Pharmacology & Therapeutics 222 (2021) 107792

Contents lists available at ScienceDirect

Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/pharmthera

Medicinal chemistry, pharmacology, and therapeutic potential of α-conotoxins antagonizing the α9α10 nicotinic acetylcholine receptor

Xiao Li [a,b,1], Han-Shen Tae [c,1], Yanyan Chu [a,b,d], Tao Jiang [a,b], David J. Adams [c,*], Rilei Yu [a,b,d,*]

[a] Key Laboratory of Marine Drugs, Chinese Ministry of Education, School of Medicine and Pharmacy, Ocean University of China, 5 Yushan Road, Qingdao 266003, China
[b] Laboratory for Marine Drugs and Bioproducts, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266003, China
[c] Illawarra Health and Medical Research Institute (IHMRI), University of Wollongong, Wollongong, New South Wales 2522, Australia
[d] Innovation Platform of Marine Drug Screening & Evaluation, Qingdao National Laboratory for Marine Science and Technology, Qingdao, Shandong 266100, China

ARTICLE INFO

Available online 10 December 2020

Keywords:
α-conotoxin
α9α10 nicotinic acetylcholine receptor
chemical modification
binding site determination
therapeutic potential

ABSTRACT

α-Conotoxins are disulfide-rich and well-structured peptides, most of which can block nicotinic acetylcholine receptors (nAChRs) with exquisite selectivity and potency. There are various nAChR subtypes, of which the α9α10 nAChR functions as a heteromeric ionotropic receptor in the mammalian cochlea and mediates postsynaptic transmission from the medial olivocochlear. The α9α10 nAChR subtype has also been proposed as a target for the treatment of neuropathic pain and the suppression of breast cancer cell proliferation. Therefore, α-conotoxins targeting the α9α10 nAChR are potentially useful in the development of specific therapeutic drugs and pharmacological tools. Despite dissimilarities in their amino acid sequence and structures, these conopeptides are potent antagonists of the α9α10 nAChR subtype. Consequently, the activity and stability of these peptides have been subjected to chemical modifications. The resulting synthetic analogues have not only functioned as molecular probes to explore ligand binding sites of the α9α10 nAChR, but also have the potential to become candidates for drug development. From the perspectives of medicinal chemistry and pharmacology, we highlight the structure and function of the α9α10 nAChR and review studies of α-conotoxins targeting it, including their three-dimensional structures, structure optimization strategies, and binding modes at the α9α10 nAChR, as well as their therapeutic potential.

Contents

1. Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated cation-selective channels that were initially shown to participate in synaptic transmission at the neuromuscular junction (Hogg, Raggenbass, & Bertrand, 2003; Unwin, 2013) and are broadly distributed throughout both central and peripheral nervous systems (Bertrand & Terry, 2018; Zoli, Pucci, Vilella, & Gotti, 2018). To date, 17 subunits (α1–α10, β1–β4, γ, δ, and ε) have been identified and they assemble into distinct nAChR subtypes (Albuquerque, Pereira, Alkondon, & Rogers, 2009). Individual subunits contain a large N-terminal extracellular domain (ECD), an ion conducting transmembrane domain (TMD), and an

* Corresponding authors.
E-mail addresses: djadams@uow.edu.au (D.J. Adams), ryu@ouc.edu.cn (R. Yu).
[1] These authors contribute equally to this work.

https://doi.org/10.1016/j.pharmthera.2020.107792
0163-7258/

Abbreviations

ACh	acetylcholine;
CCI	chronic constriction nerve injury;
ECD	extracellular domain;
ICD	intracellular domain;
IC_{50}	half-maximal inhibitory concentration;
KO	knock out;
nAChR	nicotinic acetylcholine receptor;
NMR	nuclear magnetic resonance;
PDB	protein data bank;
PNL	partial sciatic nerve ligation;
TMD	transmembrane domain

intracellular domain (ICD) that provides an interface for communication with the cellular milieu (Gharpure, Noviello, & Hibbs, 2020; Thompson, Lester, & Lummis, 2010) (Fig. 1). A series of nAChR subtypes are involved in vital physiological processes, such as neurotransmitter release, synaptic transmission and neuronal integration (Zoli et al., 2018).

Dysfunctional nAChRs associated with genetic and epigenetic factors, altered regulation of expression levels, or cell metabolism and cofactors are implicated in a variety of human diseases and health conditions (Bertrand & Terry, 2018; Dani & Bertrand, 2007; Dineley, Pandya, & Yakel, 2015; Zoli, Pistillo, & Gotti, 2015). These conditions include neuropathic pain (Hone & McIntosh, 2018; Hone, Servent, & McIntosh, 2017; Umana, Daniele, & McGehee, 2013), cognitive deficits in schizophrenia and Alzheimer's disease (Hurst, Rollema, & Bertrand, 2013; Parikh, Kutlu, & Gould, 2016), Parkinson's disease (Jurado-Coronel et al., 2016; Quik, Zhang, McGregor, & Bordia, 2015), epilepsy (Becchetti, Aracri, Meneghini, Brusco, & Amadeo, 2015), tobacco addiction (Benowitz, 2009; Hurst et al., 2013), and cancer (Chen, Cheuk, Shin, & Kwong, 2019; Dang, Meng, & Song, 2016).

1.1. The α9α10 nAChR

Phylogenetic analysis of the nAChR genes from a broad selection of vertebrate species shows that among the members of the nAChR family, the subfamily comprised of the *CHRNA9* and *CHRNA10* genes forms the earliest branch, suggesting that the α9 and α10 subunits are evolutionarily distinct from other nAChR subunits (Franchini & Elgoyhen, 2006; Pedersen et al., 2019). The α9α10 nAChR is critical for mediating synaptic transmission from the medial olivocochlear to the cochlear hair cells (Elgoyhen & Katz, 2012) and has been implicated in a series of pathological conditions including, neuropathic pain and tumor proliferation (Chen et al., 2019; Hone et al., 2017).

Compared to other subtypes, the α9α10 nAChR has a number of unique biophysical and pharmacological characteristics. The relevant divalent to monovalent cation permeability of the rat α9α10 nAChR to Ca^{2+}, Ba^{2+} and Mg^{2+} is high (e.g. $P_{Ca}/P_{Na} \cong 9$) and ACh-evoked currents were blocked in a voltage-dependent manner by higher concentrations (>0.5 mM) of these cations (Gómez-Casati, Fuchs, Elgoyhen, & Katz, 2005; Weisstaub, Vetter, Elgoyhen, & Katz, 2002). Pharmacologically, nicotine, a classic agonist of neuronal nAChRs, is an antagonist of the α9α10 nAChR, with a half-maximal inhibitory concentration (IC_{50}) of 3.9 μM for the rat subtype (Verbitsky, Rothlin, Katz, & Elgoyhen, 2000). Similarly, nicotinic receptor agonists of other nAChR subtypes, such as cytisine and epibatine, are antagonists of the α9α10 nAChR (Verbitsky et al., 2000). Muscarine, a selective agonist of muscarinic AChRs, also inhibits rat α9α10 nAChRs with an IC_{50} of 41 μM (Elgoyhen et al., 2001; Vincler & McIntosh, 2007). These unique pharmacological properties of α9α10 nAChR are intriguing but also bring challenges for understanding its function.

1.2. The α9α10 nAChR in hearing

Physiologically, the α9α10 nAChR is best known for mediating the inhibition of hair cell function within the auditory sensory organ (Elgoyhen et al., 2001; Elgoyhen, Katz, & Fuchs, 2009). The organ of Corti in the mammalian cochlea is responsible for the transmission of sound waves into electrical signals, via the outer and inner hair cells. Synapses of both hair cells are regulated by the α9α10 nAChR, which is functionally coupled to the activation of small-conductance, Ca^{2+}-dependent potassium channels (Elgoyhen & Katz, 2012; Glowatzki & Fuchs, 2000; Gómez-Casati et al., 2005; Katz et al., 2004; Wedemeyer et al., 2018). This is also important developmentally; a recent study using an α9 knock-in mouse model has highlighted that transient efferent innervation

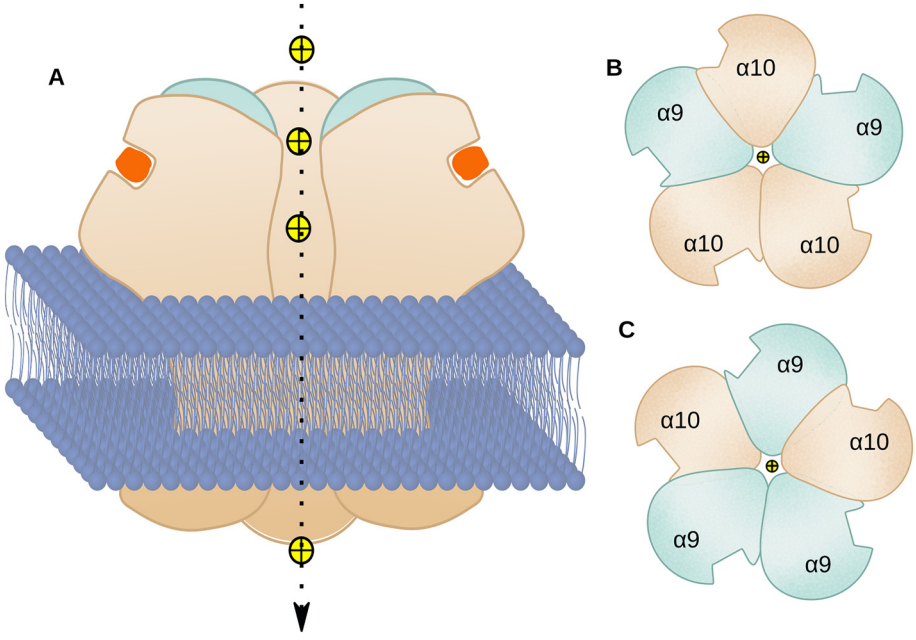

Fig. 1. Representative model of the α9α10 nAChR. (A) Side sectional view of the α9α10 nAChR (brown and teal) structure embedded in the cell membrane (blue). (B,C) Top view of the α9α10 nAChR structure in different stoichiometries. Binding of agonists (orange) to the binding sites located at the extracellular domain (ECD) opens the gate in the transmembrane domain (TMD), allowing cations (yellow) through the open channel.

to the cochlea is required for the correct establishment of the auditory system (Di Guilmi et al., 2019; Wedemeyer et al., 2018).

The outer hair cells of the adult rat cochlea express both α9 and α10 mRNAs; however, only α9 is detected in the inner cells after the onset of hearing and no ACh-mediated responses were observed in electrophysiological experiments (Katz et al., 2004; Morley & Simmons, 2002). It is also reported that cholinergic responses are insufficient to drive normal olivocochlear inhibition in α10 knockout (KO) rats (Vetter et al., 2007). The α9 subunit was initially suggested to form a homomeric pentamer (Elgoyhen, Johnson, Boulter, Vetter, & Heinemann, 1994) but the α10 subunit, which shares high sequence homology with the α9 subunit, has been functionally co-assembled *in vitro* with the α9 subunit to form α9α10 heteropentamers (Elgoyhen et al., 2001; Sgard et al., 2002). Co-injection of α9 and α10 mRNAs into *Xenopus laevis* oocytes boosts functional nAChR expression by ~100-fold compared to injection of α9 mRNA alone (Elgoyhen et al., 2001). These findings suggest that the α10 subunit is necessary in the formation of optimally functional receptors in the outer hair cells despite α9 alone being able to form a functional homomeric receptor *in vitro* (Elgoyhen et al., 1994). Moreover, the pharmacological properties of nAChRs expressed in hair cells closely match the α9α10 heteropentamer rather than the α9 homopentamer, suggesting that the native receptor is composed of α9 and α10 subunits (Gómez-Casati et al., 2005).

A functional role of α9-containing nAChRs in auditory transmission is supported by studies on KO mice and use of heterologous receptor systems. The α9 subunit is implicated in selective attention, as suggested by poor performance of KO animals to visual stimuli in the presence of auditory distractors (Jorratt, Delano, Delgado, Dagnino-Subiabre, & Terreros, 2017). Additionally, heterologous α9α10 nAChRs have been proposed as a possible target mediating solvent-induced ototoxicity (van Kleef, Vijverberg, & Westerink, 2008). Taken together, the α9α10 nAChR plays an essential role in the efferent auditory system and may be considered as a potential therapeutic target for different hearing disorders, including noise-induced hearing loss, vertigo, and tinnitus (Lustig, 2006).

1.3. The α9α10 nAChR in inflammation, stress and regulation of neuropathic pain

Inflammation involves the recruitment of immune cells and release of mediators such as chemokines and cytokines in response to tissue injury, stress, and infection, and there is increasing evidence implicating inflammatory mechanisms in neuropathic pain. Neuropathic pain arises due to a lesion or disease of the somatosensory nervous system, which includes aberrant neuroinflammation in the peripheral and central nervous systems affected by autoimmune disorders. Additionally, traumatic neuropathy itself is associated with excessive inflammation, which may be involved in the development and persistence of neuropathic pain.

Anti-inflammatory efferent signals from the brain are mediated by the vagus nerve through the cholinergic anti-inflammatory pathway (Pavlov, Chavan, & Tracey, 2018). Activation of the vagal efferent fibers releases ACh that interacts with nAChRs of the immune cells, inhibiting pro-inflammatory cytokines release. Transcripts and proteins of the α9 and α10 subunits have been reported in a variety of immune cells (Lustig, Peng, Hiel, Yamamoto, & Fuchs, 2001; Peng et al., 2004), including purified populations of T-cells (CD3+, CD4+, CD8+ and the Jurkat, MT2 and CEM T-cell lines), B-cells (CD19+, CD80+ and EBV-immortalized B-cells), peripheral blood lymphocytes, monocytes, macrophages, dendritic cells, and microglia.

In mouse monocytes, α9-containing receptors are postulated to mediate the effect of nicotine in attenuating both cell proliferation and production of pro-inflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-12), while stimulating the production of the anti-inflammatory cytokine IL-10 (St-Pierre et al., 2016). Moreover, nicotine has been suggested to confer neural protection in a mouse model of brain inflammation, possibly involving the α9 subunit (Simard et al., 2013), by reducing the infiltration of pro-inflammatory monocytes and neutrophils into the central nervous system (Jiang et al., 2016).

Additionally, in human monocyte-derived U937 cells, the α9 and/or α10 subunits are postulated to attenuate phosphocholine-induced release of IL-1β, interestingly, via the metabotropic functions of the receptor, as phosphocholine itself failed to elicit ionotropic activities from both homo/heterologous α9 and α9α10 nAChRs (Richter et al., 2016; Zakrzewicz et al., 2017). In summary, α9 and/or α10 subunits may play an important role in regulating immune cell populations and cytokine production, and consequently, the manifestation of neuropathic pain.

The α9α10 nAChR is also expressed in adrenal and pituitary glands (Colomer et al., 2010; Sgard et al., 2002; Zemkova, Kucka, Bjelobaba, Tomic, & Stojilkovic, 2013), suggesting a potential role in the stress response. In cold-stressed rats, α9α10 nAChRs dominantly contribute to ACh-induced currents in adrenal chromaffin cells and expression levels of α9 nAChR transcript and protein are upregulated (Colomer et al., 2010). A role for the α9α10-nAChR in behavioural and physiological stress responses, was also demonstrated by comparing the stress- and affect-related phenotypes of wild-type (WT) and α9-nAChR KO mice (Mohammadi, Burton, & Christie, 2017). Naïve KO mice exhibited largely normal behaviour on standard tests of affective behaviour. However, after sub-chronic restraint stress α9 KO mice showed significantly decreased stress-induced arousal and increased anxiety-like behaviour when compared to WT animals. Physiologically, corticosterone responses were muted in KO mice after an acute stressor, but exaggerated in response to the same stressor after undergoing sub-chronic stress. Moreover, α9 KO mice exhibited unusual patterns of circadian activity and a dysfunction in reward seeking after a period of reduced access to reward (Mohammadi et al., 2017). These findings demonstrate a role of the α9α10-nAChR in the regulation of stress responses, circadian activity and in reward-related affective behaviours.

Evidence to support the involvement of α9α10 nAChRs in pain comes primarily from *in vivo* pharmacological studies. The inhibition of α9α10 nAChRs by small molecules (e.g. tetrakis-quaternary ammonium compound ZZ-204G) and a class of α-conotoxins (e.g. Vc1.1, RgIA, PeIA and GeXIVA; see section 2) has been shown to alleviate the symptoms of pain (Del Bufalo, Cesario, Salinaro, Fini, & Russo, 2014; Di Cesare Mannelli et al., 2014; Holtman et al., 2011; Hone & McIntosh, 2018; McIntosh, Absalom, Chebib, Elgoyhen, & Vincler, 2009; Pacini et al., 2016). However, there is evidence that α9α10 nAChR inhibition is neither necessary nor sufficient for analgesia (Mohammadi & Christie, 2015). In a chronic constriction nerve injury (CCI) model of neuropathic pain, α9-nAChR KO mice displayed normal responses to noxious mechanical and thermal stimuli, and chronic cold mechanical allodynia also developed normally. However, α9 KO mice exhibited a distinct phenotype in the development of mechanical hyperalgesia; WT mice continued to display elevated mechanical hyperalgesia 21 days after injury, whereas α9 KO mice showed substantially reduced hyperalgesia following CCI surgery (Mohammadi & Christie, 2014). In a separate study, conducted in the oxaliplatin model of peripheral neuropathy with a different strain of α9 KO mice, oxaliplatin produced robust cold allodynia in WT animals, but the expression of cold-allodynia in α9 KO mice was attenuated in both magnitude and symptom duration (Romero et al., 2017). Together, these studies suggest that germline deletion of α9 nAChRs *per se* can attenuate the development and progression of neuropathic pain and that there may not be a close phenotypic correlation between KO mice and pharmacological treatment in WT animals.

1.4. The α9α10 nAChR in cancer

Certain cancers are linked to selective overexpression of membrane receptors and ion channels, including nAChRs. Some nAChR subunits

are associated with the pathogenesis of cancers, such as α5, α9, and α10 which have been reported in various breast cancer cell lines and in human breast tumors, and α9 expression is significantly elevated in tumors compared to normal cells (Lee et al., 2010). Additionally, transcripts of α9 have also been reported in cervical and lung cancer cell lines and tumors (Liu, Qian, Sun, Zhangsun, & Luo, 2019; Mucchietto et al., 2018; Wang, Zhang, Gu, Bao, & Bao, 2014).

Contribution of the α9 subunit to cancer cell survival was critically shown by the failure of transiently *CHRNA9*-silenced human breast cancer cells to grow in cultures (Lee et al., 2010). Moreover, exposure to the primary active component of cigarettes, nicotine, promotes α9-mediated oncogenesis of human breast epithelial cells possibly by stimulating cyclin D3 overexpression (Chen et al., 2011). Treatment of cultured breast cell lines with nicotine stimulates a cellular proliferation and migration signalling cascade involving protein kinase C and cdc42 (cell division control protein 42 homolog), and consequently promotes cancer progression (Guo et al., 2008). Further research shows that long-term exposure to low-dose of nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) will cause non-malignant breast epithelial cell transformation through activation of the α9-nAChR-mediated signalling pathway (Fararjeh et al., 2019). Although nicotine acts as an antagonist of the ionotropic α9 receptor (Verbitsky et al., 2000), the activation effects of nicotine have been postulated to be contributed by the metabotropic α9 nAChR as nicotine does not elicit current responses from homomeric α9 and/or α9α10 nAChRs (Richter et al., 2016). In addition to breast cancer, nicotine can also induce melanoma cell proliferation through stimulation of the α9-nAChR-mediated AKT and ERK signalling pathways (Nguyen et al., 2019). In colorectal cancer, it has been suggested that parasympathetic nerves may promote the progression of tumors through α9 nAChR (Zhou et al., 2018). The role of the α9 subunit in the development of breast and lung cancers has been suggested (Chen et al., 2011; Huang et al., 2017; Lee et al., 2010; Mucchietto et al., 2018; Wang et al., 2014), whereby exposure of healthy and cancerous cells to nicotine modulates the expression and function of nAChRs, leading to tumor progression, invasion, metastasis, and resistance to apoptosis.

2. Conotoxins

Antagonists of the α9α10 nAChR can potentially be developed as therapeutic compounds for the treatment of physiological conditions associated with this subtype. A series of azaaromatic quaternary ammonium analogues have been proposed as possible agents for the treatment of neuropathic and tonic inflammatory pain (Wala, Crooks, McIntosh, & Holtman, 2012; Zheng et al., 2011). Moreover, potent anti-glioblastoma agents have been designed by hybridizing the anti-proliferative onium-alkyloxy-stilbene based structures of α7 and α9 nAChR antagonists with a pro-oxidant, mitocan (Bavo et al., 2018). However, these non-peptide, small molecular compounds have potential off-target effects, thus limiting their therapeutic applications. The discovery of highly potent and selective nAChR-targeting peptides in the venom of *Conus* marine snails (conotoxins) has opened up new opportunities for developing novel peptide therapeutics and molecular probes to study the structure and function of various nAChR subtypes (Clark, Fischer, Nevin, Adams, & Craik, 2006; Ellison et al., 2006; Luo et al., 2015). Molluscs of the genus *Conus* comprise more than 750 species (Jin et al., 2019) and these carnivorous cone snails hunt various prey types, ranging from worms to fish, and other molluscs, including cone snails (Luo et al., 2015; Olivera et al., 1985). Each cone snail venom contains a unique cocktail of hundreds to thousands of bioactive peptides called conotoxins (Akondi et al., 2014; Olivera et al., 1990). Conotoxins can be categorized into different subfamilies according to their signal peptide sequence, cysteine framework pattern, and biological target (Vetter & Lewis, 2012). Based on their signal peptide, the conotoxins have been grouped into different subfamilies such as A-, D-, I-, J-, M-, O-, P-, S-, T and others (Kaas, Yu, Jin, Dutertre, & Craik, 2012; Robinson & Norton, 2014). In conotoxins, cysteine residues stabilize the secondary structure and facilitate the folding of the peptides (Gongora-Benitez, Tulla-Puche, & Albericio, 2014) and the unique arrangement of these residues is also used to group the peptides for which, to date, over 30 frameworks have been reported (Kaas et al., 2012). These bioactive peptides can be divided further into α-, δ-, γ-, κ-, μ-, ω-, ι-, ρ-, φ and other classes, depending on their pharmacological targets (Akondi et al., 2014; Kaas, Westermann, Halai, Wang, & Craik, 2008; Lewis, Dutertre, Vetter, & Christie, 2012), which include membrane receptors, ion channels, G protein-coupled receptors, transporters (Myers, Cruz, & Olivera, 1993; Nilsson et al., 2005; Sadeghi, McArthur, Finol-Urdaneta, & Adams, 2017; Sharpe et al., 2001), and enzymes (Pennington, Czerwinski, & Norton, 2018; Vetter & Lewis, 2012). This makes them valuable as novel drug leads for neuroscience research and disease treatment (Dutton & Craik, 2001; Halai & Craik, 2009).

Among the different classes of conotoxins, the α-conotoxins are the most studied due to their abundance and pharmacological importance (Abraham & Lewis, 2018; Giribaldi & Dutertre, 2018). The majority of these α-conotoxins act as competitive antagonists of muscle and neuronal nAChRs (Lewis et al., 2012). Classical α-conotoxins comprise 12–40 amino acids with four cysteine residues (C1 to C4) that adopt a CC−C−C framework. Depending on the disulfide bridge-contributing residues, there are three possible isomers, namely globular (C1−C3, C2−C4), ribbon (C1−C4, C2−C3) and bead (C1−C2, C3−C4) (Lebbe, Peigneur, Wijesekara, & Tytgat, 2014). The peptide segments between C2 and C3, and between C3 and C4 are referred to as loops, the length of which are used to sub-classify the α-conotoxins (Jin et al., 2019). Based on the number of amino acid residues in each loop, the α-conotoxins are further classified as 3/5, 4/3, 4/4, 4/6, 4/7, and 4/8 subtypes (Abraham & Lewis, 2018; Dutton & Craik, 2001).

2.1. Conotoxin antagonists of the α9α10 nAChR

To date, at least nine α-conotoxins that target the α9α10 nAChR have been described (Table 1) using proteomic analysis of crude cone snail venom or via transcriptomic screening of the venom duct. Interestingly, their structures are widely divergent, with different sequence lengths and various formations of disulfide bonds. The structures of typical α-conotoxins from four different *Conus* species are shown in Fig. 2.

α-PeIA that is isolated from the venom of *C. pergrandis*, consists of 16 amino acid residues and contains two disulfide bonds (McIntosh et al., 2005). It was determined to have a high potency for α9α10 nAChRs and has a 260-fold higher selectivity for rat α9α10 nAChR (IC_{50} = 6.9 nM) than rat α7 nAChR. Although PeIA can discriminate between α7 and α9α10 nAChRs, it also blocks rat α3β2 and chimeric rat α6/α3β2β3 subtypes with relatively high potency (McIntosh et al., 2005).

The 16-residue Vc1.1 from the Australian molluscivorous cone snail, *C. victoriae* (Satkunanathan et al., 2005), has an amidated C-terminus, two disulfide bonds in a C1−C3 and C2−C4 arrangement, and a typical α-helical motif (Safavi-Hemami et al., 2011; Sandall et al., 2003) (Fig. 2). Vc1.1 has been reported to attenuate both mechanical hyperalgesia and allodynia in neuropathic pain models in rats (Napier et al., 2012; Satkunanathan et al., 2005) and it was reported that Vc1.1 accelerates functional recovery of injured neurons (Livett et al., 2006; Satkunanathan et al., 2005). Furthermore, previous studies also suggested that the analgesic potency of Vc1.1 is higher than that of ziconotide (Clark et al., 2006). However, *in vitro* studies showed that Vc1.1 is less potent at inhibiting human α9α10 nAChR (IC_{50} = 1 μM) compared with rat α9α10 nAChRs (IC_{50} = 20–100 nM) (Table 1) and consequently its use was suspended after phase II human clinical trials (Del Bufalo et al., 2014).

To date, α-RgIA from *C. regius* is the shortest conotoxin (13 amino acids) with potency at α9α10 nAChRs, with two disulfide bonds but devoid of C-terminus amidation (Ellison et al., 2006). Residues Arg9,

Table 1
Conotoxins targeting the α9α10 nAChR.

Conotoxin	*Conus* species	Sequence	IC_{50} (nM) at α9α10	
			Rat	Human
α-PeIA	*C. pergrandis*	GCCSHPACSVNHPELC*	6.9 (McIntosh et al., 2005) 33 (Hone et al., 2012)	22.2 (Yu et al., 2018)
α-Vc1.1	*C. victoriae*	GCCSDPRCNYDHPEIC*	19 (Clark et al., 2006) 64.2 (Nevin et al., 2007) 109 (Halai et al., 2009) 70 (Yu, Kompella, Adams, Craik, & Kaas, 2013) 28.3 (Cai et al., 2018)	1,000 (Yu et al., 2018)
α-RgIA	*C. regius*	GCCSDPRCRYRCR	5.2 (Vincler et al., 2006) 4 (Azam & McIntosh, 2012) 2.6 (Ren et al., 2019)	494 (Azam & McIntosh, 2012) 1,400 (Ren et al., 2019)
αB-VxXXIVA	*C. vexillum*	VRCLEKSGAQPNKLFRPPCCQKGPSFARHSRCVYYTQSRE	3900 [globular] >30,000 [ribbon] 1200 [bead] (Luo et al., 2013)	ND
αO-GeXIVA	*C. generalis*	TCRSSGRYCRSPYDRRRRYCRRITDACV*	22.7 [globular] 7 [ribbon] 4.6 [bead] (Luo et al., 2015)	198.6 [globular] 35.1 [ribbon] (Wu et al., 2017) 116 [globular] 20.3 [ribbon] 47.3 [bead] (Zhangsun et al., 2017)
αS-GVIIIB	*C. geographus*	SGSTCTCFTSTNCQGSCECLSPPGCYCSNNGIRQPGCSCTCPGTG*	9.8 (Christensen, Bandyopadhyay, Olivera, & McIntosh, 2015)	ND
αD-GeXXA	*C. generalis*	DVHRPCQSVRPGRVWGKCCLTRLCSTMCCARADCTCVYHTWRGHGCSCVM	1.2 (Xu et al., 2015)	28 (Xu et al., 2015)
αO-GeXXVIIA	*C. generalis*	ALMSTGTNYRLLKTCRGSGRYCRSPYDCRRRYCRRISDACV*	ND	16.2 (Jiang et al., 2017)
αD-Lt28.1	*C. litteratus*	LHCHEISDLTPWILCSPEPLCGGKGCCAQEVCDCSGPACTCPPCL	3,092 (Lu et al., 2017)	ND

* = amidated C-terminal; C = cysteine residue; [ ] = disulfide bond isomer; IC_{50} = half-maximal inhibitory concentration; ND = not determined

Tyr10, and Arg13 play important roles in the potency and selectivity for α9α10 nAChR (Ellison et al., 2008). Similar to Vc1.1, RgIA is reported as a potent analgesic in a CCI rat model and for nerve injury associated inflammation (Di Cesare Mannelli et al., 2014). Results from more recent studies also suggest that the administration of RgIA4 (an analogue of RgIA) can effectively reduce oxaliplatin-dependent hypersensitivity to mechanical and thermal noxious and non-noxious stimuli (Christensen et al., 2017). However, Vc1.1 and RgIA were also proposed to exert their effects by activating G protein-coupled γ-aminobutyric acid type B ($GABA_B$) receptors (Berecki, McArthur, Cuny, Clark, & Adams, 2014; Callaghan et al., 2008; Castro et al., 2017; Cuny et al., 2012; Huynh, Cuny, Slesinger, & Adams, 2015; Mohammadi & Christie, 2015; Sadeghi et al., 2017)

VxXXIVA is a member of the B-subfamily discovered in *C. vexillum*. Different from the majority of conotoxins whose precursor sequence is composed of a signal segment, a "pro" region and a mature sequence, the precursor peptide of VxXXIVA lacks a "pro" region. The mature αB-VxXXIVA contains 40 amino acid residues and has a rare C-CC-C cysteine framework. The ribbon disulfide isomer shows the highest potency at rat α9α10 nAChRs, with an IC_{50} of 1.2 μM (Luo et al., 2013).

GeXIVA is a new O-superfamily α-conotoxin identified from *C. generalis* that consists of 28 amino acid residues. Unlike most O-superfamily conotoxins, which contain six cysteine residues, GeXIVA only has four cysteine residues, forming three possible disulfide isomers (Luo et al., 2015). The most active disulfide isomer is the bead isomer (Wu et al., 2017; Zhangsun et al., 2017) with an IC_{50} of 4.6 nM at the rat α9α10 nAChR. The ribbon isomer is equipotent but has an additional helical motif from positions 12–18, which contributes to a more rigid structure that forms significant interactions with the α9α10 nAChR (Luo et al., 2015) (Fig. 2). Dissociation kinetics studies of GeXIVA at the α9α10 nAChR suggested that the binding site of GeXIVA does not overlap with that of RgIA, and a potential binding site is located at the principal α9 and complementary α10 subunit interface (Luo et al., 2015). Similar to RgIA and Vc1.1, GeXIVA also significantly reduces mechanical hyperalgesia in CCI rats and has no impact on the motor performance of the tested animals (Luo et al., 2015). Furthermore, GeXIVA can relieve and reverse oxaliplatin-induced mechanical and cold allodynia after single and repeated intramuscular injections in rats (Wang et al., 2019).

Most conotoxins targeting the α9α10 nAChR are extracted from mollusc- or worm-hunting cone snails. However, αS-GVIIIB, which is composed of 45 amino acid residues and contains 10 cysteine residues, was isolated from the venom of the fish-hunting *C. geographus*. αS-GVII IB potently blocks the rat α9α10 nAChR, with an IC_{50} of 9.8 nM and >100-fold selectivity over other subtypes. Both GVIIIB and RgIA share a binding site contributed by the interface between the principal and complementary components of the α10 and α9 subunits, respectively (Christensen et al., 2015).

αD-conotoxins occur naturally as dimers with complex disulfide connections (Loughnan et al., 2006), including GeXXA from the venom of *C. generalis*, and each GeXXA monomer contains 50 residues, including 10 cysteines (Fig. 2). GeXXA inhibits various nAChR subtypes but it is most potent at the rat (IC_{50} = 1.3 nM) and human

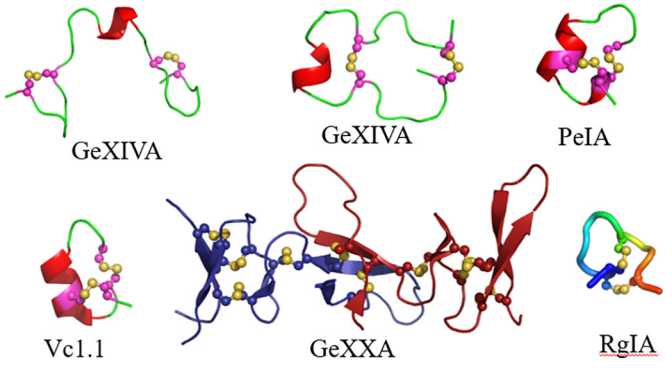

Fig. 2. Structures of typical α/αO/αD-conotoxins targeting the α9α10 nAChR. Nuclear magnetic resonance (NMR) structures of the bead and ribbon isomers of αO-GeXIVA (Luo et al., 2015). Crystal structures of α-PeIA (PDB code 5JME) and αD-GeXXA (PDB code 4X9Z), and NMR structures of α-Vc1.1 (PDB code 2H8S) and α-RgIA (PDB code 2JUT). GeXIVA and GeXXA were discovered in *C. generalis*. PDB, protein data bank.

(IC_{50} = 28 nM) α9α10 nAChR. The species difference in potency is attributed to residue His7 of the rat α10 subunit and uniquely, GeXXA inhibition at the α9α10 nAChR is attained possibly via cooperative binding of the monomeric C-terminal domains to the top surface of the receptor (Xu et al., 2015).

In addition to GeXIA and GeXXA, the venom of *C. generalis* contains a third conotoxin, GeXXVIIA, with potent activity at the α9α10 nAChR. The precursor sequence of GeXXVIIA is similar to GeXIVA, and it also belongs to the O-superfamily. The native form of αO-GeXXVIIA is a disulfide-linked 5-cysteine-containing homodimer. In comparison to the folded monomer, the linear monomer of this toxin has the highest inhibitory activity at the human α9α10 nAChR, with an IC_{50} of 16.2 nM, indicating that the binding of the linear GeXXVIIA peptide to nAChR is only dependent on the sequence of the peptide, and the disulfide bonds have no contribution to its potency. GeXXVIIA is neither a competitive antagonist nor an open channel pore blocker by entering the membrane electric field of nAChR, thus representing a new class of nAChR allosteric inhibitor (Jiang et al., 2017).

The D-superfamily conotoxin Lt28.1 was cloned from *C. literatus* and comprises 45 amino acids with 10 cysteine residues (Lu et al., 2017). Lt28.1 selectively inhibits the rat α9α10 nAChR despite relatively low potency (IC_{50} = 3 μM) (Lu et al., 2017).

3. Conotoxin optimization strategies

Although conotoxins possess favorable pharmacological activities, they also have a number of shortcomings, such as short half-life, instable disulfide bonds, limited modes of administration and reduced potency at human nAChRs, and all of these hinder translational application of conotoxins as drug leads or pharmacological tools (Carstens et al., 2011; Romero et al., 2017; Tabassum et al., 2017). Therefore, in order to improve the activity, selectivity and stability of conotoxins, a series of strategies have been applied, including scanning mutation, disulfide bond modification, backbone cyclization and polymerization (Craik & Adams, 2007) (Fig. 3).

Among the α-conotoxins active at the α9α10 subtype, Vc1.1 and RgIA have attracted significant research interest due to their short length and relatively stable structure. They share the same cysteine framework, in which loop 1 between C2 and C3 is crucial for binding whereas loop 2 between C3 and C4 is primarily responsible for subtype selectivity (Halai et al., 2009; Luo et al., 2015). Considering that inhibition of the α9α10 nAChR can effectively alleviate neuropathic and chronic visceral pain in animal pain models (Dutton & Craik, 2001; Giribaldi & Dutertre, 2018; Janes, 2005), as well as suppress tumor growth (Sun et al., 2020; Sun et al., 2020), structure-activity analysis of α9α10 nAChR-selective α-conotoxins is therefore critical for the development of therapeutic drug leads and pharmacological tools. Here, we summarize successful modification strategies applied to α-conotoxins targeting the α9α10 nAChR, which can provide guidance for future peptide optimization.

3.1. Amino acid sequence scanning

In the field of peptide research, alanine scanning mutagenesis is a well-known technique for identifying key residues for structural stability

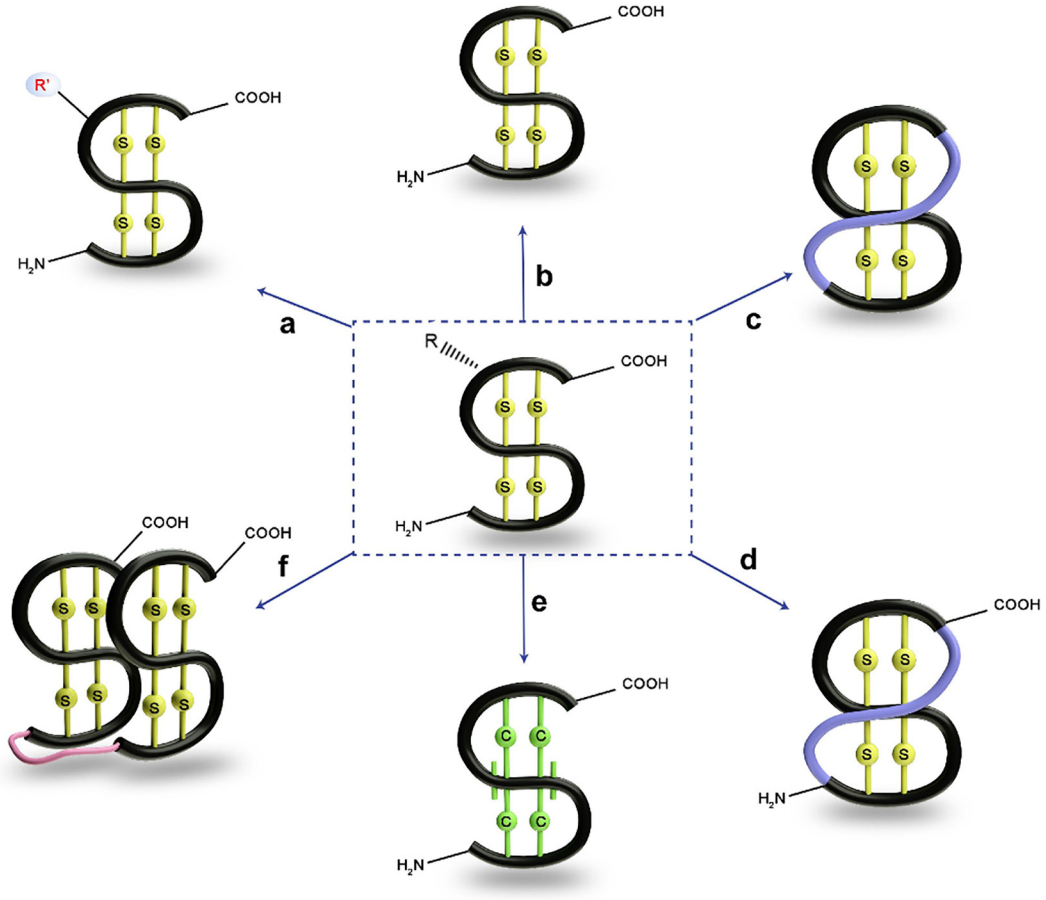

Fig. 3. Overview of the chemical modification strategies that have been applied to α-conotoxins inhibiting the α9α10 nAChR. a) Substitution or modification of side chain residues for activity and selectivity improvement. b) Replacement of L-amino acids with D-amino acids increases the enzyme stability of the peptide. c) Backbone cyclization increases the enzyme stability of the peptide. d) Side chain cyclization improves the stability of the peptide and maintains potency. e) A dicarba bridge substituted for the native cysteine framework improves peptide stability and tunes the selectivity of the peptide to certain receptors. f) Dimerization of the peptide improves the potency of α-conotoxins.

and bioactivity (Halai et al., 2009; Morrison & Weiss, 2001). Besides alanine scanning, researchers have also utilized aspartic and/or lysine scanning in order to explore the effects of side-chain charge on peptide-receptor interactions (Halai et al., 2009). In addition, D-type amino acid scanning in which L-amino acids are replaced with D-amino acids, has also been applied to facilitate the development of more stable analogues (Ren et al., 2019).

Halai et al. (2009) performed extensive Ala scanning in conjunction with Lys and Asp scanning to explore the effects of neutral, positive, and negative charge substitutions, respectively, at all non-cysteine residues of Vc1.1. Most analogues had a dramatic reduction in activity at the α9α10 nAChR, attributed to overall structural distortion and/or disruption in the interactions between amino acids of Vc1.1 and the receptor. In contrast, introduction of a positive charge at position 4 (Ser) and hydrophobic residues at position 9 (Asn), resulted in analogues with more potent activity at the α9α10 nAChR than the native peptide (Halai et al., 2009). Additionally, Pro residues of Vc1.1 are essential to maintain structural stability of the peptide. The Pro residue at position 6 is involved in defining both the ligand conformation and receptor binding affinity, whereas the Pro at position 13 facilitates the native folding of Vc1.1. Overall, residues at position 5 to 7 are crucial for Vc1.1 binding to the α9α10 nAChR, whereas residues at position 11 to 15 are required for receptor selectivity (Halai et al., 2009).

RgIA is a member of the 4/3 family of α-conotoxins, as is ImI (Ellison et al., 2006; Quiram, Jones, & Sine, 1999). Interestingly, although they have a similar sequence and structure, their nAChR subtype targets are quite different. RgIA is a selective antagonist of the human and rat α9α10 subtype, whereas ImI preferentially blocks the human α7 and α3β2 subtypes. Despite this, the activity of both RgIA and ImI at their respective nAChR subtypes is conferred by the conserved Asp-Pro-Arg (DPR) motif in loop1 (Ellison et al., 2006; Kennedy et al., 2020; Quiram & Sine, 1998). While loop 1 is responsible for latching the α-conotoxins to the receptor, residues at position 9 (Ala in ImI and Arg in RgIA) are responsible for the nAChR subtype selectivity (Ellison et al., 2006).

Susceptibility to proteolytic degradation is an unfavorable property that greatly limits the clinical applications of α-conotoxins. Almost all naturally occurring peptides are built from L-amino acids, but D-amino acid-containing peptides have been described from a number of organisms (Quiram & Sine, 1998). Due to the limited prevalence of D-amino acids in nature, they are less prone to enzymatic degradation, thus D-amino acid scanning is a valuable strategy for prolonging the bioavailability and bioactivity of α-conotoxins and revealing the critical residues involved in conotoxin-target interactions. RgIA is a potent selective antagonist of α9α10 nAChRs (Table 1), however, compared to other α-conotoxins, RgIA is more susceptible to proteolysis due to the higher abundance of arginine residues (Ren et al., 2019). Thus, Ren et al. (2019) generated a series of RgIA D-amino acid scanning analogues and tested their activity on α9α10 nAChR ACh-evoked currents and stability against enzymatic activity in serum. The change of amino acid chirality in RgIA decreased its potency at α9α10 nAChRs, except for [D-Arg13]RgIA, which was proposed to be located outside the binding site and with high mobility. Despite the reduced potency of other D-Arg analogues, the serum stability of these peptides including [D-Arg13]RgIA was notably improved (Ren et al., 2019).

In a recent study, Huynh, Harvey, Gajewiak, Craik, and McIntosh (2020) implemented amino acid substitution to define the chemical properties of RgIA that contribute to its activity against α9α10 nAChRs. The results indicated that the primary contribution to activity are the side chain properties of RgIA residues, rather than backbone conformation. It was also found that replacement of Arg13 with Tyr significantly increased the potency of RgIA for human α9α10 nAChRs by 243-fold and the discrepancy between human and rats was eliminated (Huynh et al., 2020).

3.2. Unnatural amino acid substitutions

Non-coded (unnatural) amino acids with unique side chain properties are commonly employed in structure-function studies of conotoxins. Recently, Chu et al. (2019) described unnatural 2,4-diamino butyric acid (Dab) analogues of Vc1.1 ([S4Dab, N9A]Vc1.1 and [S4Dab, N9W]Vc1.1) with ~20-fold increases of potency compared to unmodified Vc1.1 at the human α9α10 nAChR. Another example is RgIA4, an analogue of RgIA with two unnatural amino acids (citrulline at position 9 and 3-iodotyrosine at position 10), which has >300 fold higher potency at the human α9α10 nAChR compared to the parent peptide (RgIA4 IC_{50} = 1.5 nM (Romero et al., 2017) vs RgIA IC_{50} = 500–1400 nM) (Table 1). A unique highlight of RgIA4 is essentially the absence of a species potency gap between human and rodent α9α10 nAChRs (IC_{50} = ~1-2 nM) (Christensen et al., 2017; Romero et al., 2017) in contrast to the native peptide. Most importantly, RgIA4 has an analgesic effect without tolerance in a rat chemotherapy-induced neuropathic pain model (Christensen et al., 2017; Romero et al., 2017).

3.3. Peptide backbone cyclization

A major caveat to the potential of conotoxins as therapeutics is their susceptibility to *in vivo* degradation, which limits their effectiveness when delivered orally (Clark, Akcan, Kaas, Daly, & Craik, 2012; Clark et al., 2010). However, the discovery of natural cyclic peptides that are stable *in vivo* introduces a new dimension for improving the bioavailability of linear peptides (Clark et al., 2005; Franke et al., 2017).

Synthetic peptide cyclization can be achieved by performing an amidation reaction between the amino group of the N-terminus and the carboxyl group of C-terminus of the linear peptide. However, this technique may cause structural distortion that can result in reduced activity of the peptide. To resolve this issue, it is common to insert a linker consisting of amino acids between the two terminal ends of the peptide and optimization of the linker length is critical as it affects the properties of cyclic conotoxins (Armishaw, Dutton, Craik, & Alewood, 2010; Carstens et al., 2016; Clark et al., 2010; Halai et al., 2011). Cyclization of RgIA generated peptides that are more stable in serum than the linear RgIA, and constructs with 6- and 7-residue linkers consisting of glycine and alanines, were most stable. However, only the cyclic peptide with a 7-residue linker had comparable potency to linear RgIA for inhibiting α9α10 nAChRs. An NMR structure study also suggested that a linker of three or four residues leads to perturbations in the peptide backbone fold, and a linker of five residues or more introduces negligible restraint on the flexibility of the peptides, which is necessary for binding to the receptor (Halai et al., 2011).

An orally active cyclized Vc1.1 (cVc1.1), with the connecting N- and C-termini linked via a GGAAGG-linker (4 glycines and 2 alanines), was ~10-fold less potent at α9α10 nAChRs than the linear Vc1.1 (Clark et al., 2010). Structurally, cVc1.1 has an α-helical region from residues Pro6 to Asn11, similar to the native Vc1.1. However, cVc1.1 also contains a new helix formed by residues Pro13 to Cys16 and a type I β-turn from residues Gly1 to Ser4, suggesting that cVc1.1 is somewhat more rigid than Vc1.1. This increased rigidity results in dramatically improved stability of cVc1.1 in human serum and simulated intestinal fluid. More importantly, cVc1.1 induced a strong analgesic effect in a rat CCI model when administered orally (Clark et al., 2010), and recent research also suggests that cVc1.1 inhibits colonic nociceptors from healthy mice (Castro et al., 2018). High stability, good activity and oral efficacy make cVc1.1 a significant milestone in the transformation of conotoxins into drugs. In another example, the ribbon isomer of GeXIVA was cyclized with a linker of two glycine residues (Wu et al., 2017). Compared to the linear peptide, the cyclic construct is equipotent at inhibiting the human α9α10 nAChR, with slightly improved serum stability. As a result of its high structural flexibility and Arg-rich sequence

(9 Arg in total), GeXIVA is highly susceptible to endoproteases and/or denaturation of side chains, which are hardly negated by backbone cyclization (Wu et al., 2017). Although cyclization did not improve the properties of the peptide, the introduction of the short linker resulted in selective yield of the ribbon disulfide isomer without the need for orthogonal protection during the chemical synthesis.

3.4. Peptide side-chain cyclization

Linear peptides can also be cyclized via amino acid side chain-to-side chain connectivity. Zheng et al. (2020) introduced a key lactam linkage at the termini through side chain cyclization and successfully developed an RgIA4 analogue with improved serum stability while retaining potency at the human α9α10 nAChR (Zheng et al., 2020). The NMR structure of this analogue overlays well with that of RgIA4, demonstrating that the cyclization does not perturb the overall conformation of backbone and key side-chain residues. Structurally, the extra lactam bond provides an additional conformational constraint to the bioactive conformation and suppresses disulfide scrambling, leading to considerable serum stability improvements. Furthermore, the analogue exhibited a strong analgesic effect in a chemotherapy-induced neuropathic pain model (Zheng et al., 2020).

3.5. Disulfide bond replacement

Disulfide bonds play a vital role in maintaining the stable structure and biological activity of conotoxins. However, multiple disulfide bonds are inherently susceptible to shuffling under reducing physiological environments, generating a heterogeneous peptide population. Disulfide bond isomers (globular, ribbon and bead) of α-conotoxins are shown to have different structural properties with a range potencies that inhibit nAChRs (Gehrmann, Alewood, & Craik, 1998; Rabenstein & Weaver, 1996). Several disulfide bond replacement strategies have been applied to generate conotoxins with improved potential as drug leads, including the replacement of disulfide bonds with other cross-links and deletion by substitution of Cys with non-cross-linking residues (Green et al., 2014; MacRaild et al., 2009). A dicarba bridge was used as a substitute for the native cysteine framework of Vc1.1 and RgIA in early research, and region-selective replacement of a disulfide bond with a dicarba bridge showed the potential to tune the innate biological activity of a peptide for one receptor over another (Kennedy et al., 2020). For instance, the [3,16]-dicarba Vc1.1 isomer retains activity at the α9α10 nAChR whereas the [2,8]-dicarba Vc1.1 isomer only acts on G protein-coupled $GABA_B$ receptors. Similarly, the [3,12]-dicarba RgIA analogue retains inhibition at the α9α10 nAChR but not at the N-type calcium channel, whereas [2,8]-dicarba analogues display opposite target selectivity. Use of dicarba-substituted Vc1.1 and RgIA analogues demonstrated that the C1−C3 bridge is important for inhibiting the α9α10 nAChR, whereas the C2−C4 bridge is responsible for $GABA_B$ receptor modulation (Chhabra et al., 2014; Van Lierop et al., 2013). Indeed, not only do these analogues provide more stable peptides, they are also valuable as probes for conotoxin structure-activity relationship studies at different targets.

Another strategy was to replace the C1-C3 disulfide bond of cVc1.1 with non-covalent bond residues in [C2H,C8F]cVc1.1 (Yu et al., 2015). Compared to cVc1.1, [C2H,C8F]cVc1.1 was marginally less potent at inhibiting the human α9α10 nAChR despite increased peptide hydrophobicity. The linear [C2H,C8F]Vc1.1 was unable to block the α9α10 nAChR although it is structurally similar to Vc1.1, thus suggesting that the C1−C3 disulfide bond of α-conotoxins plays a vital role in the inhibition of nAChRs, and that the linker of [C2H,C8F]cVc1.1 may inadvertently compensate for the activity loss resulting from disulfide bond deletion (Tabassum et al., 2017). Both cVc1.1 and [C2H,C8F]cVc1.1 also reduce nociception in a mouse model of chronic visceral pain, therefore, taking into account the structural simplicity and conformational stability of this cyclic Vc1.1 analogue, [C2H,C8F]cVc1.1 is a candidate for the treatment of pain associated with gastrointestinal conditions (Castro et al., 2018; Yu et al., 2015).

Recently, Xu et al. (2020) explored the effect of replacing all or some of the disulfide bridges in GeXIVA with Ala or Ser residues. Interestingly, most analogues retained a potency similar to GeXIVA for inhibiting the human α9α10 nAChR. One analogue, [C2A,C9A,C20S, C27S]GeXIVA, was twice as active as the native peptide, suggesting that the disulfide bridges are not necessary for the activity of GeXIVA at α9α10 nAChR (Xu et al., 2020).

3.6. Multimerization of conotoxins

Multiple monomeric conotoxins attached to a single molecular scaffold may be more potent because they concomitantly occupy multiple binding sites at nAChRs. Wan et al. (2015) used a chain of 9 poly(ethylene glycol) molecules (PEG9) as a linker to synthesize dimer- and tetramer-ImI, which increased its potency at the human α7 nAChR by more than 100-fold (Wan et al., 2015). Recently, PEG9-dimeric constructs of PeIA, Vc1.1 and RgIA# ([ΔR13]RgIA) were designed and upon dimerization, all three constructs had significantly improved potency compared to their monomeric counterparts at the human α9α10 nAChR. Interestingly, dimeric RgIA# also had significantly improved potency at the human α7 nAChR. Considering the α7 and α9α10 subtypes are involved in the development of tumors, dimeric RgIA# may be valuable as a molecular probe in cancer research. Surprisingly, dimeric RgIA# retains its activity at the α9α10 nAChR when either PEG6 or PEG13 is used as the linker, suggesting that PEG6 alone is sufficiently long for an α-conotoxin dimer to simultaneously bind two adjacent binding sites (Liang et al., 2020). This study provides a general method to dramatically increase the activity of conotoxins. In addition, it has been reported that the introduction of PEG is beneficial for the stability of peptides (Lawrence & Price, 2016).

Overall, various chemical modification strategies have been established to modify α-conotoxins targeting α9α10 nAChR in order to obtain analogues with higher activity and stability. These strategies may also promote exploration of the mechanism of action of conotoxins on α9α10 nAChR and enhance the versatility of these peptides as drug leads for analgesic therapy.

4. Binding sites and interactions at the α9α10 nAChR

The nAChR ligand binding site is located in the extracellular domain, contributed by the interface of adjacent subunits (Fig. 4A,B). Each binding site is composed of a principal (+) component formed by three highly conserved loops (loops A–C) from one subunit, and a complementary (−) component consisting of a β-sheet (loops D and E) and loop F from an adjacent subunit (Brejc et al., 2001; Dellisanti, Yao, Stroud, Wang, & Chen, 2007; Unwin, 2005). Owing to the lack of a crystal structure of the α9α10 nAChR pentameric extracellular domain, the stoichiometry and binding site of this receptor can only be explored by means of electrophysiology experiments and computational methods.

Due to their relatively simple, well-defined structure and similar pharmacological properties, Vc1.1 and RgIA are often used to explore the binding pockets of α9α10 nAChR. Previous studies showed that RgIA is a competitive antagonist and binds at the ACh-binding site, that is, the orthosteric site (Ellison et al., 2008; Kryukova et al., 2018). Using computational techniques, Perez, Cassels, and Zapata-Torres (2009) postulated that RgIA binds to the human α9(+)α10(−) interface, with residues Pro197 and Asp198 of the α9 subunit (+) component and Glu58 and Asp114 of the α10 subunit (−) component contributing to the interaction (Perez et al., 2009). However, this conclusion was challenged by an experimental study aimed at explaining the differential sensitivity to RgIA by rat and human α9α10 nAChRs. It was proposed that RgIA species differences could be attributed to the Thr residue at position 56 of the α9 subunit (−) component, therefore suggesting the α10(+)α9(−) interface is more likely to be the binding

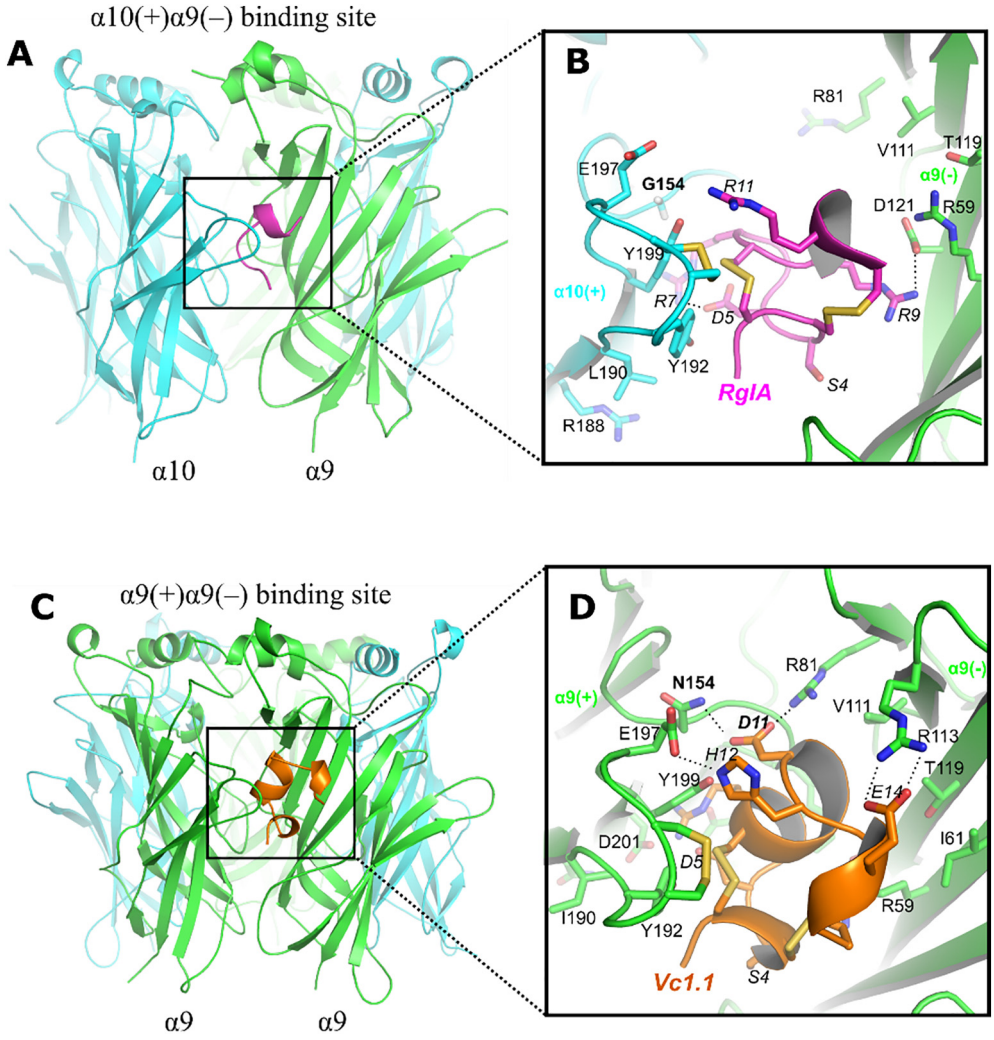

Fig. 4. Binding modes of α-conotoxins to the human α9α10 nAChR. (A) Binding of RgIA at the human α10(+)-α9(−) interface. (B) Expanded view of the interactions between RgIA and residues of the α10(+)α9(−) binding site. (C) Binding of Vc1.1 at the human α9(+)-α9(−) interface. (D) Expanded view of the interactions between Vc1.1 and residues of the α9(+)α9(−) binding site. Dashed lines indicate hydrogen bonds between the α-conotoxins and α9α10 nAChR residues. Residues from the RgIA and Vc1.1 are italicized. Modified and adapted with permission from Yu et al. (2018).

site of RgIA (Azam & McIntosh, 2012). A subsequent study demonstrated none of the residues predicted by Perez et al. (2009) are crucial for RgIA binding at the rat a9(+)a10(−) site. However, mutations of homologous residues in opposing subunits resulted in a 19- to 1700-fold loss of RgIA potency (Azam et al., 2015). They also modeled the complexes of the rat α9α10 extracellular domain with RgIA and ACh using the crystal structure of the human α9 nAChR ECD (Zouridakis et al., 2014) and both experimental and computational methods provided additional support for the binding of RgIA at the α10(+)α9(−) interface (Fig. 4C).

Using a combination of computational modeling and electrophysiology experiments, Yu et al. (2013) proposed the α10(+)α9(−) interface as the binding site for Vc1.1 and that the Thr residue at position 59 of the (−) site confers Vc1.1 preferential selectivity for rat over human α9α10 nAChRs.

Heteromeric nAChRs can assemble in two possible stoichiometries (2α,3α/β or 3α,2α/β) and stoichiometric variants of the α4β2 subtype have been reported in the central nervous system (DeDominicis et al., 2017). Studies of α4β2 nAChRs in native cells and heterologous systems suggest both stoichiometries have distinct functional and pharmacological properties (Carbone, Moroni, Groot-Kormelink, & Bermudez, 2009; DeDominicis et al., 2017; Mazzaferro et al., 2011). Initially for the α9α10 nAChR, Plazas, Katz, Gómez-Casati, Bouzat, and Elgoyhen (2005) concluded that nAChRs expressed in *Xenopus* oocytes formed a stoichiometry consisting of two α9 and three α10 subunits ($(\alpha9)_2(\alpha10)_3$) (Plazas et al., 2005). However, using Vc1.1 to probe the function of α9α10 nAChRs assembled from biased α9 to α10 subunit ratios, Indurthi et al. (2014) proposed that heterologous rat receptors exist as a mixed population of $(\alpha9)_2(\alpha10)_3$ and $(\alpha9)_3(\alpha10)_2$ nAChRs (Fig. 4A, B). The biphasic concentration-response of Vc1.1 suggests the presence of a high-sensitivity α9(+)α9(−) binding site in addition to low sensitivity α10(+)α9(−) and/or α9(+)α10(−) binding sites, which are present in both stoichiometries (Indurthi et al., 2014). Additionally, the authors inferred that Vc1.1 but not RgIA could act at the α9(+)α9(−) interface.

Yu et al. (2018) explored the molecular determinants conferring binding preference of Vc1.1 at the human α9α10 nAChR. They demonstrated that the hydrogen bond between Asp11 of Vc1.1 and Asn154 in the α9 subunit (+) component confers the stoichiometric-dependent activity of Vc1.1. By contrast, the corresponding Gly154 in the α10(+) subunit is incapable of forming such interactions with Vc1.1. In addition, this work also demonstrated that the α9(+)α9(−) interface is a high affinity binding site for Vc1.1 (Yu et al., 2018) (Fig. 4D). Following this study, Chu et al. (2019) revealed in-depth details of Vc1.1 and α9

(+)α9(−) interactions. The Ser4 residue of Vc1.1 forms hydrogen bonds with two aspartic acid residues of the α9 subunit (Asp166 and Asp169), and the Vc1.1 Tyr10 residue forms hydrogen bonds with Asn107 and Asp119 in the binding pocket.

The binding site of GeXIVA has not as yet been identified, but competitive binding studies between GeXIVA and RgIA excluded the possibility of the α10(+)α9(−) site as a binding site of GeXIVA (Luo et al., 2015). Further study has indicated that the GeXIVA should act directly on the orthosteric site rather than the allosteric site or channel pore of the α9α10 nAChR (Kryukova et al., 2018). In a subsequent study, Xu et al. (2020) built computational models of disulfide-deficient analogues of GeXIVA for each probable orthosteric binding site, and all of the molecular models suggested a rational explanation for the limited effects of the two disulfide bonds on the affinity of GeXIVA. However, there were minor differences in the binding mode of the GeXIVA α-helix between the binding sites: for instance, the helix was tipped outward at the α9(+)α10(−) binding site, whereas at the α10(+)α10(−) binding site, Pro12 did not participate in the helix formation upon binding (Xu et al., 2020). Further investigations are required to clarify the exact binding site(s) for GeXIVA.

The high potency of GeXXVIIA for the human α9α10 nAChR and its unique structure also encouraged researchers to explore its mechanism of action (Jiang et al., 2017). Jiang et al. (2017) ruled out the possibility that the linear analogue of GeXXVIIA (GeXXVIIA-L) is a competitive antagonist or a channel pore blocker of the human α9α10 nAChR. Most likely, GeXXVIIA-L is a novel class of nAChR allosteric inhibitor and investigation of the mechanism of action may lead to the development of novel α9α10 nAChR-targeting drugs (Jiang et al., 2017).

A study by Boffi et al. (2017) explored the contribution of different subunit interfaces of α9α10 nAChRs. Mutations of Tyr190 and the disulfide bond in loop C comparably affected the function of both subunits, indicating that α9 and α10 subunits equally contribute to the (+) components of the α9α10 nAChR. However, the introduction of a W55T mutation in loop D impaired binding and function of the rat α9 subunit but not the α10 subunit, suggesting that the contribution of α9 and α10 subunits to the (−) components is non-equivalent (Boffi et al., 2017). Molecular dynamics simulation of RgIA binding to the α9α10 nAChR drew the same conclusion. Zouridakis et al. (2019) determined the crystal structure of the extracellular domain of monomeric α9 nAChR in complex with RgIA and further analyzed the binding modes of RgIA at α9(+)α9(−), α9(+)α10(−), and α10(+)α9(−) binding sites. The study supports favorable binding of RgIA at α9(+)/α9(−) or α10(+)/α9(−) sites rather than the α9(+)/α10(−) interface, in accordance with mutational data (Zouridakis et al., 2019). The results of these two studies may be helpful in designing improved α-conotoxin analogues targeting the α9α10 nAChR.

Although research on the interactions of Vc1.1 and RgIA at the α9α10 nAChR has made substantial progress, there is a burgeoning need to employ biophysical methods such as X-ray crystallography or cryo-electron microscopy to study them at an atomic level. Until then, computational techniques continue to play a dominant role in deciphering the mechanism of action of conotoxins at nAChRs.

5. Therapeutic potential of conotoxins targeting the α9α10 nAChR

5.1. Pain relief

Damage to the somatosensory nervous system may result in the induction of neuropathic pain (Bordet & Pruss, 2009), a complex and debilitating syndrome that affects millions of people worldwide. Conventional opioid analgesics lack analgesic potency for neuropathic pain, and opioid usage is restricted by tolerance, addiction, and unintentional overdose (Khademi, Kamangar, Brennan, & Malekzadeh, 2016). Thus, α-conotoxins Vc1.1, RgIA, and GeXIVA warrant attention not only because of their simple structures and capacity to alleviate neuropathic pain in animal models, but, of particular interest, for their ability to produce enduring analgesia and accelerate the recovery of damaged nerves through immune-mediated mechanisms (Hone et al., 2017; Mohammadi & Christie, 2015).

Partial sciatic nerve ligation (PNL) and CCI models are typically used to evaluate the effectiveness of α-conotoxins in alleviating neuropathic pain (Bennett & Xie, 1988; McIntosh et al., 2009; Seltzer, Dubner, & Shir, 1990; Vincler & McIntosh, 2007). Subcutaneous administration of Vc1.1 in doses of 24, 80, 160, 240 or 800 μg/kg in rats with CCI dose-dependently alleviated mechanical allodynia by 54 – 80%, with a peak analgesic effect of 1 h, and the highest concentrations produced an extended effect lasting up to 24 h after administration (McIntosh et al., 2009; Vincler & McIntosh, 2007). In another experiment, Vc1.1 was administered daily for 7 days by intramuscular injection near the site of injury at doses of 0.036 μg, 0.36 μg and 3.6 μg to rats with CCI and at a dose of 0.36 μg in rats with PNL (Livett et al., 2009; Satkunanathan et al., 2005). Vc1.1 significantly reversed mechanical hyperalgesia in both CCI and PNL rats at 1 h, 3 h and 24 h post-administration, and research on long-term effects showed that Vc1.1 attenuated mechanical hyperalgesia for up to a week following cessation of treatment, without tolerance (McIntosh et al., 2009; Satkunanathan et al., 2005). More interestingly, administration of 0.36 μg and 3.6 μg Vc1.1 to the contralateral hind leg also reversed mechanical hyperalgesia, indicating that Vc1.1 is stable during systemic circulation (Satkunanathan et al., 2005).

Vc1.1 has also been tested in a model of diabetic neuropathy induced by streptozotocin (Khan, Chen, & Pan, 2002). Subcutaneous administration of Vc1.1 at doses of 300 μg/kg significantly reduced mechanical allodynia for a prolonged period of at least 6 h but an acute analgesic effect was not observed on mechanical hyperalgesia at the same dose in this model. In addition, Vc1.1 was also shown to be an effective analgesic against pain resulting from an inflammatory injury. A dose of 2.4 mg/kg Vc1.1 significantly alleviated mechanical hyperalgesia induced by an intraplantar injection of complete Freund's adjuvant, with a peak analgesic effect at 1–1.5 h. Intramuscular injection of RgIA (2 and 10 nmol) to CCI rats also resulted in significant reduction of mechanical hyperalgesia, and repeated injection of RgIA reduced the development of pain even at 24 h after the last injection (Di Cesare Mannelli et al., 2014). A recently characterized αO-conotoxin, GeXIVA, also exhibited extremely strong activity in the CCI model. GeXIVA[1,2] or GeXIVA [1,4] at doses of 0.5 nmol, 1 nmol and 2 nmol per rat, was injected intramuscularly for one or two weeks. GeXIVA[1,2] at all doses dramatically reversed allodynia and persisted for up to 6 h after a single administration, whereas GeXIVA[1,4] only showed an analgesic effect at doses of 1 nmol and 2 nmol. Notably, both GeXIVA[1,2] and GeXIVA[1,4] at doses of 1 nmol produced an effect comparable to 500 nmol morphine but no addiction or motor side effects were observed during treatment. Analogous to Vc1.1, the short term effect of GeXIVA[1,2] and GeXIVA [1,4] was sustained for 24 h after injection, and the pain relief effect persisted for two weeks after the last injection (Li et al., 2016).

The analgesic effects of these α9α10 nAChR antagonists lasted for up to 24 h post-administration, which is longer than their half-life in serum. Moreover, when α-conotoxins were administered daily, analgesic effects remained for several days after the cessation of treatment and changes in the underlying disease pathology of nerve injury and repair were observed (Di Cesare Mannelli et al., 2014; Satkunanathan et al., 2005). However, such extended and cumulative anti-nociceptive effects are inconsistent with the rapid off-rate kinetics for these conotoxins (Ellison et al., 2006; Luo et al., 2015), and thus some researchers have attributed the cumulative analgesic and restorative effects of these α9α10 nAChR antagonists to immunological effects, and histological analysis supported such a notion.

Rats subjected to CCI exhibit a number of morphological changes in the injured nerve including edema, axon demyelination, axon degeneration, and increased infiltration of immune cells into the site of injury (Basbaum, Gautron, Jazat, Mayes, & Guilbaud, 1991; Sommer, Lalonde,

Heckman, Rodriguez, & Myers, 1995). In addition, the cell bodies of these axons in the dorsal root ganglion (DRG) show signs of damage. RgIA increased the number of nerve fibers, myelin thickness and axon diameter while reducing edema in CCI rats, and prevented the activation of microglia and astrocytes in the dorsal horn of the spinal cord (Di Cesare Mannelli et al., 2014). Moreover, accumulating evidence indicates that immune cells play a crucial role in the development of neuropathic pain following nerve injury and α9α10 nAChRs have been reported to be expressed in a variety of immune cells (Grace, Hutchinson, Maier, & Watkins, 2014; Peng et al., 2004). Both Vc1.1 and RgIA have been demonstrated to reduce the number of immune cells infiltrating into sciatic nerves, including CD86+ macrophages and CD2+ lymphocytes (Di Cesare Mannelli et al., 2014; Satkunanathan et al., 2005). The possible analgesic mechanism of GeXIVA has also been considered to involve inhibiting infiltration of immune cells into injured nerves, although more definitive evidence is needed (Li et al., 2016). These observations highlight that antagonism of α9α10 nAChRs expressed in immune cells is a potential mechanism that accounts, in part, for the analgesic and anti-inflammatory properties of these α9α10 nAChR antagonists.

Although conotoxins targeting the α9α10 nAChR can exert certain analgesic and anti-inflammatory effects through immunomodulatory mechanisms, the precise analgesic target is still controversial. Nevin et al. (2007) showed that Vc1.1 analogues (vc1a and [P6O] Vc1.1), which retained their potency at α9α10 nAChRs, produced no analgesia in PNL rats. Additionally, it was reported that α9 KO mice display a similar pain phenotype to wild-type mice and differences were detected only for chronic mechanical hyperalgesia (Mohammadi & Christie, 2014). These studies suggest that inhibition of α9α10 nAChR is insufficient for the analgesic activity of Vc1.1.

On this basis, conotoxin inhibition of the high voltage-activated calcium channels through activation of G protein-coupled $GABA_B$ receptors has been proposed as the possible analgesic mechanism (Benke, 2020; Callaghan et al., 2008; Klimis et al., 2011; Sadeghi et al., 2017). The $GABA_{B1}$ subunit is required for $GABA_B$ receptor function and the $GABA_{B1}$-KO model was generated in order to elucidate the role that $GABA_B$ receptors play in pain modulation (Goudet et al., 2009). $GABA_{B1}$ KO mice exhibited hyperalgesia in several behavioral tests, including the hot plate, tail flick, and paw pressure tests (Schuler et al., 2001); however, there was no difference in neuropathic and chronic inflammatory pain between conditional mouse mutants lacking a functional $GABA_{B(1)}$ subunit specifically in nociceptors (SNS-$GABA_{B1}^{-/-}$ mice) and wild-type littermates in a spared nerve injury model or with intraplantar formalin injection (Gangadharan et al., 2009). Furthermore, in both radioligand binding and functional experiments, Vc1.1 and RgIA failed to interact with human $GABA_B$ receptors expressed in *Xenopus* oocytes (McIntosh et al., 2009). In addition, use of the typical $GABA_B$ agonist baclofen leads to tolerance to the anti-nociceptive effects and the analgesic effects appear to be centrally mediated (Enna & Bowery, 2004; Goudet et al., 2009; Kent, Park, & Lindsley, 2020), whereas the action of these analgesic peptides is almost entirely peripherally mediated and more importantly, without tolerance.

In summary, the α-conotoxins Vc1.1, RgIA and GeXIVA represent novel peptide compounds with unique pharmacological actions and analgesic effects. These α9α10 antagonists not only produce analgesia, but also accelerate the functional recovery of damaged nerves through immunological modulation, which makes them candidates for the treatment of neuropathic pain.

5.2. Treatment of inflammatory disorders

The α9α10 nAChR-selective α-conotoxin antagonists were shown to have analgesic effects in animal models of neuropathic and inflammatory pain, but knowledge of the role of α9α10 nAChRs in experimental colitis is limited. A recent study reported the role of α9α10 nAChRs in the dextran sodium sulfate (DSS) experimental animal colitis model (AlSharari, Toma, Mahmood, McIntosh, & Damaj, 2020). Compared to the DSS-control group, a dose of RgIA of 0.2 nmol/mouse significantly reversed the disease activity index score, loss of body weight, and total histological damage score, as well as the colonic level of TNF-α. Moreover, RgIA dramatically rescued the shortening of colon length. These findings suggest a novel therapeutic application for RgIA and other α9α10 nAChR antagonists in the treatment of possibly other inflammatory disorders.

5.3. Antitumor activity

The nAChRs have attracted increased attention due to their major role in tumorigenesis and cancer progression (Dang et al., 2016). Breast cancer is the highest-incidence cancer among women worldwide (Bray et al., 2018), and the expression of α9 nAChR is closely correlated with the development of breast cancer (Lee et al., 2010). The α9 nAChR subunit is upregulated in various breast cancer cell lines compared with the normal epithelial cell line (Zhangsun, et al., 2020), and stimulation of the α9 nAChR led to cancer cell proliferation, angiogenesis and tumor metastasis (Ho, Lee, & Wu, 2011). Previous studies indicated that garcinol and green tea polyphenol(-)-epigallocatechin-3-gallate can inhibit nicotine-induced breast cancer cell proliferation through the downregulation of the α9 nAChR, further delaying the development of breast cancer cells *in vivo* (Chen et al., 2011; Tu et al., 2011). Luteolin and quercetin have also been shown to suppress tumor growth by downregulating the a9 nAChR subunit expressed on the human breast cancer cell surface (Shih et al., 2010). These results suggest that the regulation of α9-containing nAChR expression plays an important role in the process of breast cancer development, and that inhibition of α9-containing nAChRs could be a potential therapeutic strategy for cancer treatment.

The αO-conotoxin GeXIVA has been used to investigate the effects of inhibiting α9α10 nAChR on different human breast cancer cell lines due to its high potency and selectivity for the receptor (Sun, Bao, et al., 2020). Recently, Sun, Zhangsun, et al. (2020) confirmed that the α9 nAChR subunit is overexpressed in different breast cancer cell lines and that GeXIVA concentration-dependently inhibited the growth of cancer cells, with IC_{50} values ranging from 35 to 130 μM (Sun, Bao, et al., 2020). GeXIVA was most effective on the MDA-MB-157 cell line (Sun, Bao, et al., 2020, Sun, Zhangsun, et al. (2020)), significantly suppressing the proliferation and migration of cells by downregulating the α9-nAChR subunit. Moreover, flow cytometry analysis showed that GeXIVA promoted apoptosis of breast cancer cells (Sun, Bao, et al., 2020). Importantly, this study also showed growth inhibition of α9-nAChR KO MDA-MB-157 cells, thus confirming the involvement of the α9 subunit in cancer cell proliferation (Sun, Bao, et al., 2020).

In addition to breast cancer cells, the effects of GeXIVA were also determined on SiHa and CaSki cervical cancer cell lines, in which the expression of both α9 and α10 subunits are upregulated. GeXIVA markedly inhibited the proliferation of SiHa and CaSki cell lines, while it was only mildly cytotoxic for normal cells (Liu et al., 2019). Given that targeted treatment of cervical cancer is still in the early stages of research, inhibition of α9α10 nAChR may be useful for cervical cancer-targeted therapy.

In addition, α9α10 nAChRs have also been shown to mediate nicotine-induced proliferation of A549 adenocarcinoma lung tumors cells and this effect can be inhibited using RgIA4 (Mucchietto et al., 2018). These results highlight the pathophysiological role of α9α10 nAChR in promoting non-small cell lung carcinoma cell growth and provides strong evidence for an antitumor effect of RgIA4.

The nAChRs are strongly implicated in the development of certain cancers, but there are currently few studies on the antitumor effects of conotoxins targeting the α9α10 nAChR (Ho et al., 2011; Lee et al., 2010; Mucchietto et al., 2018; Sun, Bao, et al., 2020, Sun, Zhangsun, et al. (2020)). In the treatment of breast, cervical and lung cancer, both the α9α10 nAChR and conotoxins that specifically target it present

an opportunity for further development and subsequent potential translation to the clinic.

6. Outlook

The α9α10 nAChR has attracted attention as a therapeutic target due to its involvement in key physiological and pathological conditions including hearing, inflammatory disorders, neuropathic pain, and cancer. However, the precise role of the α9α10 nAChR in some of these conditions remains to be explored. Moreover, structural determination of the intact α9α10 nAChR is long overdue and will assist in the development of therapeutics. Conotoxin antagonists of the α9α10 subtype represent a new class of therapeutics with high potency and selectivity, thus minimizing off-target effects, which is also possible due to the mainly peripheral distribution of the receptor. These peptides are effective analgesics in animal pain models and have anti-proliferative effects on cancer cells, warranting further development for future potential clinical translation.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the grant from the National Science and Technology Major Project for Significant New Drugs Development (No. 2018ZX09735004), Fundamental Research Funds for the Central Universities (201762011 and 201941012), the National Key Research and Development Program (2019YFC0312601), National Natural Science Foundation of China (NSFC) (No. 81502977.), and an Australian Research Council (ARC) Discovery Project Grant (DP150103990 awarded to D.J.A.).

References

Abraham, N., & Lewis, R. J. (2018). Neuronal nicotinic acetylcholine receptor modulators from cone snails. *Marine Drugs 16*, 208.

Akondi, K. B., Muttenthaler, M., Dutertre, S., Kaas, Q., Craik, D. J., Lewis, R. J., & Alewood, P. F. (2014). Discovery, synthesis, and structure activity relationships of conotoxins. *Chemical Reviews 114*, 5815.

Albuquerque, E. X., Pereira, E. F. R., Alkondon, M., & Rogers, S. W. (2009). Mammalian nicotinic acetylcholine receptors: From structure to function. *Physiological Reviews 89*(1), 73–120.

AlSharari, S. D., Toma, W., Mahmood, H. M., McIntosh, J. M., & Damaj, M. I. (2020). The α9α10 nicotinic acetylcholine receptors antagonist α-conotoxin RgIA reverses colitis signs in murine dextran sodium sulfate model. *European Journal of Pharmacology 883*, 173320.

Armishaw, C. J., Dutton, J. L., Craik, D. J., & Alewood, P. F. (2010). Establishing regiocontrol of disulfide bond isomers of α-conotoxin Iml via the synthesis of N-to-C cyclic analogs. *Biopolymers 94*(3), 307–313.

Azam, L., & McIntosh, J. M. (2012). Molecular basis for the differential sensitivity of rat and human α9α10 nAChRs to α-conotoxin RgIA. *Journal of Neurochemistry 122*, 1137–1144.

Azam, L., Papakyriakou, A., Zouridakis, M., Giastas, P., Tzartos, S. J., & McIntosh, J. M. (2015). Molecular interaction of α-conotoxin RgIA with the rat α9α10 nicotinic acetylcholine receptor. *Molecular Pharmacology 87*, 855–864.

Basbaum, A. I., Gautron, M., Jazat, F., Mayes, M., & Guilbaud, G. (1991). The spectrum of fiber loss in a model of neuropathic pain in the rat: An electron microscopic study. *Pain 47*(3), 359–367.

Bavo, F., Pucci, S., Fasoli, F., Lammi, C., Moretti, M., Mucchietto, V., et al. (2018). Potent antiglioblastoma agents by hybridizing the onium-alkyloxy-stilbene based structures of an α7-nAChR, α9-nAChR antagonist and of a pro-oxidant mitocan. *Journal of Medicinal Chemistry 61*(23), 10531–10544.

Becchetti, A., Aracri, P., Meneghini, S., Brusco, S., & Amadeo, A. (2015). The role of nicotinic acetylcholine receptors in autosomal dominant nocturnal frontal lobe epilepsy. *Frontiers in Physiology 6*, 22.

Benke, D. (2020). $GABA_B$ receptors and pain. *Current Topics in Behavioral Neurosciences*. Berlin, Heidelberg: Springer. https://doi.org/10.1007/7854_2020_130.

Bennett, G. J., & Xie, Y. K. (1988). A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain 33*(1), 87–107.

Benowitz, N. L. (2009). Pharmacology of nicotine: Addiction, smoking-induced disease, and therapeutics. *Annual Review of Pharmacology and Toxicology 49*, 57–71.

Berecki, G., McArthur, J. R., Cuny, H., Clark, R. J., & Adams, D. J. (2014). Differential Cav2.1 and Cav2.3 channel inhibition by baclofen and α-conotoxin Vc1.1 via $GABA_B$ receptor activation. *Journal of General Physiology 143*(4), 465–479.

Bertrand, D., & Terry, A. V. J. (2018). The wonderland of neuronal nicotinic acetylcholine receptors. *Biochemical Pharmacology 151*, 214–225.

Boffi, J. C., Marcovich, I., Gill-Thind, J. K., Corradi, J., Collins, T., Lipovsek, M. M., et al. (2017). Differential contribution of subunit interfaces to α9α10 nicotinic acetylcholine receptor function. *Molecular Pharmacology 91*, 250–262.

Bordet, T., & Pruss, R. M. (2009). Targeting neuroprotection as an alternative approach to preventing and treating neuropathic pain. *Neurotherapeutics 6*(4), 648–662.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians 68*(6), 394–424.

Brejc, K., Van Dijk, W. J., Klaassen, R. V., Schuurmans, M., Van Der Oost, J., Smit, A. B., & Sixma, T. K. (2001). Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature 411*, 269–276.

Cai, F., Xu, N., Liu, Z., Ding, R., Yu, S., Dong, M., et al. (2018). Targeting of N-type calcium channels via $GABA_B$-receptor activation by α-conotoxin Vc1.1 variants displaying improved analgesic activity. *Journal of Medicinal Chemistry 61*(22), 10198–10205.

Callaghan, B., Haythornthwaite, A., Berecki, G., Clark, R. J., Craik, D. J., & Adams, D. J. (2008). Analgesic α-conotoxins Vc1.1 and Rg1A inhibit N-type calcium channels in rat sensory neurons via $GABA_B$ receptor activation. *Journal of Neuroscience 28*, 10943–10951.

Carbone, A. -L., Moroni, M., Groot-Kormelink, P. -J., & Bermudez, I. (2009). Pentameric concatenated $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ nicotinic acetylcholine receptors: Subunit arrangement determines functional expression. *British Journal of Pharmacology 156*(6), 970–981.

Carstens, B. B., Clark, R. J., Daly, N. L., Harvey, P. J., Kaas, Q., & Craik, D. J. (2011). Engineering of conotoxins for the treatment of pain. *Current Pharmaceutical Design 17*, 4242–4253.

Carstens, B. B., Swedberg, J., Berecki, G., Adams, D. J., Craik, D. J., & Clark, R. J. (2016). Effects of linker sequence modifications on the structure, stability, and biological activity of a cyclic α-conotoxin. *Biopolymer 106*(6), 864–875.

Castro, J., Grundy, L., Deiteren, A., Harrington, A. M., O'Donnell, T., Maddern, J., et al. (2018). Cyclic analogues of α-conotoxin Vc1.1 inhibit colonic nociceptors and provide analgesia in a mouse model of chronic abdominal pain. *British Journal of Pharmacology 175*(12), 2384–2398.

Castro, J., Harrington, A. M., Garcia-Caraballo, S., Maddern, J., Grundy, L., Zhang, J., et al. (2017). α-Conotoxin Vc1.1 inhibits human dorsal root ganglion neuroexcitability and mouse colonic nociception via $GABA_B$ receptors. *Gut 66*(6), 1083–1094.

Chen, C. -S., Lee, C. -H., Hsieh, C. -D., Ho, C. -T., Pan, M. -H., Huang, C. -S., et al. (2011). Nicotine-induced human breast cancer cell proliferation attenuated by garcinol through down-regulation of the nicotinic receptor and cyclin D3 proteins. *Breast Cancer Research and Treatment 125*(1), 73–87.

Chen, J., Cheuk, I. W. Y., Shin, V. Y., & Kwong, A. (2019). Acetylcholine receptors: Key players in cancer development. *Surgical Oncology 31*, 46–53.

Chhabra, S., Belgi, A., Bartels, P., van Lierop, B. J., Robinson, S. D., Kompella, S. N., et al. (2014). Dicarba analogues of α-conotoxin RgIA. Structure, stability, and activity at potential pain targets. *Journal of Medicinal Chemistry 57*(23), 9933–9944.

Christensen, S. B., Bandyopadhyay, P. K., Olivera, B. M., & McIntosh, J. M. (2015). αS-Conotoxin GVIIIB potently and selectively blocks α9α10 nicotinic acetylcholine receptors. *Biochemical Pharmacology 96*, 349–356.

Christensen, S. B., Hone, A. J., Roux, I., Kniazeff, J., Pin, J. P., Upert, G., et al. (2017). RgIA4 potently blocks mouse α9α10 nAChRs and provides long lasting protection against oxaliplatin-induced cold allodynia. *Frontiers in Cellular Neuroscience 11*, 219.

Chu, X., Tae, H. -S., Xu, Q., Jiang, T., Adams, D. J., & Yu, R. (2019). α-Conotoxin Vc1.1 structure-activity relationship at the human α9α10 nicotinic acetylcholine receptor investigated by minimal side chain replacement. *ACS Chemical Neuroscience 10*, 4328–4336.

Clark, R. J., Akcan, M., Kaas, Q., Daly, N. L., & Craik, D. J. (2012). Cyclization of conotoxins to improve their biopharmaceutical properties. *Toxicon 59*(4), 446–455.

Clark, R. J., Fischer, H., Dempster, L., Daly, N. L., Rosengren, K. J., Nevin, S. T., et al. (2005). Engineering stable peptide toxins by means of backbone cyclization: Stabilization of the α-conotoxin MII. *Proceedings of the National Academy of Science of the United States of America 102*, 13767–13772.

Clark, R. J., Fischer, H., Nevin, S. T., Adams, D. J., & Craik, D. J. (2006). The synthesis, structural characterization, and receptor specificity of the α-conotoxin Vc1.1. *Journal of Biological Chemistry 281*(32), 23254–23263.

Clark, R. J., Jensen, J., Nevin, S. T., Callaghan, B. P., Adams, D. J., & Craik, D. J. (2010). The engineering of an orally active conotoxin for the treatment of neuropathic pain. *Angewandte Chemie (International Edition) 49*(37), 6545–6548.

Colomer, C., Olivos-Oré, L. A., Vincent, A., McIntosh, J. M., Artalejo, A. R., & Guérineau, N. C. (2010). Functional characterization of α9-containing cholinergic nicotinic receptors in the rat adrenal medulla: implication in stress-induced functional plasticity. *Journal of Neuroscience 30*(19), 6732–6742.

Craik, D. J., & Adams, D. J. (2007). Chemical modification of conotoxins to improve stability and activity. *ACS Chemical Biology 2*, 457–468.

Cuny, H., de Faoite, A., Huynh, T. G., Yasuda, T., Berecki, G., & Adams, D. J. (2012). γ-Aminobutyric acid type B ($GABA_B$) receptor expression is needed for inhibition of N-type (Cav2.2) calcium channels by analgesic α-conotoxins. *Journal of Biological Chemistry 287*(28), 23948–23957.

Dang, N., Meng, X., & Song, H. (2016). Nicotinic acetylcholine receptors and cancer. *Biomedical Reports 4*(5), 515–518.

Dani, J. A., & Bertrand, D. (2007). Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annual Reviews in Pharmacology and Toxicology 47*, 699–729.

DeDominicis, K. E., Sahibzada, N., Olson, T. T., Xiao, Y., Wolfe, B. B., Kellar, K. J., & Yasuda, R. P. (2017). The $(\alpha4)_3(\beta2)_2$ stoichiometry of the nicotinic acetylcholine receptor predominates in the rat motor cortex. *Molecular Pharmacology 92*(3), 327–337.

Del Bufalo, A., Cesario, A., Salinaro, G., Fini, M., & Russo, P. (2014). Alpha9alpha10 nicotinic acetylcholine receptors as target for the treatment of chronic pain. *Current Pharmaceutical Design 20*(38), 6042–6047.

Dellisanti, C. D., Yao, Y., Stroud, J. C., Wang, Z. -Z., & Chen, L. (2007). Crystal structure of the extracellular domain of nAChR α1 bound to α-bungarotoxin at 1.94 Å resolution. *Nature Neuroscience 10*, 953–962.

Di Cesare Mannelli, L., Cinci, L., Micheli, L., Zanardelli, M., Pacini, A., McIntosh, J. M., & Ghelardini, C. (2014). α-Conotoxin RgIA protects against the development of nerve injury-induced chronic pain and prevents both neuronal and glial derangement. *Pain 155*, 1986–1995.

Di Guilmi, M. N., Boero, L. E., Castagna, V. C., Rodriguez-Contreras, A., Wedemeyer, C., Gómez-Casati, M. E., & Elgoyhen, A. B. (2019). Strengthening of the efferent olivocochlear system leads to synaptic dysfunction and tonotopy disruption of a central auditory nucleus. *Journal of Neuroscience 39*(36), 7037–7048.

Dineley, K. T., Pandya, A. A., & Yakel, J. L. (2015). Nicotinic ACh receptors as therapeutic targets in CNS disorders. *Trends in Pharmacological Science 36*(2), 96–108.

Dutton, J. L., & Craik, D. J. (2001). α-Conotoxins: Nicotinic acetylcholine receptor antagonists as pharmacological tools and potential drug leads. *Current Medicinal Chemistry 8*(4), 327–344.

Elgoyhen, A. B., Johnson, D. S., Boulter, J., Vetter, D. E., & Heinemann, S. (1994). α9: An acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell 79*(4), 705–715.

Elgoyhen, A. B., & Katz, E. (2012). The efferent medial olivocochlear-hair cell synapse. *Journal of Physiology (Paris) 106*(1–2), 47–56.

Elgoyhen, A. B., Katz, E., & Fuchs, P. A. (2009). The nicotinic receptor of cochlear hair cells: A possible pharmacotherapeutic target? *Biochemical Pharmacology 78*, 712–719.

Elgoyhen, A. B., Vetter, D. E., Katz, E., Rothlin, C. V., Heinemann, S. F., & Boulter, J. (2001). α10: A determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proceedings of the National Academy of Sciences of the United States of America 98*(6), 3501–3506.

Ellison, M., Feng, Z. -P., Park, A. J., Zhang, X., Olivera, B. M., McIntosh, J. M., & Norton, R. S. (2008). α-RgIA, a novel conotoxin that blocks the α9α10 nAChR: Structure and identification of key receptor-binding residues. *Journal of Molecular Biology 377*(4), 1216–1227.

Ellison, M., Haberlandt, C., Gómez-Casati, M. E., Watkins, M., Elgoyhen, A. B., McIntosh, J. M., & Olivera, B. M. (2006). α-RgIA: A novel conotoxin that specifically and potently blocks the α9α10 nAChR. *Biochemistry 45*, 1511–1517.

Enna, S. J., & Bowery, N. G. (2004). $GABA_B$ receptor alterations as indicators of physiological and pharmacological function. *Biochemical Pharmacology 68*(8), 1541–1548.

Fararjeh, A. -F. S., Tu, S. -H., Chen, L. -C., Cheng, T. -C., Liu, Y. -R., Chang, H. -L., et al. (2019). Long-term exposure to extremely low-dose of nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induce non-malignant breast epithelial cell transformation through activation of the a9-nicotinic acetylcholine receptor-mediated signaling pathway. *Environmental Toxicology 34*(1), 73–82.

Franchini, L. F., & Elgoyhen, A. B. (2006). Adaptive evolution in mammalian proteins involved in cochlear outer hair cell electromotility. *Molecular Phylogenetics and Evolution 41*, 622–635.

Franke, B., James, A. M., Mobli, M., Colgrave, M. L., Mylne, J. S., & Rosengren, K. J. (2017). Two proteins for the price of one: Structural studies of the dual-destiny protein preproalbumin with sunflower trypsin inhibitor-1. *Journal of Biological Chemistry 292*(30), 12398–12411.

Gangadharan, V., Agarwal, N., Brugger, S., Tegeder, I., Bettler, B., Kuner, R., & Kurejova, M. (2009). Conditional gene deletion reveals functional redundancy of $GABA_B$ receptors in peripheral nociceptors in vivo. *Molecular Pain 5*, 68.

Gehrmann, J., Alewood, P. F., & Craik, D. J. (1998). Structure determination of the three disulfide bond isomers of α-conotoxin GI: A model for the role of disulfide bonds in structural stability. *Journal of Molecular Biology 278*, 401–415.

Gharpure, A., Noviello, C. M., & Hibbs, R. E. (2020). Progress in nicotinic receptor structural biology. *Neuropharmacology 171*, 108086.

Giribaldi, J., & Dutertre, S. (2018). α-Conotoxins to explore the molecular, physiological and pathophysiological functions of neuronal nicotinic acetylcholine receptors. *Neuroscience Letters 679*, 24–34.

Glowatzki, E., & Fuchs, P. A. (2000). Cholinergic synaptic inhibition of inner hair cells in the neonatal mammalian cochlea. *Science 288*(5475), 2366–2368.

Gómez-Casati, M. E., Fuchs, P. A., Elgoyhen, A. B., & Katz, E. (2005). Biophysical and pharmacological characterization of nicotinic cholinergic receptors in rat cochlear inner hair cells. *Journal of Physiology 566*(Pt 1), 103–118.

Gongora-Benitez, M., Tulla-Puche, J., & Albericio, F. (2014). Multifaceted roles of disulfide bonds. Peptides as therapeutics. *Chemical Reviews 114*, 901–926.

Goudet, C., Magnaghi, V., Landry, M., Nagy, F., Gereau, R. W., & Pin, J. -P. (2009). Metabotropic receptors for glutamate and GABA in pain. *Brain Research Review 60*(1), 43–56.

Grace, P. M., Hutchinson, M. R., Maier, S. F., & Watkins, L. R. (2014). Pathological pain and the neuroimmune interface. *Nature Reviews Immunology 14*(4), 217–231.

Green, B. R., Zhang, M. -M., Chhabra, S., Robinson, S. D., Wilson, M. J., Redding, A., et al. (2014). Interactions of disulfide-deficient selenocysteine analogs of μ-conotoxin BuIIIB with the α-subunit of the voltage-gated sodium channel subtype 1.3. *FEBS Journal 281*(13), 2885–2898.

Guo, J., Ibaragi, S., Zhu, T., Luo, L. -Y., Hu, G. -F., Huppi, P. S., & Chen, C. Y. (2008). Nicotine promotes mammary tumor migration via a signaling cascade involving protein kinase C and cdc42. *Cancer Research 68*, 8473–8481.

Halai, R., Callaghan, B., Daly, N. L., Clark, R. J., Adams, D. J., & Craik, D. J. (2011). Effects of cyclization on stability, structure, and activity of α-conotoxin RgIA at the α9α10 nicotinic acetylcholine receptor and $GABA_B$ receptor. *Journal of Medicinal Chemistry 54*(19), 6984–6992.

Halai, R., Clark, R. J., Nevin, S. T., Jensen, J. E., Adams, D. J., & Craik, D. J. (2009). Scanning mutagenesis of α-conotoxin Vc1.1 reveals residues crucial for activity at the α9α10 nicotinic acetylcholine receptor. *Journal of Biological Chemistry 284*, 20275.

Halai, R., & Craik, D. J. (2009). Conotoxins: Natural product drug leads. *Natural Product Reports 26*, 526–536.

Ho, Y. S., Lee, C. H., & Wu, C. H. (2011). The α9-nicotinic acetylcholine receptor serves as a molecular target for breast cancer therapy. *Journal of Experimental & Clinical Medicine 3*(6), 246–251.

Hogg, R. C., Raggenbass, M., & Bertrand, D. (2003). Nicotinic acetylcholine receptors: from structure to brain function. *Reviews in Physiology, Biochemistry and Pharmacology 147*, 1–46.

Holtman, J. R., Dwoskin, L. P., Dowell, C., Wala, E. P., Zhang, Z., Crooks, P. A., & McIntosh, J. M. (2011). The novel small molecule α9α10 nicotinic acetylcholine receptor antagonist ZZ-204G is analgesic. *European Journal of Pharmacology 670*(2-3), 500–508.

Hone, A. J., & McIntosh, J. M. (2018). Nicotinic acetylcholine receptors in neuropathic and inflammatory pain. *FEBS Letters 592*(7), 1045–1062.

Hone, A. J., Scadden, M., Gajewiak, J., Christensen, S., Lindstrom, J., & McIntosh, J. M. (2012). α-Conotoxin PeIA[S9H,V10A,E14N] potently and selectively blocks $\alpha6\beta2\beta3$ versus $\alpha6\beta4$ nicotinic acetylcholine receptors. *Molecular Pharmacology 82*(5), 972–982.

Hone, A. J., Servent, D., & McIntosh, J. M. (2017). α9-Containing nicotinic acetylcholine receptors and the modulation of pain. *British Journal of Pharmacology 175*, 1915–1927.

Huang, L. -C., Lin, C. -L., Qiu, J. -Z., Lin, C. -Y., Hsu, K. -W., Tam, K. -W., et al. (2017). Nicotinic acetylcholine receptor subtype alpha-9 mediates triple-negative breast cancers based on a spontaneous pulmonary metastasis mouse model. *Frontiers in Cellular Neuroscience 11*, 336.

Hurst, R., Rollema, H., & Bertrand, D. (2013). Nicotinic acetylcholine receptors: From basic science to therapeutics. *Pharmacology and Therapeutics 137*(1), 22–54.

Huynh, P. N., Harvey, P. J., Gajewiak, J., Craik, D. J., & McIntosh, J. M. (2020). Critical residue properties for potency and selectivity of α-conotoxin RgIA towards α9α10 nicotinic acetylcholine receptors. *Biochemical Pharmacology 181*, 114124.

Huynh, T. G., Cuny, H., Slesinger, P. A., & Adams, D. J. (2015). Novel mechanism of voltage-gated N-type (Cav2.2) calcium channel inhibition revealed through α-conotoxin Vc1.1 activation of the $GABA_B$ receptor. *Molecular Pharmacology 87*(2), 240–250.

Indurthi, D. C., Pera, E., Kim, H. -L., Chu, C., McLeod, M. D., McIntosh, J. M., et al. (2014). Presence of multiple binding sites on α9α10 nAChR receptors alludes to stoichiometric-dependent action of the α-conotoxin. Vc1.1. *Biochemical Pharmacology 89*, 131–140.

Janes, R. W. (2005). α-Conotoxins as selective probes for nicotinic acetylcholine receptor subclasses. *Current Opinion in Pharmacology 5*(3), 280–292.

Jiang, S., Tae, H. -S., Xu, S., Shao, X., Adams, D. J., & Wang, C. (2017). Identification of a novel O-conotoxin reveals an unusual and potent inhibitor of the human α9α10 nicotinic acetylcholine receptor. *Marine Drugs 15*(6), 170.

Jiang, W., St-Pierre, S., Roy, P., Morley, B. J., Hao, J., & Simard, A. R. (2016). Infiltration of $CCR^{2+}Ly6C^{high}$ proinflammatory monocytes and neutrophils into the central nervous system is modulated by nicotinic acetylcholine receptors in a model of multiple sclerosis. *Journal of Immunology 196*(5), 2095–2108.

Jin, A. -H., Muttenthaler, M., Dutertre, S., Himaya, S. W. A., Kaas, Q., Craik, D. J., et al. (2019). Conotoxins: Chemistry and Biology. *Chemical Reviews 119*(21), 11510–11549.

Jorratt, P., Delano, P. H., Delgado, C., Dagnino-Subiabre, A., & Terreros, G. (2017). Difference in perseverative errors during a visual attention task with auditory distractors in alpha-9 nicotinic receptor subunit wild type and knock-out mice. *Frontiers in Cellular Neuroscience 11*, 357.

Jurado-Coronel, J. C., Avila-Rodriguez, M., Capani, F., Gonzalez, J., Moran, V. E., & Barreto, G. E. (2016). Targeting the nicotinic acetylcholine receptors (nAChRs) in astrocytes as a potential therapeutic target in Parkinson's disease. *Current Pharmaceutical Design 22*(10), 1305–1311.

Kaas, Q., Westermann, J. C., Halai, R., Wang, C. K., & Craik, D. J. (2008). ConoServer, a database for conopeptide sequences and structures. *Bioinformatics 24*, 445.

Kaas, Q., Yu, R., Jin, A. H., Dutertre, S., & Craik, D. J. (2012). ConoServer: Updated content, knowledge, and discovery tools in the conopeptide database. *Nucleic Acids Research 40*, D325.

Katz, E., Elgoyhen, A. B., Gómez-Casati, M. E., Knipper, M., Vetter, D. E., Fuchs, P. A., & Glowatzki, E. (2004). Developmental regulation of nicotinic synapses on cochlear inner hair cells. *Journal of Neuroscience 24*(36), 7814–7820.

Kennedy, A. C., Belgi, A., Husselbee, B. W., Spanswick, D., Norton, R. S., & Robinson, A. J. (2020). α-Conotoxin peptidomimetics: Probing the minimal binding motif for effective analgesia. *Toxins 12*(8), 505.

Kent, C. N., Park, C., & Lindsley, C. W. (2020). Classics in chemical neuroscience: Baclofen. *ACS Chemical Neuroscience 11*(12), 1740–1755.

Khademi, H., Kamangar, F., Brennan, P., & Malekzadeh, R. (2016). Opioid Tterapy and its side effects: A review. *Archives of Iranian Medicine 19*(12), 870–876.

Khan, G. M., Chen, S. -R., & Pan, H. -L. (2002). Role of primary afferent nerves in allodynia caused by diabetic neuropathy in rats. *Neuroscience 114*(2), 291–299.

van Kleef, R. G. D. M., Vijverberg, H. P. M., & Westerink, R. H. S. (2008). Selective inhibition of human heteromeric α9α10 nicotinic acetylcholine receptors at a low agonist concentration by low concentrations of ototoxic organic solvents. *Toxicology in Vitro 22*(6), 1568–1572.

Klimis, H., Adams, D. J., Callaghan, B., Nevin, S., Alewood, P. F., Vaughan, C. W., et al. (2011). A novel mechanism of inhibition of high-voltage activated calcium channels by α-conotoxins contributes to relief of nerve injury-induced neuropathic pain. *Pain 152*, 259–266.

Kryukova, E. V., Ivanov, I. A., Lebedev, D. S., Spirova, E. N., Egorova, N. S., Zouridakis, M., et al. (2018). Orthosteric and/or allosteric binding of α-conotoxins to nicotinic acetylcholine receptors and their models. *Marine Drugs 16*(12), 460.

Lawrence, P. B., & Price, J. L. (2016). How PEGylation influences protein conformational stability. *Current Opinion in Chemical Biology 34*, 88–94.

Lebbe, E. K. M., Peigneur, S., Wijesekara, I., & Tytgat, J. (2014). Conotoxins targeting nicotinic acetylcholine receptors: An overview. *Marine Drugs 12*(5), 2970–3004.

Lee, C. -H., Huang, C. -S., Chen, C. -S., Tu, S. -H., Wang, Y. -J., Chang, Y. -J., et al. (2010). Overexpression and activation of the α9-nicotinic receptor during tumorigenesis in human breast epithelial cells. *Journal of the National Cancer Institute 102*(17), 1322–1335.

Lewis, R. J., Dutertre, S., Vetter, I., & Christie, M. J. (2012). *Conus* venom peptide pharmacology. *Pharmacological Reviews 64*, 259–298.

Li, X., Hu, Y., Wu, Y., Huang, Y., Yu, S., Ding, Q., ... Luo, S. (2016). Anti-hypersensitive effect of intramuscular administration of αO-conotoxin GeXIVA[1,2] and GeXIVA[1,4] in rats of neuropathic pain. *Progress in Neuro-Psychopharmacology & Biological Psychiatry 66*, 112–119.

Liang, J., Tae, H. -S., Xu, X., Jiang, T., Adams, D. J., & Yu, R. (2020). Dimerization of α-conotoxins as a strategy to enhance the inhibition of the human α7 and α9α10 nicotinic acetylcholine receptors. *Journal of Medicinal Chemistry 63*(6), 2974–2985.

van Lierop, B. J., Robinson, S. D., Kompella, S. N., Belgi, A., McArthur, J. R., Hung, A., et al. (2013). Dicarba α-conotoxin Vc1.1 analogues with differential selectivity for nicotinic acetylcholine and $GABA_B$ receptors. *ACS Chemical Biology 8*, 1815–1821.

Liu, Y., Qian, J., Sun, Z., Zhangsun, D., & Luo, S. (2019). Cervical cancer correlates with the differential expression of nicotinic acetylcholine receptors and reveals therapeutic targets. *Marine Drugs 17*(5), 256.

Livett, B. G., Khalil, Z., Gayler, K. R., Down, J. G., Sandall, D. W., & Keays, D. A. (2009). Alpha-conotoxin peptides with analgesic properties. US Patent 7348400

Livett, B. G., Sandall, D. W., Keays, D., Down, J., Gayler, K. R., Satkunanathan, N., & Khalil, Z. (2006). Therapeutic applications of conotoxins that target the neuronal nicotinic acetylcholine receptor. *Toxicon 48*, 810–829.

Loughnan, M., Nicke, A., Jones, A., Schroeder, C. I., Nevin, S. T., Adams, D. J., et al. (2006). Identification of a novel class of nicotinic receptor antagonists: Dimeric conotoxins VxXIIA, VxXIIB, and VxXIIC from *Conus vexillum*. *Journal of Biological Chemistry 281*, 24745–24755.

Lu, J., Zhang, K., Wang, S., Sun, T., Yu, S., Dai, Q., & Liu, Z. (2017). Cloning, expression and functional characterization of a D-superfamily conotoxin Lt28.1 with previously undescribed cysteine pattern. *Peptide 94*, 64–70.

Luo, S., Christensen, S., Zhangsun, D., Wu, Y., Hu, Y., Zhu, X., et al. (2013). A novel inhibitor of α9α10 nicotinic acetylcholine receptors from *Conus vexillum* delineates a new conotoxin superfamily. *PLoS One 8*, e54648.

Luo, S., Zhangsun, D., Harvey, P. J., Kaas, Q., Wu, Y., Zhu, X., et al. (2015). Cloning, synthesis, and characterization of αO-conotoxin GeXIVA, a potent α9α10 nicotinic acetylcholine receptor antagonist. *Proceedings of the National Academy of Sciences of the United States of America 112*(30), E4026–E4035.

Lustig, L. R. (2006). Nicotinic acetylcholine receptor structure and function in the efferent auditory system. *The Anatomical Record Part A 288*(4), 424–434.

Lustig, L. R., Peng, H., Hiel, H., Yamamoto, T., & Fuchs, P. A. (2001). Molecular cloning and mapping of the human nicotinic acetylcholine receptor α10 (CHRNA10). *Genomics 73*(3), 272–283.

MacRaild, C. A., Illesinghe, J., van Lierop, B. J., Townsend, A. L., Chebib, M., Livett, B. G., et al. (2009). Structure and activity of (2,8)-dicarba-(3,12)-cystino α-Iml, an α-conotoxin containing a nonreducible cystine analogue. *Journal of Medicinal Chemistry 52*, 755–762.

Mazzaferro, S., Benallegue, N., Carbone, A., Gasparri, F., Vijayan, R., Biggin, P. C., ... Bermudez, I. (2011). Additional acetylcholine (ACh) binding site at α4/α4 interface of (α4Å2)2α4 nicotinic receptor influences agonist sensitivity. *Journal of Biological Chemistry 286*(35), 31043–31054.

McIntosh, J. M., Absalom, N., Chebib, M., Elgoyhen, A. B., & Vincler, M. (2009). Alpha9 nicotinic acetylcholine receptors and the treatment of pain. *Biochemical Pharmacology 78*(7), 693–702.

McIntosh, J. M., Plazas, P. V., Watkins, M., Gómez-Casati, M. E., Olivera, B. M., & Elgoyhen, A. B. (2005). A novel α-conotoxin, PeIA, cloned from *Conus pergrandis*, discriminates between rat α9α10 and α7 nicotinic cholinergic receptors. *Journal of Biological Chemistry 280*, 30107–30112.

Mohammadi, S., & Christie, M. J. (2014). α9-Nicotinic acetylcholine receptors contribute to the maintenance of chronic mechanical hyperalgesia, but not thermal or mechanical allodynia. *Molecular Pain 10*, 64.

Mohammadi, S. A., Burton, T. J., & Christie, M. J. (2017). α9-nAChR knockout mice exhibit dysregulation of stress responses, affect and reward-related behaviour. *Behavioural Brain Research 328*, 105–114.

Mohammadi, S. A., & Christie, M. J. (2015). Conotoxin interactions with α9α10-nAChRs: Is the α9α10-nicotinic acetylcholine receptor an important therapeutic target for pain management? *Toxins 7*(10), 3916–3932.

Morley, B. J., & Simmons, D. D. (2002). Developmental mRNA expression of the α10 nicotinic acetylcholine receptor subunit in the rat cochlea. *Developmental Brain Research 139*(1), 87–96.

Morrison, K. L., & Weiss, G. A. (2001). Combinatorial alanine-scanning. *Current Opinion in Chemical Biology 5*(3), 302–307.

Mucchietto, V., Fasoli, F., Pucci, S., Moretti, M., Benfante, R., Maroli, A., et al. (2018). α9- and α7-containing receptors mediate the pro-proliferative effects of nicotine in the A549 adenocarcinoma cell line. *British Journal of Pharmacology 175*(11), 1957–1972.

Myers, R. A., Cruz, L. J., & Olivera, B. M. (1993). *Conus* peptides as chemical probes for receptors and ion channels. *Chemical Reviews 93*, 1923–1936.

Napier, I. A., Klimis, H., Rycroft, B. K., Jin, A. H., Alewood, P. F., Motin, L., ... Christie, M. J. (2012). Intrathecal α-conotoxins Vc1.1, AuIB and MII acting on distinct nicotinic receptor subtypes reverse signs of neuropathic pain. *Neuropharmacology 62*, 2202–2207.

Nevin, S. T., Clark, R. J., Klimis, H., Christie, M. J., Craik, D. J., & Adams, D. J. (2007). Are α9α10 nicotinic acetylcholine receptors a pain target for α-conotoxins? *Molecular Pharmacology 72*(6), 1406–1410.

Nguyen, H. D., Liao, Y. -C., Ho, Y. -S., Chen, L. -C., Chang, H. -W., Cheng, T. -C., et al. (2019). The α9 nicotinic acetylcholine receptor mediates nicotine-induced PD-L1 expression and regulates melanoma cell proliferation and migration. *Cancers 11*(12), 1991.

Nilsson, K. P., Lovelace, E. S., Caesar, C. E., Tynngard, N., Alewood, P. F., Johansson, H. M., et al. (2005). Solution structure of χ-conopeptide MrIA, a modulator of the human norepinephrine transporter. *Biopolymers 80*, 815–823.

Olivera, B. M., Gray, W. R., Zeikus, R., McIntosh, J. M., Varga, J., Rivier, J., et al. (1985). Peptide neurotoxins from fish-hunting cone snails. *Science 230*, 1338–1343.

Olivera, B. M., Rivier, J., Clark, C., Ramilo, C. A., Corpuz, G. P., Abogadie, F. C., et al. (1990). Diversity of *Conus* neuropeptides. *Science 249*, 257–263.

Pacini, A., Micheli, L., Maresca, M., Branca, J. J., McIntosh, J. M., Ghelardini, C., & Di Cesare Mannelli, L. (2016). The α9α10 nicotinic receptor antagonist α-conotoxin RgIA prevents neuropathic pain induced by oxaliplatin treatment. *Experimental Neurology 282*, 37–48.

Pedersen, J. E., Bergqvist, C. A., & Larhammar, D. (2019). Evolution of vertebrate nicotinic acetylcholine receptors. *BMC Evolutionary Biology 19*(38).

Parikh, V., Kutlu, M. G., & Gould, T. J. (2016). nAChR dysfunction as a common substrate for schizophrenia and comorbid nicotine addiction: Current trends and perspectives. *Schizophrenia Research 171*(1–3), 1–15.

Pavlov, V. A., Chavan, S. S., & Tracey, K. J. (2018). Molecular and functional neuroscience in immunity. *Annual Reviews in Immunology 36*, 783–812.

Peng, H., Ferris, R. L., Matthews, T., Hiel, H., Lopez-Albaitero, A., & Lustig, L. R. (2004). Characterization of the human nicotinic acetylcholine receptor subunit α (alpha) 9 (CHRNA9) and α (alpha) 10 (CHRNA10) in lymphocytes. *Life Sciences 76*, 263–280.

Pennington, M. W., Czerwinski, A., & Norton, R. S. (2018). Peptide therapeutics from venom: Current status and potential. *Bioorganic & Medicinal Chemistry 26*, 2738–2758.

Perez, E. G., Cassels, B. K., & Zapata-Torres, G. (2009). Molecular modeling of the α9α10 nicotinic acetylcholine receptor subtype. *Bioorganic & Medicinal Chemistry Letters 19*, 251–254.

Plazas, P. V., Katz, E., Gómez-Casati, M. E., Bouzat, C., & Elgoyhen, A. B. (2005). Stoichiometry of the α9α10 nicotinic cholinergic receptor. *Journal of Neuroscience 25*, 10905–10912.

Quik, M., Zhang, D., McGregor, M., & Bordia, T. (2015). Alpha7 nicotinic receptors as therapeutic targets for Parkinson's disease. *Biochemical Pharmacology 97*(4), 399–407.

Quiram, P. A., Jones, J. J., & Sine, S. M. (1999). Pairwise interactions between neuronal α7 acetylcholine receptors and α-conotoxin Iml. *Journal of Biological Chemistry 274*, 19517–19524.

Quiram, P. A., & Sine, S. M. (1998). Identification of residues in the neuronal α7 acetylcholine receptor that confer selectivity for conotoxin Iml. *Journal of Biological Chemistry 273*(18), 11001–11006.

Rabenstein, D. L., & Weaver, K. H. (1996). Kinetics and equilibria of the thiol/disulfide exchange reactions of somatostatin with glutathione. *Journal of Organic Chemistry 61*, 7391–7397.

Ren, J., Zhu, X., Xu, P., Li, R., Fu, Y., Dong, S., et al. (2019). D-Amino acid substitution of α-conotoxin RgIA identifies its critical residues and improves the enzymatic stability. *Marine Drug 17*(3), 142.

Richter, K., Mathes, V., Fronius, M., Althaus, M., Hecker, A., Krasteva-Christ, G., et al. (2016). Phosphocholine - An agonist of metabotropic but not of ionotropic functions of α9-containing nicotinic acetylcholine receptors. *Scientific Reports 6*, 28660.

Robinson, S. D., & Norton, R. S. (2014). Conotoxin gene superfamilies. *Marine Drugs 12*, 6058–6101.

Romero, H. K., Christensen, S. B., Di Cesare Mannelli, L., Gajewiak, J., Ramachandra, R., Elmslie, K. S., et al. (2017). Inhibition of α9α10 nicotinic acetylcholine receptors prevents chemotherapy-induced neuropathic pain. *Proceedings of the National Academy of Science of the United States of America 114*, E1825–E1832.

Sadeghi, M., McArthur, J. R., Finol-Urdaneta, R. K., & Adams, D. J. (2017). Analgesic conopeptides targeting G protein-coupled receptors reduce excitability of sensory neurons. *Neuropharmacology 127*, 116–123.

Safavi-Hemami, H., Siero, W. A., Kuang, Z., Williamson, N. A., Karas, J. A., Page, L. R., et al. (2011). Embryonic toxin expression in the cone snail *Conus victoriae*: Primed to kill or divergent function? *Journal of Biological Chemistry 286*(25), 22546–22557.

Sandall, D. W., Satkunanathan, N., Keays, D. A., Polidano, M. A., Liping, X., Pham, V., et al. (2003). A novel α-conotoxin identified by gene sequencing is active in suppressing the vascular response to selective stimulation of sensory nerves in vivo. *Biochemistry 42*, 6904–6911.

Satkunanathan, N., Livett, B., Gayler, K., Sandall, D., Down, J., & Khalil, Z. (2005). α-Conotoxin Vc1.1 alleviates neuropathic pain and accelerates functional recovery of injured neurones. *Brain Research 1059*, 149–158.

Schuler, V., Lüscher, C., Blanchet, C., Klix, N., Sansig, G., Klebs, K., et al. (2001). Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic $GABA_B$ responses in mice lacking $GABA_{B1}$. *Neuron 31*, 47–58.

Seltzer, Z., Dubner, R., & Shir, Y. (1990). A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain 43*(2), 205–218.

Sgard, F., Charpantier, E., Bertrand, S., Walker, N., Caput, D., Graham, D., et al. (2002). A novel human nicotinic receptor subunit, α10, that confers functionality to the α9-subunit. *Molecular Pharmacology 61*(1), 150–159.

Sharpe, I. A., Gehrmann, J., Loughnan, M. L., Thomas, L., Adams, D., Atkins, A., et al. (2001). Two new classes of conopeptides inhibit the α1-adrenoceptor and noradrenaline transporter. *Nature Neuroscience 4*, 902–907.

Shih, Y. -L., Liu, H. -C., Chen, C. -S., Hsu, C. -H., Pan, M. -H., Chang, H. -W., et al. (2010). Combination treatment with luteolin and quercetin enhances antiproliferative effects in nicotine-treated MDA-MB-231 cells by down-regulating nicotinic acetylcholine receptors. *Journal of Agricultural and Food Chemistry 58*(1), 235–241.

Simard, A. R., Gan, Y., St-Pierre, S., Kousari, A., Patel, V., Whiteaker, P., et al. (2013). Differential modulation of EAE by α9*- and β2*-nicotinic acetylcholine receptors. *Immunology and Cell Biology 91*(3), 195–200.

Sommer, C., Lalonde, A., Heckman, H. M., Rodriguez, M., & Myers, R. R. (1995). Quantitative neuropathology of a focal nerve injury causing hyperalgesia. *Journal of Neuropathology and Experimental Neurology 54*(5), 635–643.

St-Pierre, S., Jiang, W., Roy, P., Champigny, C., LeBlanc, É., Morley, B. J., et al. (2016). Nicotinic acetylcholine receptors modulate bone marrow-derived pro-inflammatory monocyte production and survival. *PloS One 11*(2), e0150230.

Sun, Z., Bao, J., Zhangsun, M., Dong, S., Zhangsun, D., & Luo, S. (2020). αO-Conotoxin GeXIVA inhibits the growth of breast cancer cells via interaction with α9 nicotinic acetylcholine receptors. *Marine Drugs 18*(4), 195.

Sun, Z., Zhangsun, M., Dong, S., Liu, Y., Qian, J., Zhangsun, D., & Luo, S. (2020). Differential expression of nicotinic acetylcholine receptors associates with human breast cancer and mediates antitumor activity of αO-conotoxin GeXIVA. *Marine Drugs 18*(1), 61.

Tabassum, N., Tae, H. -S., Jia, X., Kaas, Q., Jiang, T., Adams, D. J., & Yu, R. (2017). Role of CysI–CysIII disulfide bond on the structure and activity of α-conotoxins at human neuronal nicotinic acetylcholine receptors. *ACS Omega 2*(8), 4621–4631.

Thompson, A. J., Lester, H. A., & Lummis, S. C. R. (2010). The structural basis of function in Cys-loop receptors. *Quarterly Reviews of Biophysics 43*(4), 449–499.

Tu, S. -H., Ku, C. -Y., Ho, C. -T., Chen, C. -S., Huang, C. -S., Lee, C. -H., et al. (2011). Tea polyphenol (-)-epigallocatechin-3-gallate inhibits nicotine- and estrogen-induced α9-nicotinic acetylcholine receptor upregulation in human breast cancer cells. *Molecular Nutrition & Food Research 55*(3), 455–466.

Umana, I. C., Daniele, C. A., & McGehee, D. S. (2013). Neuronal nicotinic receptors as analgesic targets: It's a winding road. *Biochemical Pharmacology 86*(8), 1208–1214.

Unwin, N. (2005). Refined structure of the nicotinic acetylcholine receptor at 4Å resolution. *Journal of Molecular Biology 346*(4), 967–989.

Unwin, N. (2013). Nicotinic acetylcholine receptor and the structural basis of neuromuscular transmission: insights from *Torpedo* postsynaptic membranes. *Quarterley Reviews of Biophysics 46*, 283–322.

Verbitsky, M., Rothlin, C. V., Katz, E., & Elgoyhen, A. B. (2000). Mixed nicotinic-muscarinic properties of the α9 nicotinic cholinergic receptor. *Neuropharmacology 39*(13), 2515–2524.

Vetter, D. E., Katz, E., Maison, S. F., Taranda, J., Turcan, S., Ballestero, J., et al. (2007). The α10 nicotinic acetylcholine receptor subunit is required for normal synaptic function and integrity of the olivocochlear system. *Proceedings of the National Academy of Sciences of the United States of America 104*, 20594–20599.

Vetter, I., & Lewis, R. J. (2012). Therapeutic potential of cone snail venom peptides (conopeptides). *Current Topics in Medicinal Chemistry 12*, 1546–1552.

Vincler, M., & McIntosh, J. M. (2007). Targeting the α9α10 nicotinic acetylcholine receptor to treat severe pain. *Expert Opinion on Therapeutic Targets 11*(7), 891–897.

Vincler, M., Wittenauer, S., Parker, R., Ellison, M., Olivera, B. M., & McIntosh, J. M. (2006). Molecular mechanism for analgesia involving specific antagonism of α9α0 nicotinic acetylcholine receptors. *Proceedings of the National Academy of Sciences of the United States of America 103*, 17880–17884.

Wala, E. P., Crooks, P. A., McIntosh, J. M., & Holtman, J. R. (2012). Novel small molecule α9α10 nicotinic receptor antagonist prevents and reverses chemotherapy-evoked neuropathic pain in rats. *Anesthesia & Analgesia 115*, 713–720.

Wan, J., Huang, J. X., Vetter, I., Mobli, M., Lawson, J., Tae, H. S., et al. (2015). α-Conotoxin dendrimers have enhanced potency and selectivity for homomeric nicotinic acetylcholine receptors. *Journal of the American Chemical Society 137*, 3209–3212.

Wang, H., Li, X., Zhangsun, D., Yu, G., Su, R., & Luo, S. (2019). The α9α10 nicotinic acetylcholine receptor antagonist αO-conotoxin GeXIVA[1,2] alleviates and reverses chemotherapy-induced neuropathic pain. *Marine Drugs 17*, 265.

Wang, Y., Zhang, Y., Gu, C., Bao, W., & Bao, Y. (2014). Neuronal acetylcholine receptor subunit alpha-9 (CHRNA9) polymorphisms are associated with NSCLC risk in a Chinese population. *Medical Oncology 31*(5), 932.

Wedemeyer, C., Vattino, L. G., Moglie, M. J., Ballestero, J., Maison, S. F., Di Guilmi, M. N., et al. (2018). A gain-of-function mutation in the α9 nicotinic acetylcholine receptor alters medial olivocochlear efferent short-term synaptic plasticity. *Journal of Neuroscience 38*(16), 3939–3954.

Weisstaub, N., Vetter, D. E., Elgoyhen, A. B., & Katz, E. (2002). The a9a10 nicotinic acetylcholine receptor is permeable to and is modulated by divalent cations. *Hearing Research 167*(1–2), 122–135.

Wu, X., Huang, Y. -H., Kaas, Q., Harvey, P. J., Wang, C. K., Tae, H. -S., ... Craik, D. J. (2017). Backbone cyclization of analgesic conotoxin GeXIVA facilitates direct folding of the ribbon isomer. *Journal of Biological Chemistry 292*, 17101–17112.

Xu, P., Kaas, Q., Wu, Y., Zhu, X., Li, X., Harvey, P. J., et al. (2020). Structure and activity studies of disulfide-deficient analogues of αO-conotoxin GeXIVA. *Journal of Medicinal Chemistry 63*(4), 1564–1575.

Xu, S., Zhang, T., Kompella, S. N., Yan, M., Lu, A., Wang, Y., et al. (2015). Conotoxin αD-GeXXA utilizes a novel strategy to antagonize nicotinic acetylcholine receptors. *Scientific Reports 5*, 14261.

Yu, R., Kompella, S. N., Adams, D. J., Craik, D. J., & Kaas, Q. (2013). Determination of the α-conotoxin Vc1.1 binding site on the α9α10 nicotinic acetylcholine receptor. *Journal of Medicinal Chemistry 56*(9), 3557–3567.

Yu, R., Seymour, V. A. L., Berecki, G., Jia, X., Akcan, M., Adams, D. J., ... Craik, D. J. (2015). Less is more: Design of a highly stable disulfide-deleted mutant of analgesic cyclic α-conotoxin Vc1.1. *Scientific Reports 5*, 13264.

Yu, R., Tae, H. S., Tabassum, N., Shi, J., Jiang, T., & Adams, D. J. (2018). Molecular determinants conferring the stoichiometric-dependent activity of α-conotoxins at the human α9α10 nicotinic acetylcholine receptor subtype. *Journal of Medicinal Chemistry 61*, 4628–4634.

Zakrzewicz, A., Richter, K., Agné, A., Wilker, S., Siebers, K., Fink, B., et al. (2017). Canonical and novel non-canonical cholinergic agonists inhibit ATP-induced release of monocytic interleukin-1β via different combinations of nicotinic acetylcholine receptor subunits α7, α9 and α10. *Frontiers in Cellular Neuroscience 11*, 189.

Zemkova, H., Kucka, M., Bjelobaba, I., Tomic, M., & Stojilkovic, S. S. (2013). Multiple cholinergic signaling pathways in pituitary gonadotrophs. *Endocrinology 154*(1), 421–433.

Zhangsun, D., Zhu, X., Kaas, Q., Wu, Y., Craik, D. J., Mcintosh, J. M., & Luo, S. (2017). αO-Conotoxin GeXIVA disulfide bond isomers exhibit differential sensitivity for various nicotinic acetylcholine receptors but retain potency and selectivity for the human α9α10 subtype. *Neuropharmacology 127*, 243–252.

Zheng, G., Zhang, Z., Dowell, C., Wala, E., Dwoskin, L. P., Holtman, J. R., et al. (2011). Discovery of non-peptide, small molecule antagonists of α9α10 nicotinic acetylcholine receptors as novel analgesics for the treatment of neuropathic and tonic inflammatory pain. *Bioorganic & Medicinal Chemistry Letters 21*, 2476.

Zheng, N., Christensen, S. B., Blakely, A., Dowell, C., Purushottam, L., McIntosh, J. M., & Chou, D. H. -C. (2020). Development of conformationally constrained α-RgIA analogues as stable peptide antagonists of human α9α10 nicotinic acetylcholine receptors. *Journal of Medicinal Chemistry 63*(15), 8380–8387.

Zhou, H., Shi, B., Jia, Y., Qiu, G., Yang, W., Li, J., et al. (2018). Expression and significance of autonomic nerves and α9 nicotinic acetylcholine receptor in colorectal cancer. *Molecular Medicine Reports 17*(6), 8423–8431.

Zoli, M., Pistillo, F., & Gotti, C. (2015). Diversity of native nicotinic receptor subtypes in mammalian brain. *Neuropharmacology 96*, 302–311.

Zoli, M., Pucci, S., Vilella, A., & Gotti, C. (2018). Neuronal and extraneuronal nicotinic acetylcholine receptors. *Current Neuropharmacology 16*(4), 338–349.

Zouridakis, M., Giastas, P., Zarkadas, E., Chroni-Tzartou, D., Bregestovski, P., & Tzartos, S. J. (2014). Crystal structures of free and antagonist-bound states of human α9 nicotinic receptor extracellular domain. *Nature Structural Molecular Biology 21*, 976–980.

Zouridakis, M., Papakyriakou, A., Ivanov, I. A., Kasheverov, I. E., Tsetlin, V., Tzartos, S., & Giastas, P. (2019). Crystal structure of the monomeric extracellular domain of α9 nicotinic receptor subunit in complex with α-conotoxin RgIA: Molecular dynamics insights into RgIA binding to α9α10 nicotinic receptors. *Frontiers in Pharmacology 10*, 474.