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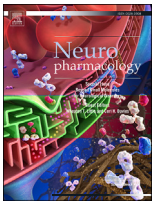

Invited review

Analgesic conopeptides targeting G protein-coupled receptors reduce excitability of sensory neurons

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ABSTRACT

Conotoxins (conopeptides) are a diverse group of peptides isolated from the venom of marine cone snails. *Conus* peptides modulate pain by interacting with voltage-gated ion channels and G protein-coupled receptors (GPCRs). Opiate drugs targeting GPCRs have long been used, nonetheless, many undesirable side effects associated with opiates have been observed including addiction. Consequently, alternative avenues to pain management are a largely unmet need. It has been shown that various voltage-gated calcium channels (VGCCs) respond to GPCR modulation. Thus, regulation of VGCCs by GPCRs has become a valuable alternative in the management of pain. In this review, we focus on analgesic conotoxins that exert their effects via GPCR-mediated inhibition of ion channels involved in nociception and pain transmission. Specifically, α-conotoxin Vc1.1 activation of $GABA_B$ receptors and inhibition of voltage-gated calcium channels as a novel mechanism for reducing the excitability of dorsal root ganglion neurons is described. Vc1.1 and other α-conotoxins have been shown to be analgesic in different animal models of chronic pain. This review will outline the functional effects of conopeptide modulation of GPCRs and how their signalling is translated to downstream components of the pain pathways. Where available we present the proposed signalling mechanisms that couples metabotropic receptor activation to their downstream effectors to produce analgesia.

Contents

Abbreviations: GPCRs, G protein-coupled receptors; RTKs, receptor tyrosine kinases; $GABA_B$, γ-aminobutyric acid type B; DAG, diacylglygerol; TRP, transient receptor potential; ASICS, acid-sensing ion channels; VGCC, voltage-gated calcium channels; GIRK channels, G protein-coupled inwardly-rectifying K^+ channel; nAChR, nicotinic acetylcholine receptor; KOR, κ-opioid receptor; NT, neurotensin; PTX, pertussis toxin; VFTD, venus fly trap domain; DRG, dorsal root ganglion; PCT, proximal carboxyl terminal; IBS, Irritable bowel syndrome; HVA, high voltage-activated; LVA, low voltage-activated.

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1. Overview

Pain is the response to an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. However, many conditions lead to the perception of pain that, when inadequately managed, can lead to adverse physical and psychological outcomes. When individuals suffer disabling conditions, the burden extends to the whole of society at an increasingly high cost to the health systems.

The current therapies for the management of pain involve modulation of G protein-coupled receptors (GPCRs), ion channels and various enzymes. Opiate drugs targeting GPCRs have long been used but have proven problematic due to the generation of many undesirable side effects. Thus, alternative avenues to pain management are a much sought after goal to deliver better health care.

A rich source of pharmacoactive compounds resides in the venom/toxin of various plants and animals. Traditionally such "venoms" have been fractionated and screened against many key targets in the pain pathways. Recently, several peptides have been discovered that target a wide variety of metabotropic receptors such as the 'G protein-coupled receptors' and 'receptor tyrosine kinases' (RTKs) that include adrenergic, γ-aminobutyric acid B ($GABA_B$), neurotensin (NT), opioid, vasopressin and insulin receptors (Callaghan et al., 2008; Craig et al., 1999; Cruz et al., 1987; Deuis et al., 2015; Safavi-Hemami et al., 2015; Sharpe et al., 2003). In this review, we discuss the current knowledge on marine cone snail venom peptides (conopeptides) targeting GPCRs. The focus will be on the functional effects of conopeptides targeting GPCRs and how their signalling is transduced to downstream components of pain pathways, such as the voltage-gated calcium channels (VGCCs). In particular, the discovery and potential use of metabotropic targeting conopeptides as potential lead analgesic compounds will be discussed.

2. Cone snails and their venom peptides

Marine cone snails are found mainly in tropical waters of the western indo-pacific ocean, with some species that have adapted to more temperate waters around South Africa, the Mediterranean and the southern California coast. The *Conus* species have a distinct, tailored venom cocktail of >200 unique peptides, dependent on their preferred prey, fish, molluscs or worms, which lead to the broad classification of cone snails as piscivorous, molluscivorous or vermivorous, respectively. Given that there are at least 800 distinct species, the genus *Conus*, encompasses hundreds of thousands of compounds many of which have been demonstrated to have therapeutic potential.

Conventionally, *Conus* venom components are distinguished as either disulfide rich conopeptides (≥2 disulfide bonds, conotoxins) or disulfide-poor conopeptides (≤1 disulfide bond). Further classification is made according to one of three other major criteria: (1) gene superfamily, (2) cysteine framework, and (3) pharmacological family (see (Kaas et al., 2010)).

Various throughput schemes have been used to identify the targets of *Conus* peptides with potential analgesic effects, traditionally including a wide array of voltage-gated ion channels (calcium (Ca_V), sodium (Na_V), and potassium (K_V) channels) and ligand-gated receptor-channels (Cys-loop receptors, ionotropic glutamate receptors, ATP-gated channels, etc.). Advances in proteomic techniques have permitted sequence mining within the previously unexplored, smaller, disulfide-poor peptides from cone snail venoms for potential hormone/neuropeptide-like bioactive substances (Robinson et al., 2017) Such 'under-utilized' portion of the cone snail venom is providing new potential analgesic compounds and helping researchers examine metabotropic receptor function and elucidate their role as analgesic targets (Lebbe and Tytgat, 2016).

α-Conotoxins are typically 12–19 amino acids long, containing two disulfide bonds with Cys^{I}-Cys^{III} and Cys^{II}-Cys^{IV} connectivity with a variable number of residues between loops, and frequently is C-terminally amidated (Table 1) (Hu et al., 1997; Millard et al., 2004). α-Conotoxins were identified by their potent activity at inhibiting ACh-evoked currents through muscle and neuronal nicotinic acetylcholine receptor (nAChR) -channels.

3. GPCRs as targets of analgesic compounds

GPCRs are integral membrane signalling proteins characterized by a seven-transmembrane-segments (7TM) architecture. They are the largest and most diverse family of proteins encoded in the mammalian genome (Takeda et al., 2002) and are divided into five main classes: Class A or rhodopsin-like receptors, Class B or secretin-like receptors, Class C or metabotropic-glutamate-like receptors, Class F or frizzled family, and Adhesion class (Alexander et al., 2015). The other two classes of GPCRs include, Class D (fungal mating pheromone receptors) and Class E (cyclic AMP receptors), have not been found in vertebrates. All mammalian classes include members with demonstrated analgesic capabilities with ~80% of individual subfamilies containing a member linked directly to nociceptive signalling (Stone and Molliver, 2009).

GPCRs convert physical and chemical stimuli into intracellular signals via the G protein (guanine nucleotide-binding proteins) signalling complex, composed of Gα, Gβ and Gγ. Ligand binding to the GPCR causes a structural rearrangement of the protein which conveys ligand binding to the intracellular heterotrimeric G protein signalling molecules, causing release of GDP, GTP activation of the Gα protein, and its concomitant release with the Gβγ dimer to modulate downstream targets (Oldham and Hamm, 2008).

Through Gα and Gβγ, GPCRs are able to communicate with ligand- and voltage-dependent ion channels in pain pathways (Stone and Molliver, 2009) including the transient receptor potential (TRP) channels, acid-sensing ion channels (ASICS), ATP-gated P2X channels, as well as voltage-gated sodium, calcium and potassium channels.

There is a broad range of evidence demonstrating inhibition of voltage-gated calcium channels (VGCC) by GPCRs as a major mechanism for modulating neurotransmitter and hormone release (Dolphin, 2003). VGCCs serve as potent drug targets in the therapy

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Table 1
α-Conotoxin inhibition of high voltage-activated calcium channels in dorsal root ganglion neurons.

α-Conotoxin	Sequence and connectivity (Loop 1, Loop 2)	HVA Ca^{2+} channel inhibition %	IC_{50} (nM)	Analgesic in animal pain model	Concentration used in animal pain models
Vc1.1	GCCSDPRCNYDHPEICNH2	30-40%[1,2,3,4]	1.7[1]	CCI[5,6,7] PNL[3,6,8] DNP[7] CVP[9] CFA[7]	CCI: 24-800 μg/kg s.c. or 0.036-3.6 μg/200 μl i.m. PNL: 1μg/kg s.c. or 0.36 μg/200 μl i.m. and 0.2 nmol i.t. DNP: 300 μg/kg s.c. CVP: 1 μM intracolonic enema CFA: 8 μg/kg-2.4 mg/kg s.c.
cVc1.1	GCCSDPRCNYDHPEICGGAAGG	45[4]	0.3[4]	CCI[4]	CCI: 0.3- 3mg/kg oral administration
Dicarba Vc1.1	GXCSDPRXNYDHPEICNH2	30-38[10]			
hcVc1.1	GHCSDPRFNYDHPEICGGAAGG	50[11]	0.86[11]		
[Ser3] Vc1.1 (1-8)	GCSSDPRC	30[2]		CVP[2]	CVP: 1-1000 nM intracolonic enema
Vc1.2	GCCSNPACMVNNPQICNH2	30[12]	47.5[12]		
Rg1A	GCCSDPRCRYR----CR	40[1,13]	40.7[13]	CCI[5,7,14]	CCI: 0.2-2 nmols/200 μl i.m.
cRg1A	GCCSDPRCRYR----CGGAAGG	45[13]	4.3[13]		
Dicarba Rg1A	GXCSDPRXRYR----CR	35[15]			
AuIB	GCCSYPPCFATNPD-CNH2	45[3]	1.5[3]	PNL[3,8]	PNL: 0.36-36 μg/kg i.m. or 0.2-2 nmols i.t.
PeIA	GCCSHPACSVNHPELCNH2	40[16]	1.1[16]		
Pn1.2	GCCSHPPCFLNNPDYC	22[2]			
Pu1.2	GGCCSYPPCIANNPL-C	27[2]			
[Ser4] Pu1.2 (1-9)	GGCSSYPPC	19[2]			
Kn1.2	PGCCNNPACVKHR---C	13[2]			
Tx1.2	PQCCSHPACNVDHPEIC	8[2]			

IC_{50} values and % inhibition (up to 1 μM) were determined for HVA calcium channel currents from rat DRG neurons. Conserved cysteines are in green. The position of the two loops and disulphide scaffold are shown (top). X (yellow) indicates the dicarba scaffold substitutions. The sequence in red and black lines represents the linker in cyclized version of the peptide. List of abbreviations.
Chronic constriction injury (CCI); Partial nerve ligation (PNL); Diabetic neuropathic pain (DNP); Chronic visceral pain (CVP); Complete Freund's adjuvant (CFA); Subcutaneous injection (s.c.); Intrathecal injection (i.t.); Intramuscular injection (i.m.). [1](Callaghan et al., 2008); [2](Carstens et al., 2016); [3](Klimis et al., 2011); [4](Clark et al., 2010); [5](Vincler et al., 2006); [6](Satkunanathan et al., 2005); [7](McIntosh et al., 2009); [8](Napier et al., 2012); [9](Castro et al., 2017); [10](van Lierop et al., 2013); [11](Yu et al., 2015); [12](Safavi-Hemami et al., 2011); [13](Halai et al., 2011); [14](Di Cesare Mannelli et al., 2014); [15](Chhabra et al., 2014); [16](Daly et al., 2011).

of many neurological, and cardiovascular disorders (Cain and Snutch, 2011; Simms and Zamponi, 2014; Striessnig et al., 2014) and particularly, in pain management due to their regulatory role in neurotransmitter release in nociceptive pathways. These channels have been classified based on their biophysical properties into low voltage-activated (LVA) or T-type channels ($Ca_V3.1$ to $Ca_V3.3$), and high voltage-activated (HVA) including L-type ($Ca_V1.1$ to $Ca_V1.4$), P/Q-type ($Ca_V2.1$), N-type ($Ca_V2.2$) and R-type ($Ca_V2.3$) channels (Catterall et al., 2005). To date, each calcium channel family has been implicated in pain and nociception, with N- and T-type calcium channels being of particular interest as potential pain targets (Patel et al., 2017; Zamponi et al., 2015).

4. α-Conotoxin modulation of VGCCs via $GABA_BR$ activation

Callaghan et al. observed that HVA calcium channel currents from rat dorsal root ganglion (DRG) neurons were strongly attenuated in the presence of α-conotoxins Vc1.1 from *Conus victoriae* and Rg1A from *Conus regius* (Callaghan et al., 2008). HVA current inhibition by Vc1.1 and Rg1A was blocked by the $GABA_BR$-selective antagonists, phaclofen, CGP55845 and CGP54626 (Callaghan et al., 2008). The involvement of a GPCR in mediating the VGCC inhibition was demonstrated by abrogation of VGCC inhibition in the presence of either pertussis toxin (PTX) or GDPβS, as well as by blockade of Vc1.1-mediated relief from neuropathic pain in behavioural studies by the $GABA_BRs$ antagonist SCH50911 (Klimis et al., 2011). Importantly, application of Vc1.1 to heterologously expressed VGCCs does not exert direct modulation of calcium channel activity in the absence of $GABA_BRs$ (Callaghan et al., 2008).

A variety of antagonists to other membrane receptors expressed in DRG neurons such as $GABA_A$, α1 and α2-adrenergic, nicotinic and muscarinic ACh receptor, and μ-opioid receptor antagonists failed to disrupt Vc1.1 inhibition of HVA calcium channel currents. Furthermore, inclusion of the selective peptide inhibitor of c-Src tyrosine kinase, pp60c-src, in the intracellular solution prevents the inhibition of Ca_V channels by α-conotoxins (Berecki et al., 2014).

$GABA_BR$ signalling through N- and R-type calcium channels has been described (Adams and Berecki, 2013; Berecki et al., 2014). Accordingly, siRNA knockdown of functional $GABA_BR$ expression decreased Vc1.1, Rg1A and AuIB inhibition of N-type calcium channel currents in rat DRG neurons (Cuny et al., 2012). A great deal of attention has been directed towards $Ca_V2.2$ as a direct target for pain modulation, thus inhibition of $Ca_v2.2$ currents via $GABA_BR$ represents a promising alternative in the treatment of pain pathologies.

To date, other α-conotoxins such as AuIB (*Conus aulicus*), PeIA (*Conus pergrandis*) and Vc1.2 (a peptide isolated from embryonic *Conus victoriae*) share similar activity to Vc1.1 and Rg1A (α-conotoxins that inhibit VGCC via $GABA_BR$ activation are listed in Table 1). These peptides also inhibit N-type VGCCs via $GABA_BR$-mediated pathways (Daly et al., 2011; Klimis et al., 2011; Safavi-Hemami et al., 2011) and have been shown to be analgesic in animal models including chronic constriction injury (CCI), partial nerve ligation (PNL), diabetic neuropathic pain (DNP) and chronic visceral pain (CVP) (Table 1). In particular, Vc1.1-induced activation of $GABA_BR$ on the peripheral endings of colonic afferents has been

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shown to inhibit mouse colonic nociceptors and also low-threshold distension-sensitive colonic afferents with greatest effect during chronic visceral hypersensitivity (Castro et al., 2017). Furthermore, Vc1.1 significantly reverses mechanical allodynia in neuropathic rats and reduces ectopic discharge associated with injured peripheral nerves (Zhao et al., 2016). Therefore, $GABA_BR$-mediated inhibition of VGCCs is proposed to contribute to the analgesic action of α-conotoxins observed in multiple animal models of pain.

4.1. $GABA_BR$ signalling pathways to Ca_V channels

Activation of GPCRs, including the $GABA_BR$, results in the dissociation of Gα and Gβγ, allowing the free subunits to signal to a variety of downstream targets that modulate VGCCs (Ikeda, 1996). Direct binding of Gβγ to the α1 subunit of Ca_V channels mediates a fast and potent 'voltage-dependent inhibition' of channel activity, by stabilization of the closed channel (reluctant state) (Agler et al., 2003). Gβγ binding and modulation of VGCCs was confirmed through transient overexpression of Gβγ or direct application of purified βγ subunits to rat sympathetic ganglion neurons (Ikeda, 1996; Herlitze et al., 1996). Reluctant state stabilization is believed to occur by uncoupling the movement of the voltage-sensor from the opening of the channel's activation gate (Hernandez-Ochoa et al., 2007; Patil et al., 1996; Rebolledo-Antunez et al., 2009), which is transiently relieved by Gβγ unbinding at strong depolarized potentials known as 'prepulse relief' (Currie, 2010), or arguably in the physiological context, in response to high frequency trains of action potentials (Park and Dunlap, 1998).

Unlike the fast voltage-dependent inhibition of Ca_V channels by $GABA_BR$, the voltage-independent pathway occurs within tens of seconds, and is thought to be mediated via the dissociated Gα subunit without direct binding to the channel protein. Voltage-independent inhibition of Ca_V channels by $GABA_BRs$ thus involves different diffusible second messengers (phosphoinositides, tyrosine kinase, etc), and remodelling of the GPCR/channel complex (by phosphorylation, etc.). Strong membrane depolarization cannot reverse this type of inhibition hence it is deemed as voltage-independent (Dolphin, 2003). For example, baclofen (a structural analogue of GABA) binding to $GABA_BR$ inhibits N-type calcium channel in capsaicin-responsive DRG, an indicator of nociceptive neurons (Bell et al., 2004; Lipscombe and Raingo, 2007). This effect appears to occur via tyrosine kinase mediated voltage-independent inhibition of specific $Ca_V2.2$ channel splice variants (Andrade et al., 2010; Raingo et al., 2007).

4.2. Analgesic α-conotoxins: a proposed mechanism of action

$GABA_BR$ can modulate VGCCs in both voltage-dependent and -independent fashions, contingent on the GPCR agonist and the downstream effectors involved. $Ca_V2.1$ and $Ca_V2.2$ channels are major targets of direct inhibition by GPCRs but $Ca_V2.3$ channels also respond to $GABA_BR$ modulation (Page et al., 1998; Rebolledo-Antunez et al., 2009).

$GABA_BR$-mediated inhibition of $Ca_V2.1$ is voltage-dependent and thus can be relieved by strong depolarization whereas GABA and baclofen inhibit $Ca_V2.3$ in a voltage-independent manner. Cyclized α-conotoxin, c-Vc1.1, potently inhibits $Ca_V2.3$ in a voltage-independent manner without affecting $Ca_V2.1$ (Berecki et al., 2014). Vc1.1 inhibition of N-type calcium channels in DRG neurons is thus proposed to be either voltage-dependent (37b) or voltage−independent (37a) depending on the splice variant expressed (Fig. 1) (Callaghan et al., 2008; Raingo et al., 2007).

Similarly, experiments involving co-expression of human $GABA_BRs$ and human $Ca_V2.1$and $Ca_V2.3$ in HEK293T cells showed that linear and cyclized versions of Vc1.1 do not inhibit $Ca_V2.1$ but do inhibit $Ca_V2.3$ in a voltage-independent manner, an effect that can be attenuated by application of the $GABA_BR$ antagonist CGP55845 (Berecki et al., 2014). These findings confirm $GABA_BRs$ involvement in Vc1.1 and cVc1.1 inhibition of Ca_v channels. Furthermore, it has recently been shown that human DRG neurons express $GABA_BR$ and $Ca_V2.2$, and $Ca_V2.3$, which are the direct and downstream targets of Vc1.1's attenuation of neuroexcitability (Castro et al., 2017).

Remarkably, Vc1.1 and Rg1A do not appear to activate G protein-coupled inwardly-rectifying potassium (GIRK) channels heterologously co-expressed with $GABA_BRs$ in *Xenopus* oocytes (McIntosh et al., 2009) unlike other $GABA_BR$ agonists that normally activate GIRK channels (Sodickson and Bean, 1996).

Interestingly, Vc1.1 and baclofen produce opposing effects on $Ca_V2.2$ current kinetics. In HEK293 cells, Vc1.1 accelerates the rate of channel activation and shifts the inactivation curve to more hyperpolarized potentials (Huynh et al., 2015), whereas, baclofen slows the rate of activation and shifts the half-activation potential of $Ca_V2.2$ to more depolarized potentials (Huynh et al., 2015). Furthermore, α-conotoxin Vc1.1 inhibition of Ca_V channels is use- or frequency-dependent (Callaghan et al., 2008), which is advantageous for pain treatment because hyperexcitable cells are preferentially targeted.

Studies on chick DRG neurons unravelled the involvement of Gα/tyrosine kinase dependent phosphorylation of N-type channels α1 subunit $Ca_V2.2$ splice variant e37a (Schiff et al., 2000). Analysis of the $Ca_V2.3$ channel C-terminus revealed a pair of tyrosine residues (Y1761 and Y1765, human, $\alpha_{1E\text{-}c}$) phosphorylated by c-src kinases which is required for voltage-independent inhibition of $Ca_V2.3$ by GPCRs (Berecki et al., 2014). The homologous residue to position 1761 in human and rodent $Ca_V2.1$ channels is phenylalanine, which cannot be readily phosphorylated by c-src kinases and thus may explain the lack of voltage-independent inhibition in these calcium channel subtypes. Interestingly, bovine and rabbit $Ca_V2.1$ channels do present the analogous tyrosine and display voltage-independent inhibition via $GABA_BR$ (Berecki et al., 2014; Burgoyne and Weiss, 2001).

Thus, $GABA_BR$ signalling pathways affected by α-conotoxins lead to inhibition of VGCCs and ultimately produces analgesia distinct to that generated by other $GABA_BRs$ agonists such as baclofen. It is worth noting that Vc1.1 is ~1000 times more potent than baclofen at inhibiting HVA calcium channels in sensory neurons.

4.3. Molecular determinants of α-conotoxin binding to $GABA_BRs$

Although the venus fly trap domain (VFTD) of the $GABA_{B1}$ subunit is the main (orthosteric) binding site for natural $GABA_B$ ligands, there is evidence that its obligatory partner $GABA_{B2}$ contains a binding site for allosteric modulators located in its transmembrane domain, which is not associated with the N-terminal VFTD (Binet et al., 2004; Pin and Prezeau, 2007). Site-directed mutagenesis studies demonstrated that the α-conotoxin binding site does not lie within the $GABA_{B1}$ VFTD. Furthermore, it was shown that mutation or deletion of the proximal C-terminal domain (PCT) of $GABA_{B1a}$ reduced Vc1.1, but not baclofen, inhibition of $Ca_V2.2$ channels (Huynh et al., 2015). This demonstrated that $GABA_{B1a}$ PCT couples signalling to VGCCs, and it is involved in α-conotoxin activation of $GABA_BRs$. This further highlights the discovery of another agonist site on the $GABA_B$ subunits distinct from their VFTD (Fig. 1). Molecular dynamics simulations of the interaction between α-conotoxin Vc1.1 and $GABA_BR$ propose key interaction sites at the interface between the $GABA_BR$ ectodomains (Adams and Berecki, 2013).

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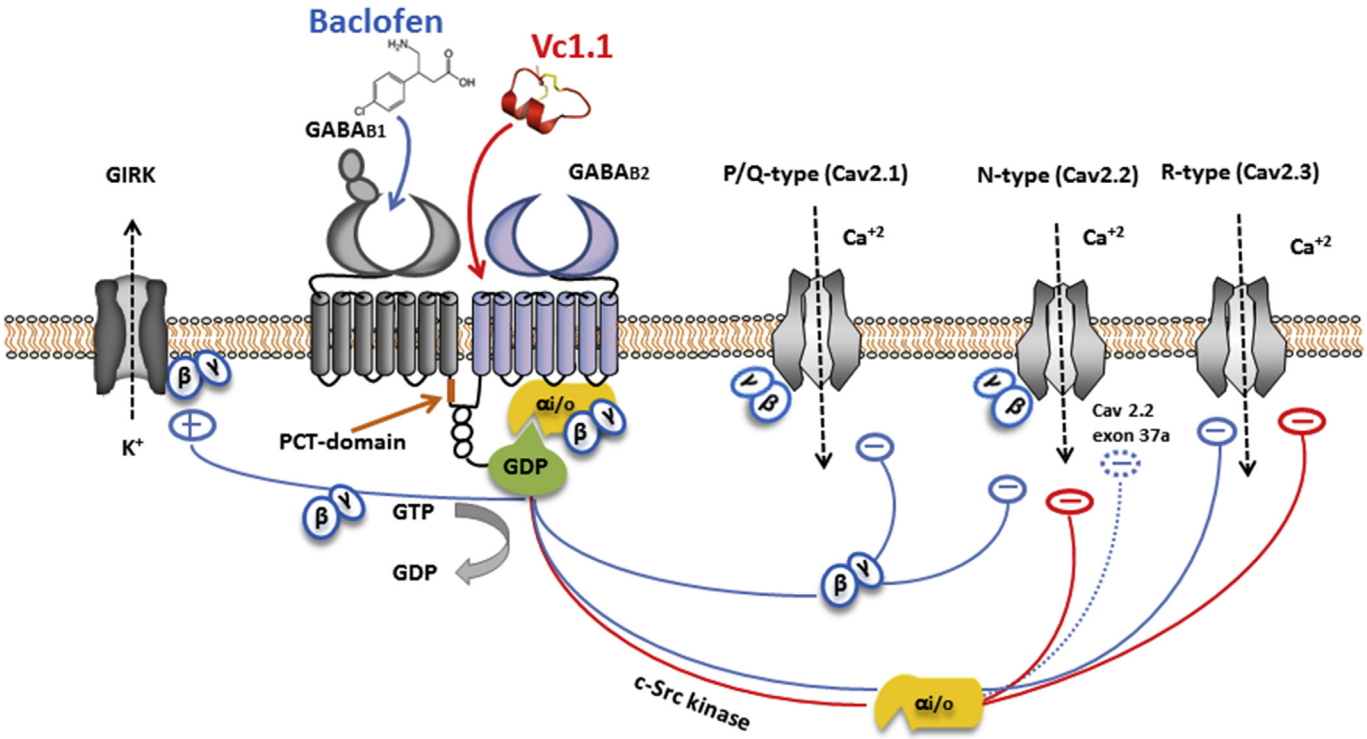

Fig. 1. $GABA_BRs$ downstream effector signalling pathways: $GABA_BRs$ signalling pathways activated by baclofen (**blue**) and α-conotoxin Vc1.1 (**red**). Baclofen binds to VFTD of $GABA_{B1}R$ subunit and activates $GABA_BRs$ resulting in dissociation of Gα and Gβγ heterodimer. Released Gβγ inhibits VGCCs largely in a voltage-dependent manner and hence depresses evoked Ca^{2+}-dependent neurotransmitter release, while also activating GIRK channels. In contrast, α-conotoxins bind to an as yet unidentified site on $GABA_BRs$ that is suggested to be at the interface between the $GABA_BRs$ ectodomains. $GABA_BR$ activation results in dissociation of Gα and Gβγ heterodimer. Released Gα subunit inhibits $Ca_V2.2$ and $Ca_V2.3$ by c-Src kinase-mediated phosphorylation of the C-terminal domain of these channels.

5. Other targets of the α-conotoxins

α-Conotoxin inhibition of neuronal nAChR subtypes has been associated with analgesic effects in animal models of neuropathic pain. For instance, α-conotoxins Vc1.1 and Rg1A inhibit α9α10 nAChRs with low nanomolar affinity and show analgesic activity in rodent models of chronic constriction injury (CCI) and partial nerve ligation (PNL) (Satkunanathan et al., 2005; Vincler et al., 2006). Vc1.1 was also shown to reduce nociceptive signalling of noxious colorectal distention with increased efficacy in chronic visceral pain conditions (Castro et al., 2017). Moreover, AuIB reduces mechanical allodynia in rat models of neuropathic pain and is an α3β4 nAChR antagonist (Napier et al., 2012), whereas α-conotoxin MII is a potent α3β2 nAChR antagonist that confers short-acting, anti-allodynic effects (Klimis et al., 2011; Napier et al., 2012).

Although early studies proposed that the analgesic effect of α-conotoxins could be mainly attributed to their inhibition of nAChRs, extensive analyses of various α-conotoxins and their analogues have shed light on alternative effectors of their analgesic actions (Livett et al., 2006; Nevin et al., 2007). Furthermore, antagonism of α9α10 nAChR is not sufficient to reverse allodynia observed in animal pain models where Vc1.1, AuIB or MII were administered (Klimis et al., 2011). A comprehensive review of α9α10 nAChRs as a pain target has recently been published (Mohammadi and Christie, 2015). Additionally, Vc1.1 and Rg1A inhibited HVA calcium channels via $GABA_BR$ activation in the α9 nAChR knockout mouse and experience analgesia upon Vc1.1 and Rg1A treatment (Callaghan and Adams, 2010). Recently, the α-conotoxin Rg1A analogue Rg1A4, a potent inhibitor of human and rat α9α10 nAChRs, has been shown to prevent chemotherapy-induced neuropathic pain (Romero et al., 2017). In this pain assay, α9 knockout mice exhibited temporary cold allodynia that was no longer relieved by Rg1A4 and a neuroprotective modulation of immune function though α9α10 nAChR is proposed as the possible mechanism of analgesia by Rg1A4. Taken together, it is clear that the analgesic effects of the α-conotoxins are mediated by complex, multifactorial pathways that require further characterization in different pain assays. These results suggest that the analgesic effects of α-conotoxins, whether through $GABA_BR$ modulation of VGCC or through nAChRs, could potentially target distinct pain signalling pathways depending on the tissue and type of pain.

6. Analgesic conopeptides targeting other GPCRs

Numerous venom-derived peptides have been reported to target GPCRs (Lewis et al., 2012; Näreoja and Näsman, 2012), however, with the exception of the α-conotoxins mentioned above, conopeptide modulation of GPCRs involved in analgesia remains largely unexplored. However, the range of available *Conus*-derived peptides is steadily increasing with the following two conopeptides taken into preclinical trials for treatment of chronic pain.

6.1. Contulakins

Neurotensin (NT) receptors are involved in a broad spectrum of neuromodulatory effects in the peripheral and central nervous systems with a key role in pain modulation, as well as Parkinson's disease, autism, blood pressure, glucose control, appetite, schizophrenia and addiction (Feng et al., 2015; Kleczkowska and Lipkowski, 2013). It has been reported that NT receptors also play role in visceral pain condition (Smith et al., 2012). To date, three main NT receptors have been characterized: receptors with high

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affinity for NT (NTS_1), receptors with lower-affinity to NT which also bind the H1 anti-histamine levocabastine (NTS_2), and intracellularly located NT receptors, Sortilin 1 (NTS_3) (Alexander et al., 2015). From these only NTS_1 and NTS_2 are recognised as GPCRs.

Contulakin-G is a 16 amino acid *O*-linked glycopeptide (sequence: ZSEEGGSNATKKPYIL) isolated from the venom of predatory marine snail, *Conus geographus* (Craig et al., 1999). Contulakin-G is a helical peptide without a disulphide bond and has two post-translational modifications: a pyroglutamate (Z), and *O*-glycosylation of threonine, Thr10. The C-terminal sequence of contulakin-G is homologous to the NT family and this peptide acts on NTS_1 and NTS_2 receptors. Contulakin-G exhibits potent analgesic activity in inflammatory and acute pain in rats following intrathecal delivery of the peptide (Allen et al., 2007; Han et al., 2008). Contulakin-G is more biologically active and stable in plasma than the synthetic non-glycosylated peptide analogue, contulakin-G-memantine (sequence: ZSEEGGSNKEKKPYIL). Currently, contulakin-G is in clinical development stage for the treatment of chronic intractable pain following intrathecal administration in spinal cord injury patients (Sang et al., 2016). Contulakin-G-memantine was shown to be a weaker agonist of the NT receptor than contulakin-G, however, it produced less receptor desensitization while retaining its penetration of the blood brain barrier. All of which makes it more attractive for clinical implementation, as neurotensin analogues capable of penetrating the blood brain barrier have been shown to be therapeutic in the treatment of pain and/or substance abuse (Boules et al., 2013; Dobner, 2006; Lee et al., 2015). The neurotensin analogues decrease both cocaine and amphetamine-induced hyperlocomotion and reward behaviour (Ferraro et al., 2016).

6.2. Conorphins

Opioid receptors (ORs) belong to the $G_{i/o}$ family of GPCRs and have important roles in various physiological functions including pain control (Dhawan et al., 1996; Stein, 2016). Opiates have been used for centuries but side effects such as addiction, tolerance and dependency greatly hinder their utility. A challenge of modern opioid pharmacology is to develop analgesic drugs with fewer side effects. Studies of μ-, δ-, and κ-OR knockout mice indicate that all opioid receptors can mediate analgesia. However, κ-opioid receptors (KOR) agonists produce analgesia without evident μ-opioid receptor (MOR) related side effects. Furthermore, they have proved to be neuroprotective and effective in suppression of the rewarding effect of opioids and cocaine (for recent review see (Lalanne et al., 2014)). Peripherally restricted, selective KOR agonists are of interest to avoid side effects associated with central activation of these receptors.

Conorphins (generic sequence: NCCRRQICC) were identified from opioid receptor screening of a conopeptide library of over 2000 distinct peptides (Brust et al., 2016). They are defined by a hydrophobic benzoprolyl moiety, a double arginine sequence, a spacer amino acid followed by a hydrophobic residue and a C-terminal vicinal disulfide moiety. Conorphin-T was selected as potent, stable and selective KOR agonist with a pharmacophore that resembles that of dynorphin A. Conorphin-T displays exceptional plasma stability and inhibits colonic sensory neurons in a mouse model of chronic visceral pain (Brust et al., 2016). This suggests a promising alternative in the treatment of visceral pain due to a substantial increase in the expression and function of KOR in colonic DRG neurons during chronic visceral hypersensitivity (Hughes et al., 2014). Conorphin-1, however, failed to produce peripheral analgesia following systemic or intraplantar administration of this peptide in inflammatory or neuropathic animal pain models (Deuis et al., 2015).

7. Perspectives

Conotoxins are an invaluable source of novel pharmacoactive molecules with the potential to become analgesic drugs. ω-Conotoxin MVIIA (Ziconotide or Prialt), a non-opioid analgesic drug, directly inhibits the N-type $Ca_V2.2$ calcium channel, and was the first FDA approved analgesic currently administered intrathecally for the management of intractable chronic pain. MVIIA has a narrow therapeutic index given the required mode of administration, however, new dosing strategies can increase its efficacy (McDowell and Pope, 2016). The detrimental secondary effects of opioid analgesic drugs are well recognised and thus there is an urgent need for potent and selective, systemically acting analgesics.

Targeting specific, peripherally expressed membrane receptors and ion channels that are involved in nociception and pain transmission is a strategy worth pursuing. The role of GPCRs such as $GABA_B$ (see Malcangio, 2017), neurotensin, κ-opioid, as well as VGCCs involved in pain pathways is well established and therefore, targeting specific GPCRs mediating VGCC inhibition peripherally may reduce the side effects associated with direct blockade of VGCCs in general.

In several animal pain models, α-conotoxins produce analgesia upon systemic administration without overt deleterious side effects. Therefore, there is considerable interest in studying these peptides as novel analgesics. $GABA_BR$ mediated inhibition of VGCCs is a primary analgesic mechanism of action of these conopeptides, however, the α-conotoxins also inhibit nAChRs and appear to modulate some pain pathways. Further studies are required to determine the molecular determinants of activity and selectivity of the α-conotoxins at their targets.

In general, *Conus* peptides targeting various GPCRs are promising alternatives for the treatment of chronic visceral pain in irritable bowel syndrome (IBS) patients that needs further investigation. Further studies involving derivatization and modification of those conopeptides affecting GPCRs will likely increase their potency, selectivity and bioavailability to expand the analgesic pharmacopeia available.

Conflict of interest statement

All authors confirm that they have no actual or potential conflict of interest.

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