic pain (Vanegas and Schaible, 2000; Yaksh, 2006; Jaggi *et al.*, 2011)

Model	Injury	Species	Effects of VGCC block	References
Neuropathic pain				
Chronic constriction injury	Four loosely constrictive ligatures around sciatic nerve	Rats and mice	$Ca_v2.2$ and Ca_v3: Dose-dependent inhibition of tactile and thermal hyperalgesia	Xiao and Bennett (1995); Yamamoto and Sakashita (1998); Dogrul *et al.* (2003); Hord *et al.* (2003); Yaksh (2006)
Diabetic neuropathy (streptozocin-induced and genetic models)	Persistent hyperglycaemia-induced changes to the nerves	Rats and mice	$Ca_v2.2$: Dose-dependent inhibition of mechanical allodynia $\alpha2$-δ subunit: Dose-dependent inhibition of static and dynamic allodynia	Calcutt and Chaplan (1997); Field *et al.* (1999)
Drug-induced (anti-cancer and anti-HIV agents)	Drug-induced injury to the nerves of the peripheral nervous system	Rats, mice, guinea pigs and rabbits	$Ca_v2.2$, Ca_v3 and $\alpha2$-δ subunit: Dose-dependent inhibition of thermal and mechanical hyperalgesia	Nozaki-Taguchi *et al.* (2001); Fukuizumi *et al.* (2003); Flatters and Bennett (2004); Lynch *et al.* (2004)
Spinal nerve ligation	Tight ligation of L5 and L6 spinal nerves or L7 spinal nerve	Rats and macaque	$Ca_v1.2$: Knockdown reversed mechanical hyperalgesia $Ca_v2.2$: Inhibition of mechanical allodynia Ca_v3 and $\alpha2$-δ subunit: Inhibition of thermal and mechanical allodynia	Chaplan *et al.* (1994); Abdi *et al.* (1998); LaBuda and Fuchs (2000); Matthews and Dickenson (2001); Cho *et al.* (2002); Scott *et al.* (2002); Abbadie *et al.* (2010); Fossat *et al.* (2010)
Partial sciatic nerve ligation	Tight ligation of one-third to half of sciatic nerve with a single ligature	Rats and mice	$Ca_v2.2$ and $\alpha2$-δ subunit: Inhibition of mechanical hyperalgesia and allodynia	Fox *et al.* (2003); Berecki *et al.* (2010)
Post-herpetic neuralgia (varicella-zoster and herpes simplex virus)	Injection of viral infected cells in the footpad	Rats and mice	$\alpha2$-δ subunit: Inhibition of mechanical hyperalgesia and allodynia	Takasaki *et al.* (2000)
Chronic compression of dorsal root ganglion	Insertion of a small rod into the L5 intravertebral foramen	Rats	$Ca_v3.2$ and $Ca_v3.3$: Inhibition of thermal hyperalgesia and allodynia	Wen *et al.* (2010)
Acute and persistent pain				
Chemically induced primary and secondary hyperalgesia	Injection of capsaicin, mustard oil or formalin (early phase) into the plantar surface of the foot or knee and ankle joints	Rats	Ca_v1 and $Ca_v2.1$: Inhibition of secondary mechanical hyperlagesia and allodynia $Ca_v2.2$: Inhibition of primary and secondary mechanical hyperalgesia and allodynia	Malmberg and Yaksh (1994); Bowersox *et al.* (1996); Sluka (1997); Nebe *et al.* (1998)
Inflammatory pain				
Carrageenan-induced inflammatory model	Injection of carrageenan into the knee joint or paw	Rats	Ca_v1, $Ca_v2.2$ and $Ca_v2.1$: Inhibition of primary and secondary mechanical hyperalgesia and allodynia	Neugebauer *et al.* (1996); Nebe *et al.* (1997); Sluka (1998)
Post-operative pain				
Post-operative pain	Longitudinal incision through the skin, fascia and muscle through the plantar surface of the hindpaw	Rats	$Ca_v2.2$: Inhibition of mechanical allodynia and heat hyperalgesia	Wang *et al.* (2000b)

et al., 1994). Ziconotide is advantageous over opioid drugs as it is a $Ca_v2.2$ antagonist, thereby avoiding the development of tolerance that is common with long-term use of morphine and other opioids (Malmberg and Yaksh, 1995).

Ziconotide has been shown to be anti-hyperalgesic in many animal pain models and later human clinical trials. In animal models, the antinociceptive effects of intrathecal ziconotide were experienced at 10 times greater potency than intrathecal morphine (Schmidtko *et al.*, 2010). Ziconotide safety and efficacy in clinical trials was assessed using both fast and slow titration rates. With fast titration, pain intensity was decreased by at least 25% when compared with placebo in patients with pain related to cancer or aids, or chronic non-malignant pain (Staats *et al.*, 2004; Wallace *et al.*, 2006; Schmidtko *et al.*, 2010). Treatment-related adverse effects occurred in 14% of all patients, with severe cognitive and neuropsychiatric adverse effects in several patients (Schmidtko *et al.*, 2010). Pain relief was decreased to 7.5% with slow dose titration in patients with chronic pain that was refractory to treatment with the current analgesics (Rauck *et al.*, 2006; Schmidtko *et al.*, 2010). Approximately 50% of patients experiencing pain relief with ziconotide classified it as a moderate to complete reduction in pain (Klotz, 2006). The incidence and severity of adverse effects was greatly reduced with slow dose titration; thus, this dosing regimen was approved by the FDA (Rauck *et al.*, 2006; Schmidtko *et al.*, 2010). Only an intrathecal route of administration has been approved for ziconotide due to the occurrence of undesired cardiovascular effects after i.v. administration (McGuire *et al.*, 1997).

Although ziconotide has been shown to be an effective anti-hyperalgesic, there are also several limitations to its use as a therapeutic. These include a narrow therapeutic index (Scott *et al.*, 2002), intrathecal route of administration (Atanassoff *et al.*, 2000; Wermeling *et al.*, 2003), potential interactions with intracellular domains or auxiliary channel subunits (Mould *et al.*, 2004) and stability issues through methionine sulfoxide formation (Schoneich *et al.*, 1997).

CVID

Other ω-conotoxins are being investigated for their therapeutic potential, including CVID (Leconotide, AM336, CNSB004) from *C. catus*, which has been shown to be more selective for $Ca_v2.2$ than MVIIA, thus producing fewer adverse effects (Lewis *et al.*, 2000; Scott *et al.*, 2002). The therapeutic potential of CVID has been extensively investigated, but as yet this research has failed to produce a marketable drug.

Unlike MVIIA, CVID can be delivered via an i.v. route of administration, with minimal adverse effects (Kolosov *et al.*, 2010). The selectivity of CVID for $Ca_v2.2$ channels was found to decrease by up to 540-fold in the presence of the β_3 subunit, which is a common subunit in sympathetic neuronal calcium channels (Lewis *et al.*, 2000). This selectivity for sensory over sympathetic neuronal calcium channels reduces the peripheral adverse effects that are common with other ω-conotoxins, such as MVIIA (Lewis *et al.*, 2000). In support of this rationale, CVID has recently been shown to be peripherally active in a rat model of diabetic neuropathic pain while causing minimal adverse effects (Kolosov *et al.*, 2010). Prior research has also shown that this peptide can access the CNS when delivered via an i.v. route of administration (Wright *et al.*, 2000; Kolosov *et al.*, 2011); therefore, CVID and peptides with similar $Ca_v2.2$ selectivity can potentially be intravenously administered for pain treatment. This may make the peptides competitive with other therapeutics for first-line treatment.

Other ω-conotoxins

Although MVIIA and CVID are effective inhibitors of $Ca_v2.2$, there are several limiting factors that provide an opportunity for the development of improved, novel ω-conotoxins. Both peptides have only a narrow therapeutic window and MVIIA administration is limited to an intrathecal route due to interaction with peripheral targets (Atanassoff *et al.*, 2000; Scott *et al.*, 2002). These peptides also suffer from significant decreases in affinity and reversibility in the presence of auxiliary subunits, which are commonly up-regulated in certain pain states (Luo *et al.*, 2001; Newton *et al.*, 2001; Mould *et al.*, 2004).

The high $Ca_v2.2$ selectivity, decreased interaction with the α2-δ subunit and improved channel recovery after block suggest that the recently identified CVIE and CVIF could overcome these limitations (Mould *et al.*, 2004; Motin and Adams, 2008; Berecki *et al.*, 2010). Additional point mutations, which have been shown to improve channel recovery, could be applied to CVIE and CVIF to allow the adverse effects to be controlled (Mould *et al.*, 2004). The discovery of other novel ω-conotoxins could further improve these properties by completely avoiding interaction with auxiliary subunits and increasing the selectivity for $Ca_v2.2$, thus decreasing the adverse effects that are commonly experienced with ω-conotoxin therapeutics. Although ω-conotoxin therapeutics are established in the clinic or currently under preclinical and clinical development, the opportunity for an improved drug candidate exists in the elusive analgesic market.

Combination therapy

Combination therapy aimed at two different molecular targets can commonly produce a superior effect to the administration of a single therapeutic alone. Synergistic acting analgesics are highly desired as a lower dose of each drug is required, thus potentially reducing the risk of adverse effects that are experienced with each drug alone. There have been several reports of the synergistic activity between ω-conotoxins and morphine. This has been shown for MVIIA (Wang *et al.*, 2000a), GVIA (Omote *et al.*, 1996) and CVID (Kolosov *et al.*, 2011).

The majority of such studies have investigated the intrathecal administration of the compounds, but more recently synergistic activity of intravenously administered CVID with morphine and flupirtine has been demonstrated. Kolosov *et al.* (2011) investigated the synergistic effects between CVID and morphine in a rat model of bone cancer pain when intravenously administered. The co-administration of CVID and morphine caused an increase in the anti-hyperalgesic action of morphine or CVID alone. Morphine (5 $mg \cdot kg^{-1}$) and CVID (20 $\mu g \cdot kg^{-1}$) alone caused a 74 and 10% reversal of hyperalgesia respectively. Administration of a combination of morphine (5 $mg \cdot kg^{-1}$) and CVID (20 $\mu g \cdot kg^{-1}$) caused a 94% reversal of hyperalgesia.

CVID has also been shown to act synergistically when given in combination with flupirtine, a K_v7 selective activator. Intravenous CVID and flupirtine produced a 25 and –6% reversal of hyperalgesia, respectively, whereas in combination, they produced an 84% reversal in a rat model of diabetic neuropathic pain (Kolosov *et al.*, 2010).

The effect of CVID administration with several other analgesics has been studied. The synergism of CVID and dexmedetomidine, an α_2-adrenoceptor agonist, was recently investigated by Blake *et al.*, (2005). α_2-Adrenoceptor agonists indirectly decrease $Ca_v2.2$-mediated neurotransmitter release, suggesting that co-administration of an α_2-adrenoceptor agonist with a $Ca_v2.2$ blocker could produce a greater analgesia than either compound administered alone. Intrathecal administration of CVID and dexmedetomidine completely inhibited allodynia in spinal nerve ligated rats when administered alone and had a synergistic effect when co-administered. The co-administration of these compounds also increased the duration of the effect of dexmedetomidine and required lower doses to achieve complete inhibition of allodynia than administration of each compound alone. The synergistic effect of opioids, α_2-adrenoceptor agonists and ω-conotoxins has also been demonstrated by Wei *et al.*, (1996).

Co-administration of ω-conotoxins with other analgesic drugs represents an evolving strategy for effective pain relief. This approach is particularly suitable for peripherally acting peptides, such as CVID, as they can be intravenously administered with opioids and other analgesics.

Future directions

$Ca_v2.2$ is an important target for analgesic development and has been targeted by marine molluscs for centuries. The resulting peptides from cone snails have been shown to have exquisite selectivity and potency for this channel, with many research groups investigating these peptides for therapeutic use and as scaffolds for rational drug design. The design efforts have so far gone unrewarded, with the chemists not able to produce a molecule that matches the activity of the native molluscan peptides. One of these peptides, MVIIA, is currently available for therapeutic use, although adverse effects and an inconvenient route of administration limit its use. Other ω-conotoxins are in various stages of development and appear promising for the treatment of various pain states. Additionally, new high-throughput techniques are rapidly emerging and provide the promise of developing superior $Ca_v2.2$ inhibitors with convenient delivery methods and fewer adverse effects. With a large number of conotoxins yet to be characterized, there is also the possibility that the cone snails that initially highlighted $Ca_v2.2$ as a pain target are one step ahead of us and have already produced a superior $Ca_v2.2$ inhibitor.

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Conflicts of interest

Professor Alewood is a co-inventor on a University of Queensland patent, covering CVIE conotoxin and related analogues.

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