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Conus Venom Peptide Pharmacology

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***Abstract*——Conopeptides are a diverse group of recently evolved venom peptides used for prey capture and/or defense. Each species of cone snails produces in excess of 1000 conopeptides, with those pharmacologically characterized (~0.1%) targeting a diverse range of membrane proteins typically with high potency and specificity. The majority of conopeptides inhibit voltage- or ligand-gated ion channels, providing valuable research tools for the dissection of the role played by specific ion channels in excitable cells. It is noteworthy that many of these targets are found to be expressed in pain pathways, with several conopeptides having entered the clinic as potential treatments for pain [e.g., pyroglutamate1-MrIA (Xen2174)] and one now marketed for intrathecal treatment of severe pain [ziconotide (Prialt)]. This review discusses the diversity, pharmacology, structure-activity relationships, and therapeutic potential of cone snail venom peptide families acting at voltage-gated ion channels (ω-, μ-, μO-, δ-, ι-, and κ-conotoxins), ligand-gated ion channels (α-conotoxins, σ-conotoxin, ikot-ikot, and conantokins), G-protein-coupled receptors (ρ-conopeptides, conopressins, and contulakins), and neurotransmitter transporters (χ-conopeptides), with expanded discussion on the clinical potential of sodium and calcium channel inhibitors and α-conotoxins. Expanding the discovery of new bioactives using proteomic/transcriptomic approaches combined with high-throughput platforms and better defining conopeptide structure-activity relationships using relevant membrane protein crystal structures are expected to grow the already significant impact conopeptides have had as both research probes and leads to new therapies.**

I. Introduction

The interrogation of chemical diversity with therapeutically relevant screens remains the dominant discovery engine for the pharmaceutical industry. Although most success with this approach has been achieved by exploring natural product extracts composed mostly of smaller organic molecules, there is a growing realization that peptides are an underused source of leads for new therapeutics. This realization is now gaining momentum following the disappointments of combinatorial chemistry, increasing failure rates of small molecules in clinical development, and the limitations of small-molecule–based approaches for many druggable targets. Sources of peptides include plant cyclotides (Henriques and Craik, 2010), phage display libraries (Pande et al., 2010), and venom peptides (Lewis and Garcia, 2003). Among these sources, venom peptides provide arguably the largest source of chemical diversity, being driven by evolutionary pressure for improved prey capture and/or defense. This diversity has arisen multiple times in nature in animals as diverse as sea anemones, jellyfish, centipedes, spiders, scorpions, cone snails, cephalopods, echinoderms, snakes, lizards, fish, platypus and arguably even fleas, mosquitoes, kissing bugs, leeches, ticks, and vampire bats (Fry et al., 2009). Ensuring their successful implementation, venom peptides have coevolved with sophisticated and specialized envenomation machinery comprising fangs, barbs, modified teeth, harpoons, nematocysts, spines, or sprays. Not surprisingly, given that most venoms are delivered intravenously, there are multiple examples of convergent evolution of a relatively small number of structural scaffolds that possess the requisite stability to remain intact, both within the venom and in plasma after envenomation. These "privileged" structural frameworks have provided the scaffolds for an explosion in sequence diversity that explains much of the chemical diversity found in present-day venoms.

Among the venomous species, cone snails within the family Conidae are unique for their ability to use a diverse array of small disulfide-bridged peptides (conopeptides or conotoxins) for prey capture (Fig. 1). Conopeptides have evolved across all phylogenetic clades and feeding strategies of cone snails from at least 16 genetically distinct superfamilies, many of which are subject to extensive post-translational modifications (Table 1). This highly evolved hunting strategy, developed in parallel with the deployment of a hollow, barbed harpoon, allows normally herbivorous molluscs to prey on animals as diverse as worms (vermivorous species), fish (piscivorous species), and other molluscs (molluscivorous species). Successful implementation of this strategy has allowed this single genus of widely distributed marine molluscs to evolve into more than 500 *Conus* species. Cone snails hunt prey mostly at night and once prey is located by smell they skillfully inject small quantities of venom (up to ~50 μl) to paralyze prey within seconds using a rich cocktail of conopeptides that are cleaved from propeptides by specialized venom endoproteases (Milne et al., 2003). Their small molecular size (typ-

[1]Abbreviations: 5-HT_3, 5-hydroxytryptamine$_3$; ACh, acetylcholine; AChBP, acetylcholine binding protein; Am2766, CKQAGESCDIFSQNCCVGTCAFICIE-NH_2; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AR, adrenergic receptor; Ca_v, calcium channel; CNS, central nervous system; Con-G, conantokin-G; DRG, dorsal root ganglion; GPCR, G-protein-coupled receptor; nAChR, nicotinic acetylcholine receptor; NET, norepinephrine transporter; NMDA, *N*-methyl-D-aspartate; NTSR, neurotensin receptor; SNX-482, GVDKAGCRYMFGGCSVNDDCCPRLGCHSLFSYCAWDLTFSD-OH; TTX, tetrodotoxin.

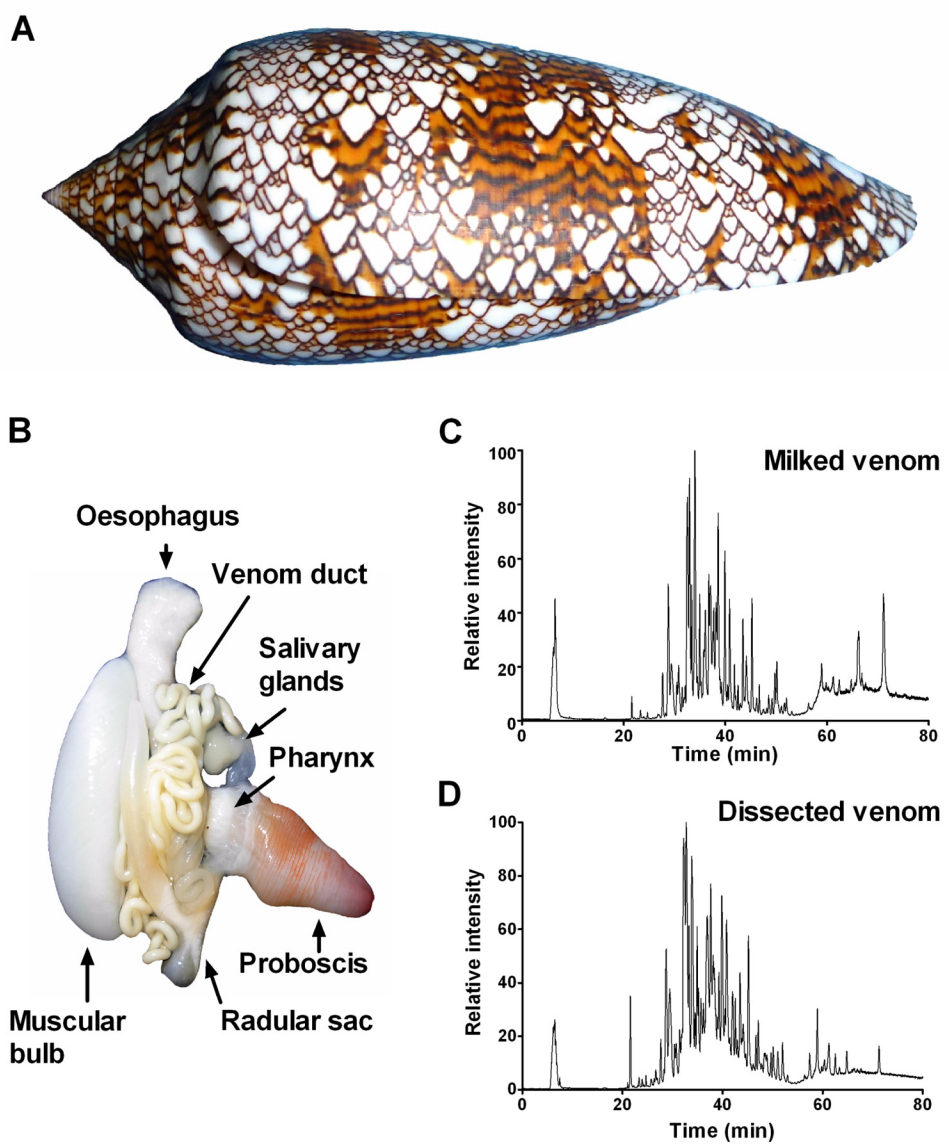

FIG. 1. The cone snail, venom apparatus, and venoms. A, shell of *C. textile*, a typical tented molluscivorous cone snail. B, dissected venom apparatus from *C. textile*, showing the long and convoluted venom duct, radular sac, proboscis, and salivary glands. C, high-performance liquid chromatography/mass spectrometry separation of venom peptides from the milked venom of *C. textile*. D, matching liquid chromatography/mass spectrometry of the venom duct from the same *C. textile*.

ically <5 kDa), relative ease of synthesis, structural stability and target specificity make them ideal pharmacological probes (Adams et al., 1999). This broadly evolved bioactivity provides a unique source of new research tools and potential therapeutic agents, with ω-MVIIA [ziconotide (Prialt; Azur Pharma Inc., Philadelphia, PA)] already approved for clinical use in the treatment of severe pain (Miljanich, 2001).

Somewhat surprisingly, many of these classes of conotoxins act on pain targets, allowing the specific dissection of key ion channels and receptors underlying pain and providing new ligands with potential as pain therapeutics (Fig. 2). Each species of cone snail produces in excess of 1000 conopeptides, with an estimated 5% overlap in conopeptides found between species (Davis et al., 2009) (see Fig. 1). At the moment, only ~0.1% of conopeptides have been characterized pharmacologically, yet many have already been identified with clinical potential, as highlighted in Table 2. The use of advanced transcriptomic/proteomic approaches combined with high throughput and multiplexed high content screens is expected to accelerate ligand and target discovery. One caveat is that many conotoxins will preferentially target prey species over related mammalian targets and are likely to be missed in screens relevant to human diseases. This review expands and updates the last comprehensive review of *Conus* spp. venoms in 2004 (Terlau and Olivera, 2004). Here we discuss the diversity, pharmacology, structure-activity relationships, and therapeutic potential of cone snail venom peptide families acting at voltage-gated ion channels (ω-, μ-, μO-, δ-, ι- and

TABLE 1

Superfamilies of conopeptides, occurrence in different Conus spp. clades and prey targets, and post-translational modifications (PTMs)

Data from Conoserver (http://www.conoserver.org).

Superfamily	Cysteine Framework	Family	Species	Clades	Diet	PTMs
A	I, II, IV, XIV	α, κ, ρ	43	E, I, II, III, V, VI, VII, X, XII, XIV, XV, XVII	V, M, P	O, *, γ, G, Z, Ys
D	XX	α	6	XII	V	O, γ
I1	VI/VII, XI	ι	11	E, I, V, X, XIV, XVII	V, M, P	O, BrW, *, D, γ
I2	XI, XII	κ	17	I, V, VI, X, XII, XIII, XVII	V, M, P	O, *, γ
I3	VI/VII, XI		3	XIV	V	
J	XIV	κ	2	IX	V	*
L	XIV	α	2	E	V, P	
M	III, IV, VI/VII, IX, XVI	α, ι, κ, μ	28	E, I, II, III, IV, V, VI, VII, X, XII, XIV, XVI, XVII	V, M, P	O, *, D
O1	VI/VII, XII	δ, γ, κ, μO, ω	53	E, I, II, III, V, VI, VII, IX, X, XI, XII, XIII, XIV, XV, XVI, XVII	V, M, P	O, BrW, *, γ
O2	VI/VII, XV	γ	15	I, V, VI, VII, X, XII, XIII, XIV, XVI	V, M, P	O, BrW, *, γ
O3	VI/VII		6	V, VII, XIV, XVI	V, M, P	
P	IX		6	V, X, XVII	V, M	*, γ
S	VIII	α, σ	6	I, II, V, XIV	V, M, P	O, BrW, *, γ
T	I, V, X, XVI	χ, ε, μ	24	E, II, III, V, VI, VII, X, XII, XIII, XIV, XVI, XVII	V, M, P	O, BrW, *, γ, G, Z
V	XV		2	XIII	V	*
Y	XVII		1	XIV	V	O

E, early clade; P, fish; M, molluscs; V, worms; *, C-terminal amidation; O, hydroproline; BrW, bromotryptophane; D, D-amino acid; γ, γ-carboxyglutamate; γ-Val, γ-hydroxyvaline; G, glycosylation; Z, pyroglutamate; Ys, sulfotyrosine.

κ-conotoxins), ligand-gated ion channels (α-conotoxins, σ-conotoxin, ikot ikot, and conantokins), GPCRs[1] (ρ-conopeptides, conopressins and contulakins), and neurotransmitter transporters (χ-conopeptides), and we discuss the potential of sodium and calcium channel inhibitors and α-conotoxins to modulate pain pathways.

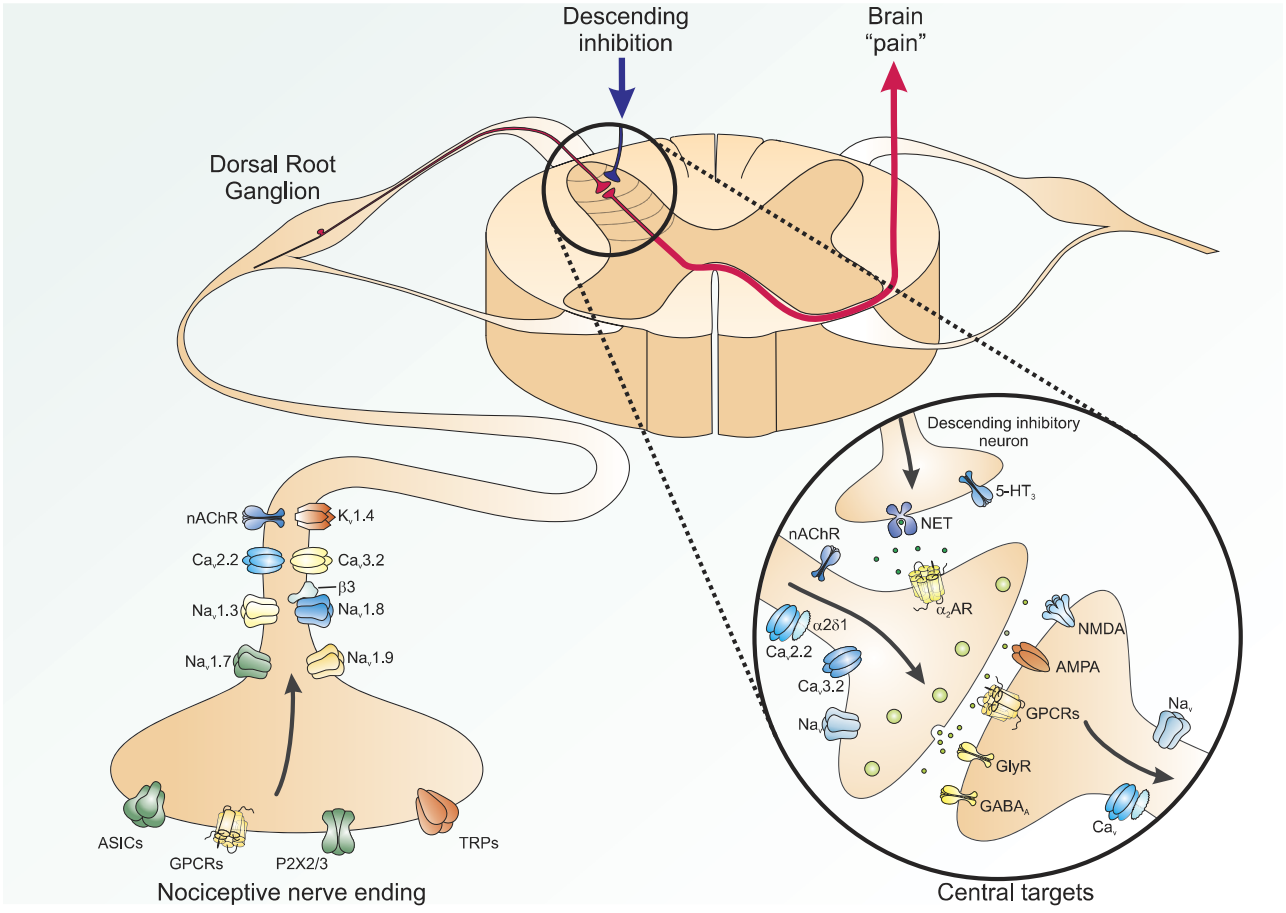

FIG. 2. Peripheral and spinal pain pathways amenable to conopeptide modulation. A number of selected targets on peripheral nociceptive terminals and afferent neurons as well as descending facilitatory and inhibitory pathways originating in the medulla are shown. Therapeutic targets with known conotoxin modulators are highlighted in blue, and those targeted by peptides derived from other species are highlighted in green. Targets with therapeutic potential but no known specific peptide modulators are shown in yellow, and targets that are modulated by venom peptides but in an undesirable manner (e.g., inhibition of $K_v1.4$ by κ-conotoxins and activation of TRPV1 by π/κ-theraphotoxin-Pc1a) are highlighted in orange. ASIC, acid-sensing ion channel; P2X2/3, purinoceptors 2X2 and 2X3; TRP, transient receptor potential channel; α2δ1, auxiliary Ca_v subunit; GlyR, glycine receptor.

TABLE 2
Clinical potential and representative sequences of major conotoxin classes defined by pharmacology
Cysteines involved in disulfide bonds (underlined) connect in discrete overlapping patterns depending on sequence (see Tables 4, 5, 7–9, and 11).

Class	Mode of Action	Name	Sequence	Clinical Potential
ω	$Ca_v2.2$ inhibitor	MVIIA	CKGKGAKCSRLMYDCCTGSCRSGKC*	Pain (intrathecal; phase IV)
μ	Na_v inhibitor	SIIIA	ZNCCNGGCSSKWCRDHARCC*	Pain (intravenous)
μO	$Na_v1.8$ inhibitor	MrVIB	ACSKKWEYCIVPIIGFIYCCPGLICGPFVCV	Pain (intrathecal/intravenous)
δ	Na_v enhancer	EVIA	DDCIKOYGFCSLPILKNGLCCSGACVGVCADL*	?
κ	K_v inhibitor	PVIIA	CRIONQKCFQHLDDCCSRKCNNRFNKCV	Cardiac reperfusion
χ	NET inhibitor	Xen2174	ZGVCCGYKLCHOC	Pain (intrathecal; phase II)
α	nAChR inhibitor	Vc1.1	GCCSDPRCNYDHPEIC*	Pain (intravenous)[a]
σ	$5HT_3$ receptor	GVIIIA	GCTRTCGGOKCTGTCTCTNSSKCGCRYNVHPSGBGCGCACS*	?
ρ	α_1-Adrenoceptor inhibitor	TIA	FNWRCCLIPACRRNHKKFC*	Cardiovascular/BPH
Conantokin	NMDA-R antagonist	Con-G	GEγγLQγNQγLIRγKSN	Pain/epilepsy (intrathecal)[a]
Conopressin	Vasopressin-R agonist	Cono-G	CFIRNCPKG*	Cardiovascular/mood
Contulakin	Neurotensin-R agonist	Cont-G	ZSEEGGSNAtKKPYIlL	Pain (intrathecal)[a]

*, C-terminal amidation.
[a] Clinical development suspended.

II. ω-Conotoxin Inhibitors of Voltage-Gated Calcium Channels

The Ca_v channels are assemblies of the various pore-forming α-subunits associated with single transmembrane $\alpha2\delta$-subunits and intracellular β-subunits (Catterall et al., 2005b, 2007) (Table 3). Calcium channel (Ca_v) inhibitors are used as antihypertensive, antiarrhythmic, and anticonvulsant agents and in chronic pain management, and other potential uses are in development (Triggle, 2007). The therapeutic potential of ω-conotoxins is based on their ability to selectively inhibit mammalian Ca_v isoforms (Table 4) expressed in ascending pain pathways (especially $Ca_v2.2$, which underlies the N-type current present in sensory neurons) and to produce antinociception in animal models of pain. Our understanding of the pharmacology of ω-conotoxins has arisen mostly from studies on GVIA, MVIIA, CVID, and MVIIC. Although these peptides are considered to function as simple pore blockers, their pharmacology is reminiscent of μO-conotoxins, because coexpression of the pore-forming α subunit with auxiliary β or

TABLE 3
Ca_v distribution, toxin inhibitors and potential effects of inhibition

Ca_v Isoform	Distribution	Selective Toxin Inhibitors	Effects/Side Effects of Inhibition	References
$Ca_v1.1$–1.4 (*L-type*) (CACNA1*S* or *C* or *D* or *F*)	Skeletal muscle (1.1), (T-tubules), Cardiac muscle (1.2–1.3), smooth muscle (1.2), CNS (1.1–1.3), DRG (1.2–1.4), retina (1.4) lymphocytes (1.2–1.4), GIT (1.2), cochlea (1.3).	None known (dihydropyridines)	Widespread effects on cardiovascular system, 1.2 implicated in neuropathic pain in spinal cord.	Kim et al., 2001; Catterall et al., 2005b, 2007; Fossat et al., 2010
$Ca_v2.1$ (*P/Q type*) (CACNA1*A*) splice variants	Brain, spinal cord, sympathetic neurons, DRG, endocrine cells, contribution to presynaptic neurotransmitter release at CNS, PNS, and neuromuscular junction.	ω-Conotoxins MVIIC ~ MVIID > CVIB > CVIC ≫ MVIIA > GVIA ≫ CVID Spider toxins: ω-agatoxin IVA	Mutations in several neurological disorders, blockers produce partial inhibition of synaptic transmission. Peripheral side effects expected from autonomic and neuromuscular block.	Bourinet et al., 1999; Nudler et al., 2003; Catterall et al., 2005b, 2007 (For selectivity see Table 4.)
$Ca_v2.2$ (CACNA1*B*) splice variants	Brain, spinal cord, sympathetic neurons, DRG, contribution to presynaptic neurotransmitter release in CNS and PNS.	ω-Conotoxins CVIE = CVID = GVIA ≫ CVIA ~ MVIIA > CVIB ~ CVIC	Blockers produce inhibition of synaptic transmission throughout the nervous system.	Catterall et al., 2005b, 2007 (For selectivity see Table 4.)
$Ca_v2.3$ (CACNA1*E*) splice variants	Brain, spinal cord, sympathetic neurons, DRG, minor role in presynaptic neurotransmitter release in CNS and PNS, synaptic plasticity.	No conotoxins Spider: SNX-482	Blockers modulate synaptic plasticity at some brain synapses.	Murakami et al., 2004; Catterall et al., 2005b, 2007; Matthews et al., 2007 (For selectivity see Table 4.)
$Ca_v3.1$ (CACNA1*G*)	Brain neurons localized to soma and dendrites. High expression in cerebellum and thalamus. Modulates action potential firing. Ovary, placenta, heart.	No conotoxins (pimozide, mibefradil, TTA-P2)	Small-molecule modulators may be useful for some CNS neurological disorders.	Catterall et al., 2005b, 2007; Yaksh, 2006; Triggle, 2007; Zamponi et al., 2009
$Ca_v3.2$ (CACNA1*H*)	CNS, DRG neurons: localized to soma and dendrites. Modulates action potential firing. Also heart, liver, kidney, lung, skeletal muscle, pancreas.	No conotoxins Scorpion: kurtoxin (pimozide, mibefradil, Z123212,[a] TTA-P2)	Pain modulation. Relaxation of coronary arteries, potential side effects from actions in other tissues.	Catterall et al., 2005b, 2007; Yaksh, 2006; Triggle, 2007; Zamponi et al., 2009; Choe et al., 2011
$Ca_v3.3$ (CACNA1*I*)	CNS neurons: localized to soma and dendrites. Modulates action potential firing.	No conotoxins (pimozide, TTA-P2, mibefradil very weak)	Presumably many side effects.	Catterall et al., 2005b, 2007; Yaksh, 2006; Triggle, 2007; Zamponi et al., 2009

[a]Z123212 (Hildebrand et al., 2011) also targets sodium channels.
PNS, peripheral nervous system; TTA-P2, 3,5-dichloro-*N*-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide.

TABLE 4

*ω-Conotoxins targeting Ca_v calcium channels (loop formula **C6C5–9CC2–4C3–6C**)*

Residues identified as being significant for affinity are underlined.

Species	Name	Diet	Sequence Disulfide Bonded (1–4/2–5/3–6) 1 2 34 5 6	Ca_v Selectivity	References
C. consors	CnVIIA	P	---**C**KGKGAO**C**TRLMYD---**CC**-HGS**C**SSSKGR**C***	2.2 > 2.1	Favreau et al., 2001
C. catus	CVIA	P	---**C**KSTGAS**C**RRTSYD---**CC**-TGS**C**RS--GR**C***	2.2 > 12.	Lewis et al., 2000
	CVIB	P	---**C**KGKGAS**C**RKTMYD---**CC**-RGS**C**RS--GR**C***	2.2 ~ 2.1 > 2.3	Lewis et al., 2000
	CVIC	P	---**C**KGKGQS**C**SKLMYD---**CC**-TGS**C**-SRRGK**C***	2.1 ~ 2.2	Lewis et al., 2000
	CVID	P	---**C**KSKGAK**C**SKLMYD---**CC**-SGS**C**SGTVGR**C***	2.2 > 2.1	Lewis et al., 2000
	CVIE-2**	P	---**C**KGKGAS**C**RRTSYD---**CC**-TGS**C**RS--LR**C***	2.2 > 2.1 > 1.2 ~ 1.3 ~ 2.3	Berecki et al., 2010
	CVIF	P	---**C**KGKGAS**C**RRTSYD---**CC**-TGS**C**RS--GR**C***	2.2 > 2.1 > 1.2 ~ 1.3 ~ 2.3	Berecki et al., 2010
C. fulmen	FVIA	P	---**C**KGTGKS**C**SRIAYN---**CC**-TGS**C**RS--GK**C***	2.2 > 2.1 > 3.2	Lee et al., 2010
C. geographus	GVIA	P	---**C**KSOGSS**C**SOTSYN---**CC**--RS**C**NOYTKR**C**Y*	2.2 > 2.1	Olivera et al., 1984; Sato et al., 1993; Kim et al., 1994; Kim et al., 1995
	GVIIA	P	---**C**KSOGTO**C**SRGMRD---**CC**--TS**C**LLYSNK**C**RRY	Mouse ~ fish > frog	Olivera et al., 1985; Abe and Saisu, 1987
	GVIIB	P	---**C**KSOGTO**C**SRGMRD---**CC**--TS**C**LSYSNK**C**RRY	Mouse ~ fish > frog	Olivera et al., 1985; Abe and Saisu, 1987
C. magus	MVIIA	P	---**C**KGKGAK**C**SRLMYD---**CC**-TGS**C**RS--GK**C***	2.2 > 2.1	Olivera et al., 1987; Kim et al., 1995; Nielsen et al., 1999a
	MVIIB	P	---**C**KGKGAS**C**HRTSYD---**CC**-TGS**C**N--RGK**C**	N.D.	Olivera et al., 1987
	MVIIC	P	---**C**KGKGAP**C**RKTMYD---**CC**-SGS**C**-GRRGK**C***	2.1 > 2.2	Hillyard et al., 1992; Nielsen et al., 1999a
	MVIID	P	---**C**QGRGAS**C**RKTMYN---**CC**-SGS**C**N--RGR**C***	2.1 ≫ 2.2	Haack et al., 1993; Monje et al., 1993; Gandía et al., 1997
C. pennaceus	PnVIA	M	--G**C**LEVDYF**C**GIPFANNGL**CC**-SGN**C**VFV---**C**TPQ	Molluscan N-type	Kits et al., 1996
	PnVIB	M	DDD**C**EPPGNF**C**GMIKIGPP-**CC**-SGW**C**FFA---**C**A	Molluscan N-type	Kits et al., 1996
C. pulicarius	PuIA	V	-RD**C**RPVGQY**C**GIPYEHNWR**CC**-SQL**C**AII---**C**VS	N.D.	Zhao and Huang, 2000
	PuIIA	V	--T**C**NTPTQY**C**TLHRH----**CC**-SLY**C**HKTIHA**C**A	N.D.	Zhao and Huang, 2000
C. radiatus	RVIA	P	---**C**KPOGSO**C**RVSSYN---**CC**--SS**C**KSYNKK**C**G	2.2	Abbott and Litzinger, 1994
C. striatus	SO3	P	---**C**KAAGKP**C**SRIAYN---**CC**-TGS**C**RS--GK**C***	Na_v ~ K_v ~ 2.2 > 2.1 > 2.3	Lu et al., 1999; Li et al., 2003b; Wen et al., 2005
	SO4	P	ATD**C**IEAGNY**C**GPTVMKI--**CC**--GF**C**SPYSKI**C**MNYPKN	N.D.	Lu et al., 1999
	SO5	P	STS**C**MEAGSY**C**GSTTRI---**CC**--GY**C**AYFGKK**C**IDYPSN	N.D.	Lu et al., 1999
	SVIA	P	---**C**RSSGSO**C**GVTSI----**CC**-GR-**C**-Y-RGK**C**T*	2.2 > 2.1	Ramilo et al., 1992
	SVIB	P	---**C**KLKGQS**C**RKTSYD---**CC**-SGS**C**GRS-GK**C***	2.1 > 2.2	Ramilo et al., 1992; Woppmann et al., 1994; Nadasdi et al., 1995
C. tulipa	TVIA	P	---**C**LSOGSS**C**SOTSYN---**CC**--RS**C**NOYSRK**C**	2.2 > 2.1	Chung et al., 1995; Wang et al., 1998
C. textile	TxO1	M	---**C**LDAGEV**C**DIFFPT---**CC**--GY**C**ILLF--**C**A	N.D.	Lu et al., 1999
	TxO2	M	---**C**YDSGTS**C**NTGNQ----**CC**-SGW**C**IFV---**C**L	N.D.	Lu et al., 1999
	TxO3	M	---**C**YDGGTS**C**DSGIQ----**CC**-SGW**C**IFV---**C**F	N.D.	Lu et al., 1999
	TxO4	M	-YD**C**EPPGNF**C**GMIKIGPP-**CC**-SGW**C**FFA---**C**A	N.D.	Lu et al., 1999
	TxO5	M	---**C**VPYEGP**C**NWLTQN---**CC**-DAT**C**VVFW--**C**L	N.D.	Lu et al., 1999
	TxO6	M	-NY**C**QEKWDY**C**PVPFLGSRY**CC**DGLF**C**TLFF--**C**A	N.D.	Lu et al., 1999
	TxVII	M	---**C**KQADEP**C**DVFSLD---**CC**-TGI**C**LGV---**C**MW	Molluscan L-type	Fainzilber et al., 1996

P, fish; M, molluscs; V, worms; *, C-terminal amidation; N.D., not determined.

α2δ subunits can significantly alter their channel affinity (Lewis et al., 2000; Mould et al., 2004; Motin et al., 2007). Such differences may have particular relevance in disease states such as pain, where α2δ subunits, particularly α2δ1, are up-regulated (see section II.C). ω-Conotoxins remain the only class of conotoxins with FDA approval to date, the registration of MVIIA (ziconotide) for the treatment of intractable pain having been approved in 2004. Unfortunately, dose-limiting neurological side effects have limited the clinical applications for ω-conotoxins.

A. Subtype Selectivity

ω-Conotoxins with defined activity at mammalian Ca_v isoforms have so far been isolated only from piscivorous cone snails, where they are likely to have evolved as part of the motor cabal (Olivera et al., 1985). Based on the behav-

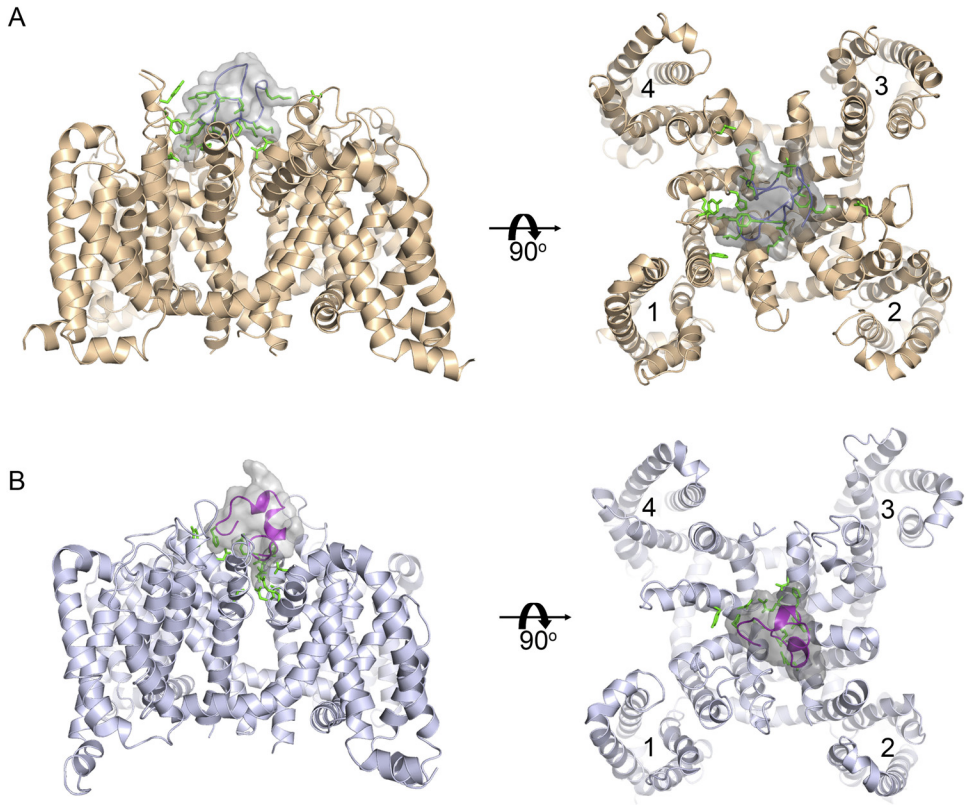

FIG. 3. Docking of conotoxins to homology models of sodium and calcium channels. A, ω-MVIIA (dark gray) docked to a homology model of $Ca_v2.2$ (light brown) built from a bacterial sodium channel crystal structure (Payandeh et al., 2011). B, μ-TIIIA (magenta) docked to a homology model of $Na_v1.4$ (light blue) built from the same bacterial sodium channel (Payandeh et al., 2011). Identified likely interacting residues are shown in sticks (green), and the order of subunits is labeled numerically. The highly flexible, unstructured loops (approximately 50~100 amino acids long) between transmembrane helices 5 and P1 in subunits 1 and 3 of both $Ca_v2.2$ and $Na_v1.4$ were removed for clarity. The docking simulation outcomes reveal large contact areas and extensive ionic interactions networks between conotoxins and all four subunits of voltage-gated ion channels that probably explain their high binding affinities.

ioral phenotype elicited after intracerebral injection in mice they were termed "shaker" peptides. It is now appreciated that ω-conotoxins represent some of the most selective known inhibitors for the neuronal Ca_v isoforms 2.2 and 2.1. Specifically, ω-conotoxins CVID, CVIE, CVIF, GVIA, and MVIIA are particularly selective for $Ca_v2.2$, whereas MVIIC and MVIID show a preference for $Ca_v2.1$ underlying P/Q-type calcium currents (Monje et al., 1993; Woppmann et al., 1994; Nadasdi et al., 1995; Nielsen et al., 1996; Flinn et al., 1999; Lewis et al., 2000; Berecki et al., 2010). The binding site of ω-conotoxins to the Ca_v subtypes has been mapped primarily to the external vestibule of the channel in the domain III S5–S6 region (Ellinor et al., 1994), consistent with preliminary results obtained from docking of ω-MVIIA to a homology model of $Ca_v2.2$ (Fig. 3A) built from the recently reported crystal structure of a bacterial sodium channel (Payandeh et al., 2011). Residues immediately outside this region seem to also contribute to $Ca_v2.2$ inhibition by GVIA, most notably Gly1326, which contributes to the reversibility of ω-conotoxin block (Feng et al., 2001). Intracellular domains (Kaneko et al., 2002; McDonough et al., 2002) are also reported to modulate binding kinetics and ω-conotoxin affinity for $Ca_v2.2$ and, in addition, the presence of subunits seems to not only modify Ca_v gating characteristics and kinetics but also the interaction of ω-conotoxins with the channel.

The high-affinity block of $Ca_v2.2$ by CVID was found to be reversible in heterologous systems expressing only the pore-forming α-subunit, but irreversible in DRG neurons, an effect that is mimicked in expression systems where the α-subunit is coexpressed with auxiliary β3 and α2δ1 subunits (Lewis et al., 2000; Mould et al., 2004; Motin et al., 2007). Likewise, inhibition of $Ca_v2.2$ by CVIE and CVIF was found to be virtually irreversible in the presence of β3 subunits but reversible in systems coexpressing the β2 subunit (Berecki et al., 2010). Intriguingly, these effects parallel modification of channel characteristics by auxiliary subunits, suggesting that altered block characteristics of ω-conotoxins in the presence of auxiliary subunits reflect altered channel kinetics. In addition to altered binding kinetics, many ω-conotoxins also have lower affinity for $Ca_v2.2$ in the presence of auxiliary subunits, especially $Ca_v\alpha2\delta1$ (Lewis et al., 2000; Berecki et al., 2010). However, the precise mechanisms contributing to these intriguing characteristics of ω-conotoxins, as well as the effect of these pharmacological differences on the therapeutic potential of these peptides, remain to be elucidated.

Although the vast majority of ω-conotoxins isolated to date are selective, to varying degrees, for mammalian

$Ca_v2.1$ and $Ca_v2.2$, only TxVII, isolated from the molluscivorous *Conus textile*, blocks molluscan dihydropyridine-sensitive channels, which are homologous to mammalian L-type voltage-gated calcium channels (Fainzilber et al., 1996). However, this ω-conotoxin seems to display phylic selectivity, as TxVII was found to be inactive at rat L-type channels expressed in PC12 cells (Fainzilber et al., 1996; Sasaki et al., 1999). Likewise, PnVIA and PnVIB were isolated from the molluscivorous *Conus pennaceus*, but in contrast to TxVII, they inhibit the molluscan dihydropyridine-insensitive high-voltage activated Ca^{2+} current (Kits et al., 1996). Not surprisingly, the sequences of these related peptides are distinct from conotoxins from piscivorous cone snails targeting mammalian channels. Indeed, molluscan channels are usually insensitive to GVIA, suggesting distinct phylum-selective pharmacologies resulting from these divergent sequences. Accordingly, ω-conotoxins from molluscivorous cone snails seem to represent a distinct subclass of ω-conotoxins with specificity for molluscan calcium channels.

Other structurally diverse ω-conotoxins include PuIA and PuIIA from the worm-hunting *Conus pulicarius*, as well as several peptides identified from the molluscivorous *C. textile* and piscivorous *Conus striatus*. The precise pharmacological target and subtype selectivity of these conotoxins remains to be determined; however, their homology to the functionally promiscuous SO3, which also inhibits sodium and potassium channels in hippocampal neurons (Li et al., 2003b; Wen et al., 2005), suggests that their pharmacological classification as ω-conotoxins may be premature.

B. Structure-Activity Relationships

The pharmacological activity of ω-conotoxins has been attributed primarily to Tyr13 and Lys2, both of which are highly conserved in ω-conotoxins derived from piscivorous cone snails (Table 4) and are proposed to form a two-point pharmacophore (Sato et al., 1993; Kim et al., 1994; Lew et al., 1997; Flinn et al., 1999) (Fig. 4). The hydroxyl group of Tyr13 in particular seems critical for high-affinity binding of ω-conotoxins to $Ca_v2.2$, although the aromatic moiety also seems to contribute to binding (Kim et al., 1994; Flinn et al., 1999). The positive charge of the second most important residue, Lys2, which is conserved in all ω-conotoxins except MVIID and TVIA, also contributes significantly to activity (Sato et al., 1993; Lew et al., 1997; Flinn et al., 1999). Detailed structure-activity relationship studies in GVIA showed that the precise location of this positive charge is less important, both lengthening and shortening of the side chain being tolerated. However, bulkier substitutions in this position greatly reduced activity (Flinn et al., 1999).

In addition, several other residues in loop 2 and 4 also contribute to varying degrees to ω-conotoxin affinity and selectivity (Gandía et al., 1997; Lew et al., 1997; Nielsen et al., 1999a). Loop 4 residues with a significant effect on ω-conotoxin affinity include Arg21 in MVIIA and Arg17,

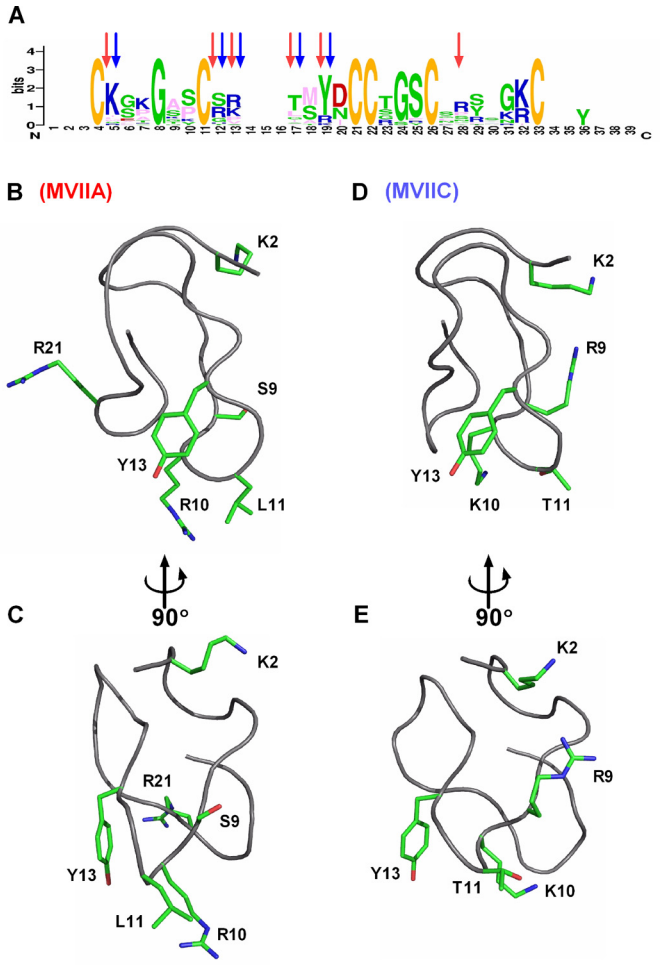

FIG. 4. Structure activity relationship of ω-conotoxins. A, consensus sequence for ω-conotoxins. B and C, structure of ω-MVIIA showing important residues for interaction with the N-type calcium channel. D and E, structure of ω-MVIIC showing important residues for interaction with the P-type calcium channel.

Tyr22, and Lys24 in GVIA, whereas the residue at position 10 in loop 2 seems to play a key role in determining subtype selectivity (Nielsen et al., 1999a). Biotinylation of Lys10 in the P/Q-selective MVIID significantly decreased binding affinity (Haack et al., 1993), and the importance of this residue for Ca_v subtype selectivity was also confirmed by several independent studies (Nadasdi et al., 1995; Nielsen et al., 1996). The residue at position 10 is consistently a lysine in ω-conotoxins with high affinity for $Ca_v2.1$, but often an arginine or hydroxyproline in $Ca_v2.2$-selective peptides (Nadasdi et al., 1995; Nielsen et al., 1996). However, it remains to be determined if the subtype selectivity of $Ca_v2.1$-selective MVIIC and MVIID are significantly altered by a K10R swap. It is noteworthy that an arginine in position 10 was reported to reduce recovery from $Ca_v2.2$ block for both MVIIA and CVID, suggesting that the effect of this residue on subtype selectivity could be due to altered recovery characteristics (Mould et al., 2004).

Although the pharmacophore of ω-conotoxins responsible for affinity and subtype selectivity at Ca_vs is becoming

increasingly clear, residues responsible for any splice variant selectivity of ω-conotoxins have not been determined to date. Nonetheless, our understanding of these structure-activity relationships are being applied to the design of small molecule, orally active ω-conotoxin peptidomimetics with the hope of engineering novel analgesic molecules based on the unique pharmacological properties of ω-conotoxins (Schroeder et al., 2004; Andersson et al., 2009).

C. Calcium Channel Inhibition by Conotoxins in Pain Management

There is strong evidence that $Ca_v2.2$ channels on nociceptive primary afferent nerves are an target for pain management. Some of the most effective nonopioid analgesics for chronic pain management, gabapentin and pregabalin, interact with Ca_v channel $\alpha2\delta$ subunits, which is thought to disrupt trafficking of $Ca_v2.2$ channels (Bauer et al., 2010). Indeed, in the spinal cord, opioids act predominantly on $Ca_v2.2$ channels via μ-opioid receptors expressed on nociceptive primary afferent nerve terminals to produce presynaptic inhibition and analgesia (Heinke et al., 2011). Many ω-conotoxins exhibit high potency and selectivity for different subtypes of voltage-gated calcium channels (Olivera et al., 1994). The development of the N-type ($Ca_v2.2$) antagonist, ω-conotoxin MVIIA (SNX-111; ziconotide), as an intrathecal analgesic to manage moderate to severe chronic pain, provided the first validation of the use of ω-conotoxins and established $Ca_v2.2$ as new target for pain therapeutics (Lewis and Garcia, 2003; Winquist et al., 2005). Although ziconotide is potent and does not induce addiction or tolerance as opioids do (Malmberg and Yaksh, 1995; Bowersox et al., 1996), its use is limited because of the need for intrathecal administration combined with significant dose-limiting neurological side effects (Penn and Paice, 2000). This side-effect profile likely arises, at least in part, from the spread of peptide from the intrathecal space to higher (supraspinal) brain centers including cerebellum and the dependence of a significant component of synaptic neurotransmission on $Ca_v2.2$ at many CNS synapses, including inhibitory spinal synapses (Penn and Paice, 2000; Schmidtko et al., 2010). However, as discussed below, other properties of ω-conotoxins could additionally contribute to their side-effect profile and therapeutic index. The distribution of Ca_v channel types (Catterall et al., 2005b, 2007), potential actions of inhibitors, as well as selective conotoxins and other inhibitors are summarized in Table 3. The role of Ca_v1 to 3 and $Ca_v\alpha2\delta$ in pain pathways and their potential for selective targeting by conotoxins is outlined below.

The Ca_v1 channel α-subunits encode L-type channels but evidence for their involvement in pain states is still very limited (Yaksh, 2006). L-type calcium channel subtypes are expressed in sensory neurons and dorsal horn of the spinal cord, where they could potentially modulate the development of neuropathic pain (Kim et al., 2001). Intrathecal L-type channel antagonists have mixed effects in pain models (see Yaksh, 2006), possibly because nonselective Ca_v1 inhibitors have little effect on afferent substance P release (Takasusuki and Yaksh, 2011) or the role of Ca_v1 changes in different models of pain. More recently, antisense knockdown and microRNA regulation of the $Ca_v1.2$ in spinal cord has been shown to reverse the development of mechanical signs of neuropathic pain, hyperexcitability of deep dorsal horn neurons, phosphorylation of cAMP response element-binding protein, and induction of cyclooxygenase-2 mRNA in the spinal cord (Fossat et al., 2010), consistent with $Ca_v1.2$ expression predominantly in neuronal somata and dendrites and its role in gene regulation and plasticity (Murakami et al., 2004; Zhang et al., 2006a). These findings suggest that specific peptide inhibitors of $Ca_v1.2$ may be useful for management and perhaps reversal of central adaptations responsible for maintenance of neuropathic pain when applied directly to the dorsal spinal cord.

$Ca_v2.1$ encodes the α-subunit of high-voltage activated P/Q-type calcium channels, with alternative splicing generating the P and Q phenotypic variants (Bourinet et al., 1999). Together with $Ca_v2.2$ and $Ca_v2.3$, P/Q-type channels contribute to the calcium influx, triggering synaptic neurotransmission at many synapses including nociceptive synapses (Heinke et al., 2004; Rycroft et al., 2007) and thus may be considered as a target for potential pain therapeutics (e.g., Vanegas and Schaible, 2000; Yaksh, 2006). However, P/Q channels are more densely expressed in non-nociceptive than in nociceptive sensory neurons and are the major Ca_v channels mediating synaptic neurotransmission at neuromuscular synapses (Uchitel et al., 1992; Westenbroek et al., 1998; Nudler et al., 2003). P/Q channel inhibitors would therefore be expected to produce neuromuscular and non-nociceptive sensory side effects after systemic or intrathecal administration. This might suggest a greater role in sensory modalities other than nociception. Indeed, intrathecal administration of the highly selective $Ca_v2.1$ inhibitor, agatoxin IVA, in a variety of pain models has produced mixed results (Malmberg and Yaksh, 1994; Vanegas and Schaible, 2000; Yaksh, 2006).

The most promising calcium channel regulating pain pathways is arguably $Ca_v2.2$, which encodes the α-subunit of high voltage activated *N*-type calcium channels. $Ca_v2.2$ inhibitors have been developed as pain therapeutics, because $Ca_v2.2$ is the predominant channel supporting nociceptive primary afferent neurotransmission in the dorsal horn of the spinal cord (Fig. 2). Although $Ca_v2.2$ is sometimes considered the only calcium channel mediating synaptic transmission at nociceptive synapses, other Ca_v channels, including P/Q-type, R-type, and to a small extent L-type, contribute ~50% of neurotransmitter release at nociceptive synapses (Heinke et al., 2004; Rycroft et al., 2007). Among a diverse group of ω-conotoxins, MVIIA was established to be highly efficacious after intrathecal administration in a range of animal pain models (Malmberg and Yaksh, 1994; Scott et al., 2002; Yaksh, 2006), although severe behavioral side effects of apparent neurological origin were observed within the effective dose-range for pain

relief (Smith et al., 2002). In humans, optimal use of ziconotide (MVIIA) requires slow and careful titration of dose and rate of intrathecal infusion. The adverse effects of ziconotide on the central nervous system include dizziness, nystagmus, confusion, abnormal gait, somnolence, speech difficulties, amblyopia, ataxia, amnesia, and abnormal thought processes (Penn and Paice, 2000; Schmidtko et al., 2010). Small-molecule $Ca_v2.2$ inhibitors may suffer from similar side-effect issues in addition to the peripheral side effects observed after intravenous administration of ziconotide. These include orthostatic hypotension, dizziness, sinus bradycardia, rhinitis, and nausea (McGuire et al., 1997). Preclinical testing of other ω-conotoxins suggest improvements are possible. For example, CVID (AM336, CNSB004, leconotide) has potency to inhibit $Ca_v2.2$ channels and relieve neuropathic pain signs in animal models comparable to that of MVIIA but has been reported to exhibit a better (approximately 5-fold) therapeutic index for analgesia versus side effects (Scott et al., 2002; Smith et al., 2002). Some of the potential reasons for improved therapeutic index and potential applications of novel ω-conotoxins are explored below.

Although CVID has similar potency at $Ca_v2.2$ as MVIIA, it is approximately 100-fold more selective for N-type than P/Q type channels (Lewis et al., 2000). This has been suggested as a reason for its improved side effect profile both in the CNS and periphery (Smith et al., 2002; Kolosov et al., 2010). However, MVIIA is already nearly 10,000-fold selective for N-type versus P/Q-type channels based on radioligand-binding studies using rat brain membrane (Lewis et al., 2000), so this might not be the explanation for better selectivity of CVID versus MVIIA and GVIA. Further systematic investigation of the relationship between N and P/Q selectivity and therapeutic index will be required to determine whether this is important.

The target residence time of a drug can strongly influence its therapeutic index (Copeland et al., 2006). The rate of reversal of inhibition of $Ca_v2.2$ by ω-conotoxins certainly influences the extent to which side effects can be managed clinically (Penn and Paice, 2000). Many ω-conotoxins, including MVIIA and GVIA, seem to bind nearly irreversibly to the N-type channel. However, reversibility can be induced by hyperpolarizations to potentials more negative than the minimum of the steady-state inactivation curve; i.e., ω-conotoxins bind with higher affinity to the inactivated state of the N-channel than the resting state (Stocker et al., 1997). Such voltage-dependent recovery from block varies for different ω-conotoxins and is strongly influenced for some by the α2δ- and β-subunits, in particular α2δ1, β2a, and β3, coexpressed with the α-subunit (Mould et al., 2004; Berecki et al., 2010). Reversibility of GVIA and MVIIA is only weakly influenced by α2δ1- and β2a- or β3-subunit coexpression, and both appear to have a narrower therapeutic index than for CVID, which is strongly influenced by the presence of these subunits (Mould et al., 2004; Berecki et al., 2010). Two more recently identified ω-conotoxins from *Conus catus*, CVIE and CVIF, show greater voltage-dependent reversal in the presence of α2δ1- and β-subunits than CVID (Berecki et al., 2010). The implications of this property for modulation of primary afferent nociceptive neurotransmission will remain uncertain until the therapeutic indices of these novel ω-conotoxins are determined. The importance of this property in nerve terminals is also uncertain because $Ca_v2.2$ channels in some nerve terminals seem to be resistant to voltage-dependent inactivation (Chan et al., 2007). Nonetheless, it is of interest that GVIA and MVIIA apparently irreversibly inhibit primary afferent synaptic neurotransmission, whereas CVID is partly reversible. CVIE and CVIF are fully reversible after removal of the peptides but show similar efficacy to CVID in pain models (Motin et al., 2007; Berecki et al., 2010). If the therapeutic indices for CVIE and CVIF were significantly better than for both CVID and MVIIA, it would suggest that reversibility of block is an important factor in therapeutic index. A novel ω-conotoxin, FVIA from *Conus fulmen*, may be useful because it is fully reversible within the normal steady-state inactivation range of $Ca_v2.2$ (−80 mV). However, its voltage- and α2δ-/β-subunit dependence and therapeutic index have not been determined (Lee et al., 2010).

$Ca_vα2δ$ subunits also strongly influence the potency of some ω-conotoxins but not others, independently of β-subunit coexpression (Mould et al., 2004; Motin et al., 2007; Motin and Adams, 2008; Berecki et al., 2010). The potency (with coexpression of β-subunits) of MVIIA, CVID, CVIF, and MVIIA are reduced approximately 100-fold by coexpression of α2δ1-subunits in oocytes, whereas the potency of CVIE is only reduced approximately 10-fold (Mould et al., 2004; Berecki et al., 2010). This may be important because α2δ1-subunit expression is profoundly upregulated in the central terminals of primary afferent nerves in neuropathic pain (Bauer et al., 2010) so that the potency of some ω-conotoxins in pain states might be strongly modulated. Consistent with such an effect, CVID and MVIIA at maximal effect doses are less effective at nociceptive primary afferent synapses in a rat inflammatory pain model (Rycroft et al., 2007). Other conotoxins and neuropathic models remain to be examined at this level.

The highly specific binding of ω-conotoxins to $Ca_v2.2$ suggests that specific interaction with splice variants may be possible. The $Ca_v2.2$ gene contains several alternative splicing sites (Lipscombe et al., 2002). If these are differentially expressed in normal nociceptive nerves or in pain states more selective ω-conotoxins may be developed to target them. The best studied e37a and e37b variants underlie expression of two mutually exclusive isoforms of N-type channels. The e37a isoform is enriched in nociceptive sensory neurons, undergoes distinct modulation by both G-protein βγ-subunits and tyrosine phosphorylation, and influences N-type calcium current density (Bell et al., 2004; Raingo et al., 2007). The e37a isoform is selectively involved in nociceptive responses in pain models (Altier et al., 2007) and confers novel pharmacological responses to opioids (Andrade et al., 2010). Because the splice site en-

codes a distinct pair of 32 amino acid sequences in the intracellular, C-terminal region of the channel, the prospects for developing selective ω-conotoxins for these isoforms may be limited given these peptides bind extracellularly (see Fig. 3A). The function and tissue distribution of other splice variants have been less extensively studied. A long C-terminal splice variant of $Ca_v2.2$ has been identified in presynaptic membranes (Gardezi et al., 2010) and other variants lacking a synaptic protein interaction site within the intracellular SII-SIII linker domain are presumably not targeted to synaptic nerve terminals (Kaneko et al., 2002). It is noteworthy that the Δ1 variant (SII-SIII domain truncation) was less sensitive to inhibition by both MVIIA and GVIA (Kaneko et al., 2002), suggesting that it may be possible to selectively target ω-conotoxins to $Ca_v2.2$ splice variants, even when sequence variations are within intracellular domains.

The efficacy of ω-conotoxins in pain management is generally considered to be due exclusively to inhibition of N-type channels at primary afferent synapses in the dorsal horn of the spinal cord. Until recently, the possibility that pain relief could be achieved with systemic administration was not considered because MVIIA produces serious systemic cardiovascular and other side effects mediated by the peripheral nervous system (McGuire et al., 1997). However, in conscious rabbits and isolated, sympathetically innervated tissues, CVID was much less potent than MVIIA at producing sympathetic and cardiovascular effects (Wright et al., 2000), suggesting it may be safer after systemic administration. Indeed, intravenous CVID has recently been shown to produce modest antihyperalgesia in pain models at doses producing no cardiovascular effects (Kolosov et al., 2010) and synergizes strongly with other analgesics, including morphine (Kolosov et al., 2010; Kolosov et al., 2011). The reasons for the greater therapeutic index of systemic CVID are not known. It could be due to greater N- versus P/Q-channel selectivity (but see selectivity section above) or to influences of auxiliary subunits expressed selectively in sympathetic neurons (Kolosov et al., 2010). Using GVIA and CVID to probe N-channels, different contributions of N-channels to vasoconstrictor versus vasodilator neurons have been noted (Jobling et al., 2004; Morris et al., 2004) but sensitivities to conotoxins differently influenced by auxiliary subunit expression, such as CVD, CVIE, and CVIF, have not been examined. Resolution of differential effects in relevant sympathetic nerve terminal preparations may produce a more rational approach to the development of safe, systemically active ω-conotoxin pain therapeutics.

In contrast to $Ca_v2.2$, $Ca_v2.3$ has not been extensively explored in pain models and no highly selective ω-conotoxins targeting this channel are known. R-type channels do contribute to nociceptive primary afferent synaptic transmission (Heinke et al., 2004; Rycroft et al., 2007), and the selective $Ca_v2.3$ inhibitor SNX-482 from the tarantula, *Hysterocrates gigas*, has been shown to be antinociceptive after intrathecal administration (Murakami et al., 2004) and to inhibit nociceptive neural responses in the spinal cord dorsal horn in vivo in a neuropathic pain model (Matthews et al., 2007).

Conotoxins acting at other calcium channel types, notably Ca_v3 subtypes, could also be developed as pain therapeutics (Yaksh, 2006; Triggle, 2007; Zamponi et al., 2009). Ca_v3 α-subunits encode the family of low-voltage-activated calcium channel currents that modulate action potential burst firing in CNS neurons and contribute to Ca^{2+}-mediated plasticity. $Ca_v3.2$ is the predominant type expressed in nociceptive and mechanosensitive sensory neurons (see Todorovic and Jevtovic-Todorovic, 2011). In neuropathic pain models, T-type channel current density is up-regulated, and this contributes to sensory neuron hyperexcitability (Jagodic et al., 2007, 2008). T-type channels are also expressed by neurons in the dorsal horn of the spinal cord (see Todorovic and Jevtovic-Todorovic, 2011) and have been shown to be crucial for induction of plasticity at nociceptive primary afferent synapses that could contribute to persistent pain (Ikeda et al., 2003, 2006). Consistent with this role, knockdown of $Ca_v3.2$ in sensory neurons reduced signs of thermal and mechanical pain in neuropathic pain models (Bourinet et al., 2005; Messinger et al., 2009). Nonselective, small-molecule T-type channel inhibitors also reduce neuropathic pain signs in animal models, and antisense knockdown experiments in sensory neurons have confirmed that this effect is mediated by $Ca_v3.2$ (Dogrul et al., 2003; Choi et al., 2007; Choe et al., 2011). Thus, evidence implicating $Ca_v3.2$ in sensory neuron hyperexcitability in neuropathic pain suggests that peripherally administered conotoxins selectively targeting $Ca_v3.2$, if they can be identified, may be useful therapeutics.

II. Conotoxins Interacting with Voltage-Gated Sodium Channels

To date, four classes of conotoxins targeting Na_v channels (μ, μO, δ, and ι conotoxins) have been isolated from cone snail venom (Cruz et al., 1985; Fainzilber et al., 1994b; McIntosh et al., 1995; Fiedler et al., 2008). Although these venom peptides exhibit activity at the same pharmacological targets, they are structurally distinct and vary in their mechanism of action, with two families producing inhibition (μ- and μO-conotoxins) and two families activation (δ- or ι-conotoxins) of Na_v channels (Table 5). The μ-conotoxins elicit Na_v inhibition through direct pore block overlapping with tetrodotoxin at site 1, consistent with preliminary docking of A15-TIIIA to a homology model of $Na_v1.4$ built from the crystal structure of a bacterial sodium channel (Fig. 3B). In contrast, μO-conotoxins seem to interfere with the voltage sensors in domain II of Na_v to restrict channel opening, δ-conotoxins cause a delay in Na_v inactivation by binding to site 6 resulting in action potential prolongation and persistent neuronal firing, whereas the ι-conotoxins enhance channel opening by shifting the voltage dependence of Na_v activation to more hyperpolar-

TABLE 5

μ- and μO-Conotoxins inhibiting $Na_v1.x$ sodium channels (μ-conotoxin loop formula CC1–8CC3–4C2–5C)

Cysteines are bolded and aligned separately for μ- and μO-conotoxins, with residues affecting sodium channel affinity underlined.

Species	Name	Diet	Sequence (Disulfide Bonded 1–4/2–5/3–6) 12 3 4 5 6	Na_{v1}.x Selectivity	Reference
μ-Conotoxins					
C. bullatus	BuIIIA	P	VTDR**CC**-K--GKRE**C**GRW-**C**RDHSR**C**-**C***	2 ~ 4 > 1 ~ 3 > 6 > 5 > 7 ~ 8	Holford et al., 2009; Wilson et al., 2011a
	BuIIIB	P	VGER**CC**KN--GKRG**C**GRW-**C**RDHSR**C**-**C***	4 > 2 > 3 > 1 > 6 > 5 > 7 ~ 8	Holford et al., 2009; Wilson et al., 2011a
	BuIIIC	P	IVDR**CC**NKGNGKRG**C**SRW-**C**RDHSR**C**-**C***	4 > ?	Holford et al., 2009
C. catus	CIIIA	P	--GR**CC**E-GPN--G**C**SSRW**C**KDHAR**C**-**C***	Amphibian skeletal/ cardiac, TTX-R	Zhang et al., 2006b
C. consors	CnIIIA	P	--GR**CC**DVPNA---**C**SGRW**C**RDHAQ**C**-**C***	2 ~ 4 > 6 ~ 5 > 3 > 1 > 7 ~ 8	Zhang et al., 2006b; Wilson et al., 2011a
	CnIIIB	P	--ZG**CC**GEPNL---**C**FTRW**C**RNNAR**C**-**C**RQQ	Amphibian TTX-R (< 25%)	Zhang et al., 2006b
C. geographus	GIIIA	P	--RD**CC**TOOKK---**C**KDRQ**C**K-OQR**C**-**C**A*	4 > 1 > 6 > 2 > 3 ~ 5 ~ 7 ~ 8	Cruz et al., 1985; Wilson et al., 2011a
	GIIIB	P	--RD**CC**TOORK---**C**KDRR**C**K-OMK**C**-**C**A*	4 > 2 > 3 > 5 > 7 > 8 > ?	Cruz et al., 1985; Lewis et al., 2007
	GIIIC	P	--RD**CC**TOOKK---**C**KDRR**C**K-OLK**C**-**C**A*	4 > 2 > N.D.	Cruz et al., 1985
C. kinoshitai	KIIIA	P	----**CC**N-------**C**SSKW**C**RDHSR**C**-**C***	2 > 4 > 6 > 1 ~ 7 > 3 > 5 > 8	Bulaj et al., 2005; Zhang et al., 2007; Wilson et al., 2011a
C. magus	MIIIA	P	--ZG**CC**NVPNG---**C**SGRW**C**RDHAQ**C**-**C***	4 > 2 > 3 > 6 ~ 1 > 5 > 7 ~ 8	Zhang et al., 2006b; Wilson et al., 2011a
C. purpurascens	PIIIA	P	-ZRL**CC**GFOKS---**C**RSRQ**C**K-OHR**C**-**C***	4 > 6 ~ 1 > 2 > 3 > 5 ~ 7 ~ 8	Shon et al., 1998a; Wilson et al., 2011a
C. pennaceus	PnIVA	M	----**CC**KYGWT---**C**-LLG**C**-SP--**C**G**C**	Molluscan	Fainzilber et al., 1995b
	PnIVB	M	----**CC**KYGWT---**C**-WLG**C**-SP--**C**G**C**	Molluscan	Fainzilber et al., 1995b
C. striatus	SIIIA	P	--ZN**CC**NGG-----**C**SSKW**C**RDHAR**C**-**C***	2 > 4 > 6 > 1 ~ 3 > 7 > 5 > 8	Bulaj et al., 2005; Yao et al., 2008; Leipold et al., 2011; Wilson et al., 2011a
	SIIIB	P	--ZN**CC**NGG-----**C**SSKW**C**KGHAR**C**-**C***	4 > 2 > ?	Schroeder et al., 2008
C. stercusmuscarum	SmIIIA	P	--ZR**CC**NGRRG---**C**SSRW**C**RDHSR**C**-**C**	4 > 2 > 1 > 3 > 6 > 5 ~ 7 > 8	West et al., 2002; Wilson et al., 2011a
C. striolatus	SxIIIA	P	---R**CC**TGKKGS--**C**SGRA**C**KN-LK**C**-**C**A*	4 > 1 > 6 > 2 > 3 ~ 5 ~ 7 ~ 8	Walewska et al., 2008; Wilson et al., 2011a
	SxIIIB	P	--ZK**CC**TGKKGS--**C**SGRA**C**KN-LR**C**-**C**A*	N.D.	Walewska et al., 2008
C. tulipa	TIIIA	P	-RHG**CC**KGOKG---**C**SSRE**C**R-OQH**C**-**C***	4 > 2 > 1 > 3 > 6 > 5 ~ 7 ~ 8	Lewis et al., 2007; Wilson et al., 2011a
μO-Conotoxins					
C. litteratus	LtVIIA	V	GE**C**LGWSNY**C**TSHSI**CC**SGE**C**ILSY**C**DIW	N.D.	Pi et al., 2007
	LtVd	V	-D**C**------**C**PAKLL**CC**NP	TTX-S > TTX-R	Liu et al., 2007a
	LtVIC	V	WP**C**KVAGSP**C**GLVSE**CC**GT-**C**NVLRNR**C**V	N.D.	Wang et al., 2008
C. marmoreus	MrVIA	M	-A**C**RKKWEY**C**IVPIIGFIY**CC**PGLI**C**GPFV**C**V	8 > 7 > 4 > 2 > TTX-S	Fainzilber et al., 1995c; McIntosh et al., 1995; Safo et al., 2000; Daly et al., 2004; Zorn et al., 2006
	MrVIB	M	-A**C**SKKWEY**C**IVPILGFVY**CC**PGLI**C**GPFV**C**V	8 > 4 > 2 ~ 3 ~ 5 ~ 7 > 9	Fainzilber et al., 1995b; Fainzilber et al., 1995c; McIntosh et al., 1995; Ekberg et al., 2006; Zorn et al., 2006
C. geographus	Conotoxin-GS	P	-A**C**SGRGSR**C**OOQ**CC**MGLR**C**GRGNPQK**C**IGAHγDV	4 > 2 > N.D.	Yanagawa et al., 1988; Hill et al., 1997

P, fish; M, molluscs; V, worms; *, C-terminal amidation; N.D., not determined; TTX-S TTX-sensitive; TTX-R, TTX-resistant.

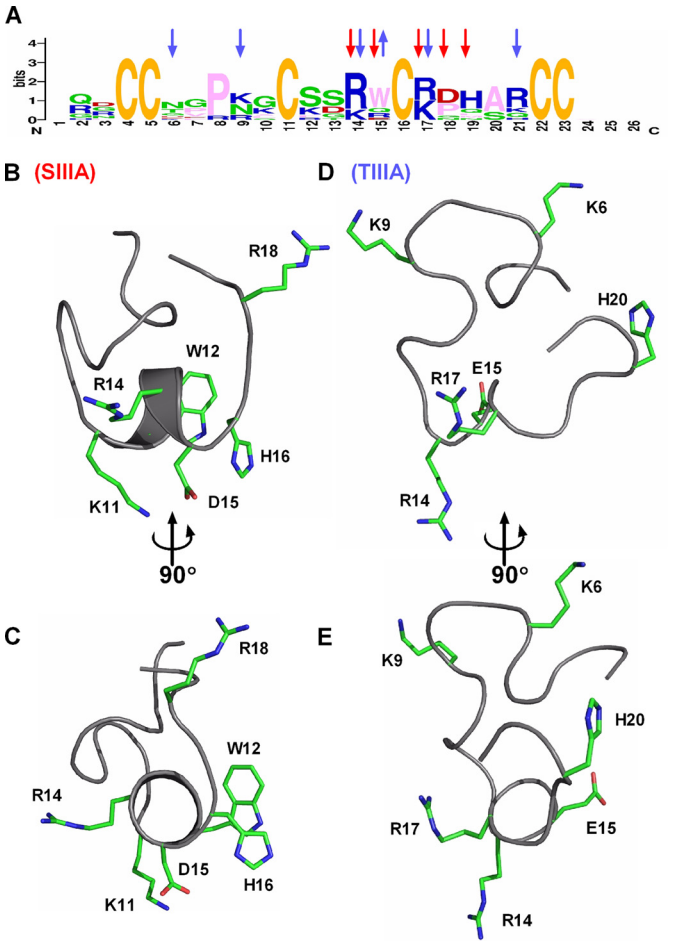

FIG. 5. Structure activity relationship of μ-conotoxins. A, consensus sequence for μ-conotoxins. B and C, structure of the smaller μ-SIIIA showing important residues for interaction with the sodium channel. D and E, structure of larger μ-TIIIA showing important residues for interaction with the sodium channel.

ized potentials. It is noteworthy that conotoxins provide some of the most subtype-selective Na_v modulators, especially for $Na_v1.4$, $Na_v1.2$, and more recently $Na_v1.8$ and have proven to be invaluable tools to dissect the physiological roles and pharmacologies of $Na_v1.1$ to $Na_v1.9$. The potential of Na_v-targeting conotoxins as drug leads is only now beginning to be explored.

A. μ-Conotoxin Inhibitors of Voltage-Gated Sodium Channels

Of the conotoxins targeting Na_v, the μ-conotoxins are the most numerous and best characterized, with 20 different μ-conotoxins described to date. μ-Conotoxins belong to the M superfamily of conopeptides and comprise 16 to 26 residues including 3 conserved disulfide bonds that stabilize their three-dimensional structure (see Table 5, Fig. 5). The μ-conotoxins are generally characterized by an overall net positive charge, which contributes to their ability to bind through electrostatic interactions to the outer vestibule of Na_v to inhibit ionic conductance (Hui et al., 2002; Hui et al., 2003; Li et al., 2003a).

μ-Conotoxins are the only venom peptides that bind to site 1 on Na_v, a site originally defined by the guanidinium pore blockers tetrodotoxin (TTX) and saxitoxin (see Fozzard and Lipkind, 2010). Although TTX (and saxitoxin) only distinguishes between TTX-sensitive ($Na_v1.1$, -1.2, -1.3, -1.4, -1.6, and -1.7) and TTX-resistant ($Na_v1.5$, -1.8, and -1.9) isoforms, μ-conotoxins display further subtype selectivity among mammalian TTX-sensitive Na_v subtypes (see Table 5). This difference in pharmacology arises because the binding sites of TTX and μ-conotoxins only partially overlap and involve multiple Na_v residues in the case of the larger μ-conotoxins (Sato et al., 1991; Dudley et al., 1995; Li et al., 1997), whereas TTX binding is crucially defined by relatively few residues deep in the pore of the Na_v (see Fozzard and Lipkind, 2010). Not surprisingly, μ-conotoxin binding to Na_v involves multiple interacting peptide side chains (see below). This larger surface area of interaction at the entrance to the channel opens up prospects to engineer further Na_v subtype selectivity, which has been considered challenging to date because of the overall high homology between Na_v subtypes in the pore region.

The first μ-conotoxins discovered were GIIIA to GIIIC, which selectively target the skeletal muscle subtype $Na_v1.4$ (Cruz et al., 1985). In recent years, the Na_v subtype selectivity of μ-conotoxins has been studied with renewed interest; many have now shown subtype selectivity for neuronal subtypes, including PIIIA, KIIIA, MIIIA, CIIIA, CnIIIA, CnIIIB, SIIIA, SmIIIA, and TIIIA. To date, no μ-conotoxins have been identified with affinity for the TTX-resistant mammalian subtypes $Na_v1.5$, -1.9, and -1.8 (Lewis et al., 2007; Schroeder et al., 2008; Leipold et al., 2011; Wilson et al., 2011a) despite a number showing activity at amphibian TTX-resistant isoforms (Zhang et al., 2006b) (see Table 5). The least susceptible isoforms include $Na_v1.3$, -1.7, and -1.5, and no μ-conotoxins have yet been found to block recombinantly expressed $Na_v1.8$ (Lewis et al., 2007; Schroeder et al., 2008; Leipold et al., 2011; Wilson et al., 2011a). Instead, the μ-conotoxins seem to either target the mammalian skeletal muscle isoform $Na_v1.4$ and/or the mammalian brain isoform $Na_v1.2$ with highest affinity. However, the modest variations in potency at the other neuronal Na_v subtypes (Table 5) may allow the development of analogs that target therapeutically relevant Na_vs.

Coexpression of the pore-forming α subunits with relevant β subunits does not seem to affect μ-conotoxin selectivity or affinity, at least in the case of KIIIA (Wilson et al., 2011a), although comprehensive studies using other μ-conotoxins are lacking. It is noteworthy that for many μ-conotoxins, Na_v subtype selectivity seems to critically depend on dissociation rate constants. For example, the high affinity of KIIIA for $Na_v1.2$ relates to its extremely slow dissociation constant, resulting in essentially irreversible high-affinity block of $Na_v1.2$ but lower affinity reversible block of other TTX-sensitive channels (McArthur et al., 2011).

The pharmacophore of the μ-conotoxins GIIIA, PIIIA, and TIIIA with five residues in loop 1 has been mapped to a critical Arg13 or Arg14 in similar positions on a turn in loop 2 (Chahine et al., 1995; Shon et al., 1998a; Lewis et al., 2007). In contrast, the pharmacophore of μ-conotoxins with one to three residues in loop 1 has been mapped to an α-helical motif formed by loops 2 and 3, with the equivalent loop 2 arginine playing only a minor role in affinity (Zhang et al., 2007; Schroeder et al., 2008). Reflecting the more extended surface of interaction with Na_v for these small μ-conotoxins, several residues have been found to contribute critically to Na_v affinity and/or selectivity of μ-conotoxins, opening up perhaps the best opportunities for the development of subtype specific inhibitors. Lys7 in KIIIA and the equivalent Lys11 in SIIIA contribute mostly to $Na_v1.4$ affinity (Zhang et al., 2007; Schroeder et al., 2008); Lys7 substitutions also affected the extent of Na_v inhibition by KIIIA, the decrease in efficacy correlating with the residue size (Zhang et al., 2010). The corresponding Arg14 in PIIIA and TIIIA also significantly decreased Na_v affinity, again most prominently at $Na_v1.4$ (Shon et al., 1998a; Lewis et al., 2007; Schroeder et al., 2008), consistent with direct binding of this residue in the pore of Na_vs (McArthur et al., 2011). Not surprisingly, substitution of the analogous residue in GIIIA (Arg13) produced a striking loss of activity at $Na_v1.4$ in this peptide (Sato et al., 1991; Becker et al., 1992), with interaction with Glu758 in domain II and Glu403 in domain I identified in $Na_v1.4$ (Chang et al., 1998).

Another residue with important implications for Na_v affinity and selectivity is Trp8 in KIIIA and the equivalent Trp12 in SIIIA (Lewis et al., 2007; Schroeder et al., 2008; McArthur et al., 2011; Van Der Haegen et al., 2011); substitution of this residue in KIIIA and SIIIA decreases affinity and favors $Na_v1.2$ selectivity over $Na_v1.4$. In contrast, the unusual negative charge (Glu15) in loop 2 of TIIIA seems to introduce an unfavorable interaction or clash, with [E15A]TIIIA having enhanced affinity, especially for $Na_v1.2$, where it is the most potent μ-conotoxin inhibitor of neuronal Na_vs identified to date (Lewis et al., 2007). The smaller μ-conotoxins KIIIA (SIIIA) are also affected by alanine substitutions at Arg10 (Arg14) and Asp11 (Asp15), which favor $Na_v1.2$ selectivity over $Na_v1.4$ (Zhang et al., 2007; Schroeder et al., 2008). It is noteworthy that the sequence of SIIIA and SIIIB differ only in residues at this position (Arg14 and Asp15 for SIIIA, and Lys14 and Gly15 for SIIIB); accordingly, the major difference between these toxins is the preferential inhibition of $Na_v1.2$ over $Na_v1.4$ by SIIIA and the reversed selectivity for SIIIB (Schroeder et al., 2008).

Several μ-conotoxin analogs have been identified that have striking effects on subtype selectivity beyond $Na_v1.2$/1.4. Most notably, KIIIA[R14A] has been found to preferentially inhibit $Na_v1.7$ over $Na_v1.2$ and $Na_v1.4$, albeit with decreased affinity overall (McArthur et al., 2011). Nonetheless, this finding indicates that it is possible to engineer smaller μ-conotoxins with tailored subtype selectivity, especially as our understanding of the precise structural requirements for Na_v subtype binding are improved after the report of the first voltage-gated sodium channel structure (see Fig. 3B).

B. μO-Conotoxin Inhibitors of Voltage-Gated Sodium Channels

The μO-conotoxins belong to the O superfamily of conopeptides and possess an ICK motif with appropriate disulfide bond connectivity (Table 5). Despite the relatively few peptides identified in this class, μO-conotoxins MrVIA and MrVIB have received considerable attention, because their analgesic effects in animal models of pain were ascribed to their relative selectivity for $Na_v1.8$ over other TTX-sensitive Na_v subtypes expressed in DRG neurons (Daly et al., 2004; Bulaj et al., 2006; Ekberg et al., 2006). Significant differences in their affinity at native $Na_v1.8$ expressed in DRG neurons and heterologously expressed $Na_v1.8$ have been reported. These presumably arise because of the presence of auxiliary β subunits, particularly β2, which has recently been shown to significantly increase the rate of $Na_v1.8$ inhibition by MrVIB and to increase its potency accordingly (Wilson et al., 2011b). It is noteworthy that μO-conotoxins can also differentiate between the TTX-resistant subtypes $Na_v1.8$ and -1.9, because the persistent $Na_v1.9$ current in DRG neurons was unaffected by MrVIB (Ekberg et al., 2006). Although MrVIA and MrVIB have been found to affect molluscan Ca_v (Fainzilber et al., 1995c), this mechanism does not seem to contribute to the analgesic effect, because mammalian Ca_v in DRG neurons were unaffected (Daly et al., 2004).

The μO-conotoxin binding site remains to be fully defined but seems to overlap at least partially with site 6, in that MrVIA was able to displace δ-conotoxin TxVIA binding (Ekberg et al., 2006). In addition, selectivity of MrVIA at $Na_v1.4$ over $Na_v1.2$ has been attributed to the SS2 loop of domain III (Zorn et al., 2006). It is noteworthy that this site is also involved in β scorpion toxin binding to site 4 (Leipold et al., 2006), and both $Na_v1.4$ and $Na_v1.8$ share a homologous residue in this domain that is distinct from the other Na_v subtypes and may contribute to the relative selectivity of MrVIA and MrVIB (Ekberg et al., 2006). Accordingly, functional overlap with the β scorpion toxin Ts1 was recently described, suggesting μO conotoxins interact with the voltage sensor of Na_vs (Leipold et al., 2007). Indeed, voltage-dependent relief of $Na_v1.4$ inhibition by MrVIB was attributed to the voltage sensor of domain II (Leipold et al., 2007), another site shared with β scorpion toxins. Similar effects were also reported for $Na_v1.8$, where the K_{off} of MrVIB was also accelerated by strong depolarizations (Wilson et al., 2011b). However, compared with $Na_v1.4$, the K_{off} of MrVIB at $Na_v1.8$ was significantly slower and required repeated depolarizations to relieve block (Wilson et al., 2011b).

In contrast to the μ-conotoxins, relatively little is known about the structure-activity relationships of μO-conotoxins, in part because only MrVIA and MrVIB, which differ

by only three residues (Ser3/Arg3; Leu14/Ile14; Val17/Ile17), have been available for pharmacological evaluation, and their effects remain to be evaluated across all Na_v subtypes in parallel. In addition, difficulties synthesizing, correctly folding, and purifying hydrophobic μO-conotoxins have thwarted attempts to identify their pharmacophore through alanine-scanning approaches. However, recent improvements in oxidative folding of synthetic MrVIB through regioselective synthesis using selenocysteines may provide an accelerated avenue for the generation of μO-conotoxin analogs, allowing residues contributing to their affinity and subtype selectivity to be identified (de Araujo et al., 2011).

C. Other Conotoxin Inhibitors of Voltage-Gated Sodium Channels

Conotoxin GS was identified as an O-superfamily conotoxin; however, in contrast to MrVIA and MrVIB, conotoxin GS seems to share a binding site with TTX and GIIIA, suggesting that this conotoxin is distinct both in structure and mode of action (Yanagawa et al., 1988; Hill et al., 1997). In addition, two novel O-superfamily conotoxins, LtVIC and LtVIIA, were identified from *Conus litteratus* using a DNA-sequencing approach (Pi et al., 2007; Wang et al., 2008). Both recombinant peptides inhibited sodium currents in DRG neurons in a manner similar to that of MrVIA and MrVIB, suggesting that they are novel μO-conotoxins, but their subtype selectivity and structure-activity relationships remain to be investigated. Even more unusual is the μT-conotoxin, LtVD, which is reported to inhibit TTX-sensitive Na_v and is thus classified as the first μ-conotoxin from the large class of small T-superfamily conopeptides (Liu et al., 2007a).

D. δ-Conotoxin Activators of Voltage-Gated Sodium Channels

The δ-conotoxins, ICK peptides structurally related to the μO- and ω-conotoxins, exhibit functional similarities to the site 3 α-scorpion toxins; they inhibit fast Na_v inactivation and shift the voltage-dependence of activation to more negative potentials, resulting in a prolongation of action potentials and persistent neuronal firing (Leipold et al., 2005). Structural analysis has shown that several hydrophobic residues conserved across the δ-conotoxins are presented on the external surface of the peptides (Shon et al., 1994), where they could potentially interact with hydrophobic residues in the S3/S4 linker of domain IV, which form part of the δ-conotoxin binding pocket, site 6 (Leipold et al., 2005).

Little is known about Na_v subtype selectivity of the δ-conotoxins. δ-Conotoxins isolated from molluscivorous cone snails, with the exception of Am2766 (Sudarslal et al., 2003; Sarma et al., 2005), target only molluscan Na_v and are inactive at mammalian targets, whereas δ-conotoxins from piscivorous cone snails seem to also inhibit inactivation of mammalian Na_v. Intriguingly, although δ-conotoxins from molluscivorous cone snails, such as TxVIA and GmVIA, showed no activity at mammalian Na_v, they still bind to mammalian tissues, suggesting that subtle differences in the δ-conotoxin binding site lead to profound effects on activity (Fainzilber et al., 1995a; Hasson et al., 1995; Shichor et al., 1996). A number of δ-conotoxins with activity at mammalian $Na_v1.2$ and $Na_v1.4$ have been described previously (Bulaj et al., 2001; Sudarslal et al., 2003; Leipold et al., 2005; Sarma et al., 2005), although detailed selectivity studies have not been conducted for the majority of these peptides. A recent report that EVIA displayed selectivity for neuronal subtypes $Na_v1.2$, 1.3 and 1.6 over muscle and cardiac subtypes $Na_v1.4$ and $Na_v1.5$ promises that subtype selectivity may be found for other δ-conotoxins (Barbier et al., 2004).

Reminiscent of the μO-conotoxins, difficulties relating to the synthesis and purification of the hydrophobic δ-conotoxins and their analogs again results in low yields of correctly folded peptide that have limited progress on both Na_v subtype-selectivity and structure-activity relationships. High sequence homology between δ-conotoxins from piscivorous cone snails has been noted, particularly in the central hydrophobic region of these peptides (Bulaj et al., 2001). It is noteworthy that the PVIA[F9A] and PVIA[I12A] analogs lost activity at mammalian Na_v, consistent with the hypothesis that these hydrophobic residues interact with hydrophobic residues that form part of site 6, whereas analogs of the adjacent residues T8A and K13A retained activity (Bulaj et al., 2001). However, the substantial reduction in affinity upon replacing Ile12 with an alanine is surprising if only hydrophobic interactions are involved, suggesting the possibility that this change may have in fact altered the structure of PVIA. It remains to be determined to what degree, if any, these residues affect Na_v subtype selectivity, or whether these findings apply to δ-conotoxins from molluscivorous cone snails that have divergent sequences.

E. ι-Conotoxin Activators of Voltage-Gated Sodium Channels

Two ι-conotoxins have recently been identified as Na_v activators with a mechanism of action distinct from that of the δ-conotoxins. ι-RXIA is a large, 46-residue peptide from the I superfamily that forms an ICK motif, whereas LtIIIA is a small, 17-residue peptide belonging to the M superfamily of conotoxins (Jimenez et al., 2003; Buczek et al., 2007; Wang et al., 2009). Despite these sequence and structural differences, both ι-conotoxins activate Na_v without significantly affecting inactivation, in contrast to the δ-conotoxins. Specifically, LtIIIA enhanced the amplitude of TTX-sensitive whole-cell Na^+ currents in DRG without any apparent effects on inactivation (Wang et al., 2009), whereas RXIA shifted the voltage dependence of activation of $Na_v1.6$ and $Na_v1.2$ to more hyperpolarized potentials without affecting inactivation (Fiedler et al., 2008). It is noteworthy that the post-translationally modified Phe44 (D-Phe) was important for activity particularly at $Na_v1.2$, suggesting participation of the disordered C-terminal tail

in binding to a yet to be defined Na_v site (Fiedler et al., 2008). RXIA was also active at $Na_v1.7$ but did not affect other Na_v subtypes (Fiedler et al., 2008), suggesting that detailed structure-activity relationship and subtype selectivity studies of the 17 ι-conotoxins identified in *Conus radiatus* (Jimenez et al., 2003) might shed further light on the mode of action of this potentially interesting family of excitatory conopeptides.

F. Sodium Channel Inhibition in Pain Management

Sodium channel (Na_v) inhibitors are used as local anesthetics, analgesics, antiarrhythmics, and anticonvulsants. They are most widely used for regional anesthesia and analgesia, as well as systemically for chronic pain management. Until very recently, the utility of small-molecule Na_v inhibitors, particularly systemic local anesthetics, has been limited both by unwanted actions on nontarget sodium channel types and difficulty in restricting distribution to target body compartments. For example, mexiletine, an orally administered lidocaine analog, is a third-line therapy for chronic pain management but has limited efficacy largely because of serious dose-limiting side effects (Dworkin et al., 2007). The severe side-effect profile of currently used Na_v inhibitors is largely due to very poor selectivity among Na_v subtypes (England and de Groot, 2009). Growth in knowledge of the diversity of Na_v types, implication of specific Na_v types in pain pathogenesis, together with highly selective expression in different tissues has provided a strong impetus to identify subtype selective Na_v antagonists for pain management.

The selectivity of conotoxins for different channel types may provide novel drugs for clinical use in regional anesthesia and for systemic or intrathecal administration in chronic pain states. The potential restriction of peptide distribution to systemic tissue compartments rather than central nervous or local application in restricted tissue compartments could provide advantages for these selective peptide Na_v antagonists. Among the conotoxin classes,

TABLE 6
Distribution of Na_v subtypes and efficacy of inhibitors

Isoform	Distribution	Potential Effects/Side Effects	Selective Toxin Inhibitors	References
$Na_v1.1$ (SCN1A)	Located primarily in neuronal soma. Found widely in CNS and peripheral neurons (including DRG) cardiac myocytes (T-tubules) (cortex, hippocampus, cerebellum), PNS: DRG, motor neurons.	Widespread effects on the nervous and cardiovascular systems.	No selective conotoxins	Catterall et al., 2005a; Catterall, 2010
$Na_v1.2$ (SCN2A)	Located primarily in axons, node of Ranvier. Found in CNS low expression in adult DRG.	Widespread effects on the nervous and cardiovascular systems.	μ-Conotoxins	Catterall et al., 2005a; Schroeder et al., 2008; Catterall, 2010; Wilson et al., 2011a
$Na_v1.3$ (SCN3A)	Widely distributed in embryonic nervous system but declines postnatally; adult rat CNS, particularly dorsal spinal cord; also upregulated after injury; adult human CNS.	Limited evidence for analgesia. If restricted to periphery, side effects should be minimal; intrathecal CNS side effects possible.	No selective conotoxins	Felts et al., 1997; Hains et al., 2003; Catterall et al., 2005a; Catterall, 2010
$Na_v1.4$ (SCN4A)	Skeletal muscle	Muscle dysfunction	μ-Conotoxins	Catterall et al., 2005a; Schroeder et al., 2008; Catterall, 2010; Wilson et al., 2011a
$Na_v1.5$ (SCN5A)	Cardiac muscle, embryonic DRG	Cardiac side effects	No selective conotoxins	Catterall et al., 2005a; Catterall, 2010
$Na_v1.6$ (SCN8A)	Highly localized in nodes of Ranvier in CNS and PNS, including DRG.	Widespread effects on the nervous and cardiovascular systems.	No selective conotoxins	Catterall et al., 2005a; Catterall, 2010
$Na_v1.7$ (SCN9A)	High expression in small sensory (DRG) neurons and autonomic neurons, also Schwann cells, CD1 + dendritic cells, and some endocrine cells (adrenal medulla).	Analgesic effects. Possible autonomic side effects but autonomic function seems normal with loss-of-function mutations; olfactory side effects.	No selective conotoxins (ProTx-II, huwentoxin-IV)	Catterall et al., 2005a; Cummins and Rush, 2007; Schmalhofer et al., 2008; Xiao et al., 2008a; Xiao et al., 2008b
$Na_v1.8$ (SCN10A)	Small- to medium-sized (presumably nociceptive) DRG neurons, also found in retina and cardiac myocytes.	Analgesic effects. Visual and cardiac side-effects likely from distributional and human mutation studies.	MrVIA, MrVIB	Ekberg et al., 2006; Cummins and Rush, 2007; O'Brien et al., 2008; Chambers et al., 2010
$Na_v1.9$ (SCN11A)	Small-diameter, mostly unmyelinated DRG neurons, also found in retina.	Potential analgesic effects. Potential visual effects.	None known	Catterall et al., 2005a; Cummins and Rush, 2007; O'Brien et al., 2008

PNS, peripheral nervous system.

some μ- and μO-conotoxins show promise as selective Na_v subtype inhibitors. A large number of μ-conotoxins display considerable channel type selectivity. Most of those isolated inhibit $Na_v1.2$ and $Na_v1.4$ most potently (Schroeder et al., 2008; Wilson et al., 2011a). Although some μ-conotoxins (e.g., KIIIA) have analgesic activity after systemic administration (Zhang et al., 2007), none to date have shown a clear Na_v selectivity profile suitable for development as pain therapeutics. By contrast, several μO-conotoxins have potentially useful selectivity profiles (see below) for the major Na_v types implicated in pain.

As summarized in Table 6, $Na_v1.7$, -1.8, -1.9, and perhaps -1.3 are potential candidate targets for pain therapeutics because of their established role in pain, selective expression in nociceptive neurons and nerve terminals, and/or their regulation in pain states (see reviews by Catterall et al., 2005a; Cummins and Rush, 2007; Dib-Hajj et al., 2009; England and de Groot, 2009; Catterall, 2010; Dib-Hajj et al., 2010). Other Na_v types are also expressed in sensory neurons, including $Na_v1.1$, -1.6, and (at low levels) -1.5 (Catterall et al., 2005a; Catterall, 2010; Dib-Hajj et al., 2010), but inhibition studies with μ-conotoxins have suggested that $Na_v1.6$ and $Na_v1.7$ are the major contributors to peripheral nerve action potentials (Wilson et al., 2011a). Widespread expression of these subtypes (except $Na_v1.7$, see below) in other excitable tissues is likely to preclude the application of inhibitors of these channels as pain therapeutics. The role of Na_v subtypes in pain pathways and their potential for selective targeting by conotoxins is outlined below.

Of the potential Na_v pain targets, $Na_v1.7$ is one of the most promising for pain management. $Na_v1.7$ is expressed almost exclusively in peripheral neurons (Catterall et al., 2005a) and selectively in nociceptive primary afferents (Raymond et al., 2004). $Na_v1.7$ gene deletion targeted to nociceptive sensory neurons ($Na_v1.8$ expressing) or knockdown reduces inflammatory pain and thermal hyperalgesia (Nassar et al., 2004) but not neuropathic pain (Nassar et al., 2005). The reason for this lack of effect in neuropathic pain is still unknown. $Na_v1.7$ expression is up-regulated in injured nerves and in sensory neuron cell bodies in inflammatory pain and may contribute to ectopic action potential discharge in painful neuromas (Dib-Hajj et al., 2010). In humans, rare loss-of-function mutations of $Na_v1.7$ produce a complete absence of pain sensation (Cox et al., 2006; Goldberg et al., 2007) and, conversely, gain-of-function mutations are associated with painful neuropathies (Fertleman et al., 2006; Han et al., 2006). Polymorphisms in $Na_v1.7$ in normal humans are also significantly correlated with pain sensitivity (Reimann et al., 2010). The mechanism responsible for the profound loss of function for $Na_v1.7$ mutations in human pain remain uncertain, but these channels are concentrated in nerve endings and amplify small depolarizations; they therefore have the potential to play a major role in coupling generator potentials in nociceptive afferent nerves with action potential generation (Cummins et al., 1998).

There are already several relatively selective small-molecule inhibitors of $Na_v1.7$ that have shown efficacy in acute, inflammatory, and neuropathic pain models (Williams et al., 2007; London et al., 2008; Bregman et al., 2011; Chowdhury et al. 2011). Although many conotoxins can also inhibit $Na_v1.7$ (see above), no highly selective antagonists have been identified to date, although analogs of the smaller μ-conotoxins have potential (see above). At the moment, spider venoms are more promising sources of selective $Na_v1.7$ inhibitors. For example, ProTx-II from the tarantula *Thrixopelma pruriens* is a potent and selective inhibitor of $Na_v1.7$ (~100-fold over other types) but shows limited efficacy in inflammatory pain models after intravenous or intrathecal administration (Schmalhofer et al., 2008). This lack of activity in pain models might suggest that selective $Na_v1.7$ inhibitors are not as useful as previously thought, but the possibility that the peptide distributes poorly from the circulation to nociceptive nerve endings cannot be excluded. Huwentoxin-IV, from the tarantula *Ornithoctonus huwena*, is also modestly selective for $Na_v1.7$ (it also inhibits $Na_v1.2$ and $Na_v1.3$), but actions in animal pain models have not been reported in detail (Xiao et al., 2008a,b).

Na_v 1.7 is also expressed in several other tissues. A major off-target location of $Na_v1.7$ expression are olfactory neurons (Ahn et al., 2011), and loss-of-function mutations can cause anosmia (Weiss et al., 2011). However, such side effects may not be of major concern to persons suffering severe pain, but loss of smell would be expected to reduce quality of life during prolonged treatment. More recently $Na_v1.7$ expression in monocyte-derived dendritic cells has been shown to modify responses to cytokines (Kis-Toth et al., 2011), suggesting potential for immune modulation and potentially immunological side effects of $Na_v1.7$ inhibitors.

The TTX-resistant channel $Na_v1.8$ is also a promising target for pain management. It is expressed predominantly in small nociceptive primary afferent nerves (Catterall et al., 2005a; Cummins and Rush, 2007; Catterall, 2010; Dib-Hajj et al., 2010). $Na_v1.8$ contributes to the action potential and repetitive firing in small, nociceptive sensory neurons (Catterall et al., 2005a; Cummins and Rush, 2007; Catterall, 2010; Dib-Hajj et al., 2010). The activity of $Na_v1.8$ in sensory neurons is strongly enhanced by inflammatory mediators, both via up-regulation and/or modulation of channel kinetics by protein kinase A and C (see Dib-Hajj et al., 2010). Both protein and mRNA for $Na_v1.8$ are up-regulated locally in injured sciatic nerves in nerve injury models (Gold et al., 2003; Thakor et al., 2009). $Na_v1.8$ knockdown (Lai et al., 2002; Gold et al., 2003) and gene deletion (Akopian et al., 1999) studies have suggested an important role in neuropathic and inflammatory pain. Perhaps the most significant phenotype in $Na_v1.8$ knockout mice is a nearly complete loss of ectopic action potential activity in experimental neuromas (Roza et al., 2003). Together with the established redistribution of $Na_v1.8$ to sites of nerve injury and neuromas in animal models and

humans (e.g., Black et al., 2008), this finding may suggest a specific therapeutic use for $Na_v1.8$ inhibitors in nerve injury-induced pain associated with neuromas.

μO-conotoxins MrVIA and MrVIB inhibit $Na_v1.8$ in sensory neurons (Daly et al., 2004; Ekberg et al., 2006) and significantly reduce mechanical and thermal pain in both neuropathic and inflammatory pain models; selectivity over motor side effects is more than 30-fold greater than found for lidocaine (Ekberg et al., 2006). It is noteworthy that potentially more stable and easier to synthesize selenocysteine analogs produced similar beneficial effects (de Araujo et al., 2011). These findings suggest that direct spinal administration of $Na_v1.8$-selective conotoxins may have a place in pain management. MrVIB has also been shown to display local anesthetic actions after intraplantar administration (Bulaj et al., 2006). Although Na_v inhibitory toxins from other species have yet to show preferential $Na_v1.8$ targeting, a chimeric scorpion toxin (drosotoxin) shows quite low potency but high selectivity for TTX-resistant ($Na_v1.8$) versus TTX-sensitive Na_v currents in rat sensory neurons (Zhu et al., 2010). Consistent with results obtained using μO-conotoxins, small-molecule antagonists preferentially targeting $Na_v1.8$ (Jarvis et al., 2007; Scanio et al., 2010) have since been reported to show efficacy in neuropathic pain models.

Although small-molecule and conotoxin inhibitors of $Na_v1.8$ have produced efficacy in pain models, there are concerns about adverse side effects. For example, in addition to their selective expression in nociceptive sensory nerves, $Na_v1.8$ (and $Na_v1.9$) is expressed in retina (O'Brien et al., 2008), where small-molecule antagonists could produce retinal side effects, although peptides are potentially excluded from this compartment. Of more concern is the recent finding that $Na_v1.8$ is expressed in cardiac myocytes and that polymorphisms in the $Na_v1.8$ gene influence QT interval, electrocardiogram conduction block, and ventricular fibrillation (Chambers et al., 2010), raising the potential for severe cardiac side effects from systemic use of $Na_v1.8$ inhibitors that would complicate long-term use.

The tetrodotoxin-resistant channel, $Na_v1.9$ is also a promising target for pain management but has been less thoroughly studied than $Na_v1.7$ or $Na_v1.8$, largely because attempts to express the channel in heterologous expression systems have been unsuccessful. It is expressed predominantly in small sensory neurons, particularly in a subclass of C-fiber nociceptors (Fang et al., 2006) and visceral sensory nerves (Rugiero et al., 2003). It has also been detected in retina (O'Brien et al., 2008). $Na_v1.9$ produces a persistent sodium current that modulates resting membrane potential and amplifies small depolarizations, contributing to excitability of small nociceptive nerves. The activity of $Na_v1.9$ is strongly enhanced by inflammatory mediators (e.g., Maingret et al., 2008; Ostman et al., 2008), although a number of $Na_v1.9$ gene deletion studies have consistently found little or no effect on neuropathic pain, except for inhibition of cold allodynia (Leo et al., 2010). In contrast, thermal pain sensitivity is consistently blunted in inflammatory pain models in $Na_v1.9$ knockout animals (Priest et al., 2005; Amaya et al., 2006). $Na_v1.9$ inhibitors may therefore be useful for inflammatory and visceral pain. Because of the difficulties in heterologously expressing $Na_v1.9$, no selective pharmacological tools, including conotoxins, have been identified to study its function in normal neurons and pain states. However, some spider and scorpion toxins including ProTxI and TsVII were recently found to interact with $Na_v1.9$ endogenously expressed in DRG neurons in a nonselective manner (Bosmans et al., 2011).

$Na_v1.3$ is also a potential target for pain therapeutics, although its role in the maintenance of pain remains controversial. It is highly expressed in embryonic DRG neurons but declines to very low levels in adult sensory neurons (Felts et al., 1997). Expression of $Na_v1.3$ in sensory neurons is strongly up-regulated by nerve injury (Black et al., 1999; Lindia and Abbadie, 2003; Fukuoka et al., 2008) and is up-regulated in humans in painful neuromas (Black et al., 2008) and the spinal cord (Hains et al., 2003). Antisense knockdown of $Na_v1.3$ in nerve-injured rats has been shown to attenuate signs of neuropathic pain (Hains et al., 2003), but in other studies, no effects of knockdown were found (Lindia et al., 2005). Moreover, both global and nociceptor-specific ($Na_v1.8$ Cre mice) deletion of $Na_v1.3$ in mice had no effect on neuropathic pain behavior or ectopic discharges from damaged nerves, suggesting $Na_v1.3$ is neither necessary nor sufficient for development of nerve-injury related pain (Nassar et al., 2006). The latter findings have strongly questioned the importance of $Na_v1.3$ in nerve injury-related pain and have reduced the impetus to develop $Na_v1.3$ specific inhibitors. Although several μ-conotoxins can inhibit $Na_v1.3$, they all interact more potently with $Na_v1.2$ or $Na_v1.4$ (Wilson et al., 2011a), and more selective analogs are required before chemical dissection of the role of $Na_v1.3$ in different pain states can be investigated.

In conclusion, the potential for identification and development of Na_v subtype-selective conotoxins remains largely untapped. Conotoxins that selectively interact with $Na_v1.7$, $Na_v1.9$, and/or $Na_v1.3$ need to identified; mixed block of these subtypes potentially produces greater efficacy than the selective block of a single subtype. It should also be noted that the potency of conotoxins may differ in injured versus uninjured neurons. As observed for ω-conotoxins (see section II.C), interactions of μO-conotoxins with Na_v channel types are also influenced by auxiliary subunit expression. $Na_v\beta2$ subunits are up-regulated in sensory neurons in neuropathic pain models (Pertin et al., 2005) and interact with Na_vs to increase the on-rate of MrVIB in heterologous systems (Wilson et al., 2011b), providing the μO-conotoxins with a window of functional selectivity. It is noteworthy that $Na_v\beta3$ subunits are also up-regulated in pain states (Shah et al., 2000), and their expression levels influence gating of $Na_v1.3$ (Cusdin et al., 2010) to potentially influence μO-conotoxin pharmacology in a disease-dependent manner.

TABLE 7

*κ-Conotoxins inhibiting K_v potassium channels (κA/M-conotoxin loop formula **C**0C6–7**CC**2–4**C**0–3**C**)*

Lower-case serine or threonine residues are glycosylated, and the lower case tryptophan is a D-amino acid. Cysteines are bolded and aligned separately for the different κ-conotoxin classes, with residues affecting potassium channel affinity underlined.

Species	Name	Diet	Sequence (Disulfide Bonding Likely 1–4/2–5/3–6) 12 3 4 5 6	Selectivity (K_vx)	References
κM- and κA- Conotoxins					
C. radiatus	RIIIJ	P	--------LOO**CC**TOO<u>K</u>KH-**C**OAOA**C**KYKO**C**---**C**KS	1.2	Chen et al., 2010
	RIIIK	P	--------<u>L</u>PS**CC**SLNL<u>R</u>L-**C**PVPA**C**<u>KR</u>NP**C**---**C**T*	TSha1 ~ *Shaker* ~ 1.2 > 1.5 ~ 1.6 ~ 1.1 ~ 1.3 ~ 1.4	Chen et al., 2010; Ferber et al., 2003, 2004; Al-Sabi et al., 2004
C. consors	CcTx	P	AOWLVPsQITT**CC**GYNOGTM**C**OS**C**M**C**TNT-**C**		Favreau et al., 1999
C. magus	MIVA	P	AOγLVVtAtTN**CC**GYNOMTI**C**OO--**C**M---**C**TYS**C**OOKRKO*	?	Santos et al., 2004
C. purpurascens	PIVE	P	----------D**CC**GVKLEM-**C**HP--**C**---L**C**DNS**C**KNYGK*	Frog, fish	Teichert et al., 2007a
	PIVF	P	----------D**CC**GVKLEM-**C**HP--**C**---L**C**DNS**C**KKSGK*	?	Teichert et al., 2007a
C. striatus	SIVA	P	ZKSLVPsVITT**CC**GYDOGTM**C**OO--**C**R---**C**TNS**C***	Frog, fish, *Shaker*	Craig et al., 1998; Santos et al., 2004
	SIVB	P	ZKELVPsVITT**CC**GYDOGTM**C**OO--**C**R---**C**TNS**C**OTKOKKO*	?	Santos et al., 2004
C. stercusmuscarum	SmIVA	P	ZTWLVPstITT**CC**GYDOGTM**C**OT--**C**M---**C**DNT**C**KOKOKKS*	?	Santos et al., 2004
	SmIVB	P	ZPWLVPstITT**CC**GYDOGSM**C**OO--**C**M---**C**NNT**C**KOKOKKS	?	Santos et al., 2004
Other κ-conotoxins					
C. planorbis	pl14a	V	---FPRPRI**C**NLA**C**RAGIGHKYPF**C**H**C**R*	1.6 > 1.1 > 1.2 ~ 1.3 ~ 1.4 ~ 1.5 ~ 2.1 ~ 3.4	Imperial et al., 2006
C. virgo	ViTx	V	SR**C**FPPGIY**C**-TSYLP**CC**WGI**CC**-ST**C**RNV**C**HLRIGK	1.1 ~ 1.3 > 1.2	Kauferstein et al., 2003
C. spurius	sr11a	V	--**C**RTEGMS**C**γγNQQ-**CC**WRS**CC**RGE**C**EAP**C**RFGP*	1.2 ~ 1.6 > 1.3	Aguilar et al., 2007, 2010
C. betulinus	BeTX	V	--**C**RAγGTY**C**-γNDSQ**CC**LNγ**CC**WGG**C**GHO**C**RHP*	BK	Fan et al., 2003
C. purpurascens	PVIIA	P	--**C**<u>R</u>IPNQ<u>K</u>**C**<u>F</u>QHLDD**CC**SRK**C**NR<u>FNK</u>**C**V*	*Shaker* K_v	Huang et al., 2005
C. striatus	Conkunitzin-S1	P	KDRPSL**C**DLPADSGSGTKAEKRIYYNSARKQ**C**LRFDYTGQG GNENNFRRTYD**C**QRT**C**L	*Shaker* Kv	Bayrhuber et al., 2005
C. ventricosus	Contryphan-Vn	P	GD**C**PwKPW**C***	Voltage-gated and Ca^{2+}-dependent K^+ channels	Massilia et al., 2003

P, fish; M, molluscs; V, worms; *, C-terminal amidation; TSha1, from trout, frog K_v, the skeletal muscle form; BK, Ca^{2+}/voltage-dependent potassium channel.

III. κ-Conotoxins Interacting with Potassium Channels

Potassium channels comprise a vastly diverse family of ion channels, with a large number of genes encoding for the pore-forming α-subunits, which are divided into channels with six, four, and two transmembrane domains. Accordingly, multiple families of κ-conotoxins targeting several different potassium channels have been isolated from cone snail venom (Table 7). Activity at potassium channels appears to constitute an important component of the "lightning-strike" cabal of piscivorous cone snails (Terlau et al., 1996); however, relatively little is known about subtype selectivity and structure-activity relationship of conotoxins targeting potassium channels, and potential therapeutic uses have not yet been determined.

A. κA-Conotoxins

The κA-conotoxins MIVA, SIVA, SIVB, SmIVA, SmIVB, PIVE, CcTx, and PIVF (Teichert et al., 2007a) are relatively short excitatory peptides that elicit potent effects after intramuscular injection in fish and direct exposure to amphibian neuromuscular preparations, but not in mammals. It remains unclear whether these observed differences are due to pharmacodynamic effects (such as restricted access to mammalian K_v channels expressed in juxtaparanodal regions of myelinated motor axons) or they reflect strong differences in subtype affinity (Teichert et al., 2007a). Although some of these peptides inhibit *Shaker* K_v channels, the precise target and structure activity relationship for many of the κA-conotoxins remains unclear.

B. κO-Conotoxins

The κO-conotoxin PVIIA has received particular attention because it was shown to exhibit cardioprotective properties in several animal models of ischemia-reperfusion injury (Lubbers et al., 2005). Arg2, Lys7, Phe9, and Phe23 were shown to contribute to voltage-dependent interactions with the *Shaker* potassium channel (Scanlon et al., 1997), which was identified as the molecular target of PVIIA and is highly homologous with mammalian K_v isoforms (Terlau et al., 1996; Shon et al., 1998b; Huang et al., 2005). These residues form part of a lysine-hydrophobic residue dyad, which is evolutionally conserved and contributes to activity at K_v channels in several other toxins, including charybdotoxin from scorpion and BgK from sea anemone (Savarin et al., 1998; García et al., 1999). Figure 6 shows a model of the interaction between PVIIA and the shaker potassium channel, revealing a likely model of interaction of the dyad comprising Lys7 and Phe9. It is surprising that the rat K_v1.1 isoform was resistant to

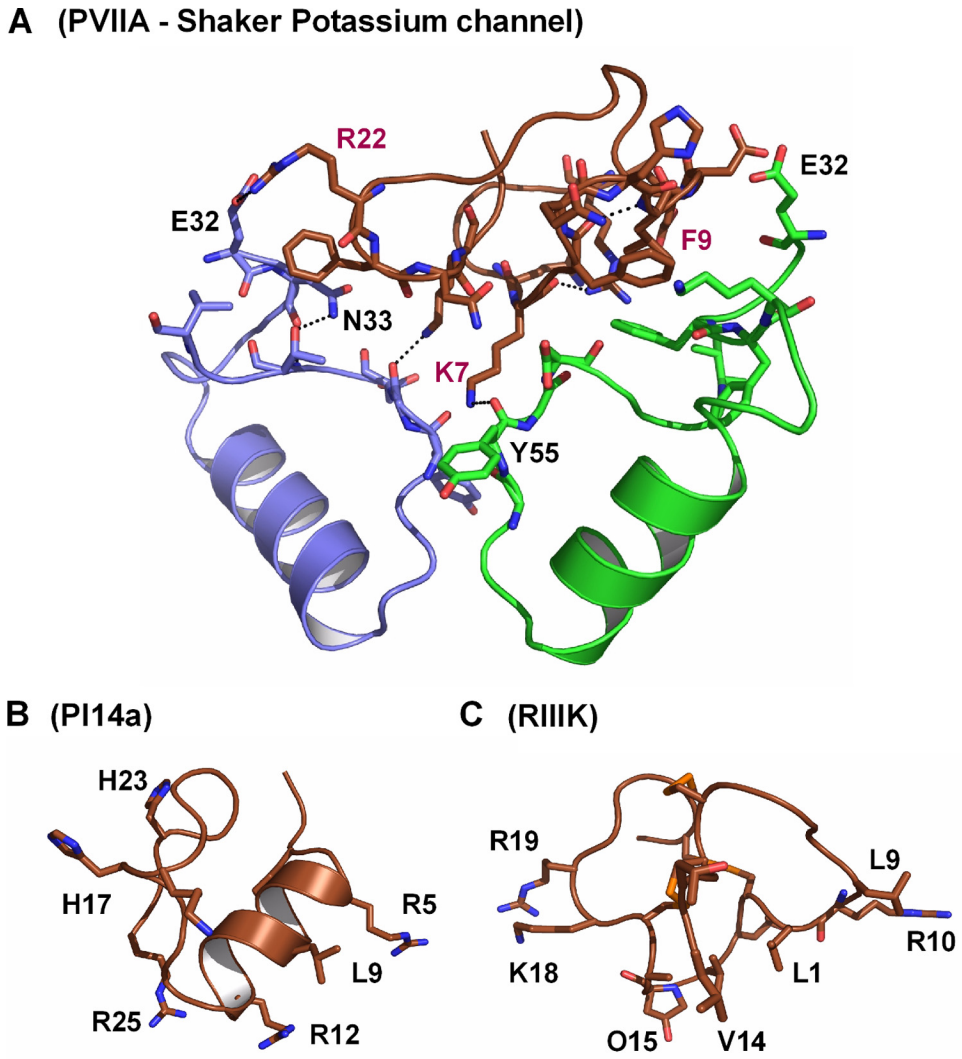

FIG. 6. Structure activity relationship of conotoxins interacting with potassium channels. A, model of the interaction between PVIIA and the shaker potassium channel, revealing a likely model of interaction of the dyad comprising Lys7 and Phe9. B, structure of Pl14a, a κJ-conotoxin. C, structure of RIIIK, a κM-conotoxin.

PVIIA (Shon et al., 1998b), indicating that other presently unidentified mammalian K_v isoforms contribute to the cardioprotective effects of PVIIA.

C. κM-Conotoxins

Like PVIIA, the κM-conotoxin RIIIJ was found to exert cardioprotective effects, presumably due to the high affinity of this peptide for mammalian $K_v1.2$ and $K_v1.2$-containing $K_v1.1$, $K_v1.5$, and $K_v1.6$ heteromultimers (Chen et al., 2010). Lys9, in conjunction with other less clearly identified residues, contributes to the high affinity of RIIIJ for $K_v1.2$. It is noteworthy that RIIIJ was less potent at $K_v1.2/K_v1.7$-heteromultimers than the related κM-conotoxin RIIIK, demonstrating that different K_v channel heteromultimers are pharmacologically distinct. Several residues, including Leu1, Arg10, Lys18, and Arg19, were found to contribute critically to activity of RIIIK at the trout *TSha1* K^+ channel, the K_v isoform most sensitive to block by RIIIK (Al-Sabi et al., 2004). It is surprising that these residues do not seem to form part of a functional dyad but interact with the channel by means of electrostatic interactions (Al-Sabi et al., 2004; Verdier et al., 2005). Based on docking interactions at the Shaker potassium channel, residues potentially involved in the pharmacophore are indicated in Fig. 6C.

D. κJ-Conotoxins

pl14a, a member of the J superfamily, is an unusual conotoxin in that it inhibits both $K_v1.6$ and several isoforms of the nicotinic acetylcholine receptor (nAChR) (Imperial et al., 2006). Homology modeling suggests that this peptide possesses both the putative functional dyad, formed by residues Lys18 and Tyr19, and a ring of basic residues comprising Arg3, Arg5, Arg12, and Arg25, reminiscent of the pharmacophore found in the κM-conotoxin RIIIK (Mondal et al., 2007). However, it remains to be determined whether these residues are involved in interactions with K_v channels as predicted. Based on docking

interactions at the Shaker potassium channel, residues potentially involved in the pharmacophore are indicated in Fig. 6B.

E. κI(2)-Conotoxins

ViTx, sr11a, and BeTX belong to the κI(2) class of conotoxins and constitute an interesting class of structurally related potassium channel modulators. ViTx inhibits $K_v1.1$ and $K_v1.3$ but not $K_v1.2$ (Kauferstein et al., 2003), whereas the recently identified sr11a inhibits $K_v1.2$ and $K_v1.6$ but not $K_v1.3$ (Aguilar et al., 2010). In contrast, BeTX displays an unusual potentiation of Ca^{2+}- and voltage-dependent BK channel current by increasing the channel open probability (Fan et al., 2003). This effect was independent of channel inactivation and was readily reversible, suggesting an interaction with an extracellular binding site (Fan et al., 2003). The molecular basis of the unique subtype selectivity of the κI(2) conotoxins remains unknown to date. The only lysine in ViTx occurs at the C terminus, suggesting that the conserved dyad motif is not involved and implicates alternate mechanisms of interaction with K_v channels (Kauferstein et al., 2003). Likewise, sr11a seems to lack a functional dyad motif; however, homology modeling identified Arg17 and Arg29 as residues possibly involved in the biological activity of this novel peptide (Aguilar et al., 2010).

F. Contryphan-Vn

Contryphan-Vn was recently identified as a D-tryptophan-containing disulfide-constrained nonapeptide with activity at both voltage-gated and Ca^{2+}-dependent K^+ channels, perhaps owing to the presence of a unique Lys-Trp dyad. However, the structural requirements for activity of Contryphan-Vn at these molecular targets, as well as its subtype-selectivity, remain to be determined (Massilia et al., 2003).

G. Conkunitzin-S1

The potassium channel-targeted toxin conkunitzin-S1 is a 60-amino acid peptide that, despite belonging to the Kunitz domain family of proteins, is stabilized by only two disulfide bonds (Bayrhuber et al., 2005). Conkunitzin-S1 was shown to inhibit the Shaker potassium channel by interacting with the ion channel pore through K427 (Bayrhuber et al., 2005).

IV. Conotoxins Interacting with Ligand-Gated Ion Channels

Cone snails have evolved multiple classes of conopeptides to target ligand-gated ion channels, including nAChR, 5-hydroxytryptamine$_3$ (5-HT$_3$), and *N*-methyl-D-aspartate (NMDA) antagonists as well as α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) enhancers. Of these, antagonists of nAChRs are the largest and most diverse and, along with the NMDA antagonists, show most potential as leads to new ligand-gated ion channel therapeutics (Table 8).

A. Conotoxin Inhibitors of Nicotinic Acetylcholine Receptors

All *Conus* spp. venoms investigated to date contain at least one conotoxin that inhibits the nAChR (McIntosh et al., 1999; Dutertre et al., 2007). Overall, seven different families of conotoxins are known to target the nAChR: α-conotoxins, αC-conotoxins, αD-conotoxins, ψ-conotoxins, αS-conotoxins, αL-conotoxins, and αJ-conotoxins (Table 8). The α-conotoxins are selective antagonists of the muscle-type (3/5) and neuronal-type (4/7, 4/4, and 4/3) nAChRs and arguably represent the largest group of characterized *Conus* spp. peptides (McIntosh et al., 1999) (Table 8). α-Conotoxins selective for neuronal subtype of nAChR have significantly contributed to their characterization both in vivo and in vitro, and some of these peptides may have therapeutic potential (Livett et al., 2006). The pharmacophore of these α-conotoxins has been thoroughly investigated and has been shown to be composed of a conserved hydrophobic patch in the first loop that determines binding and a more variable second loop that confers selectivity through pairwise interactions with different nAChR subunits (Dutertre et al., 2005).

One of the first structure-activity relationship studies on α-conotoxin was carried out on the closely related PnIA and PnIB (Fainzilber et al., 1994a). The sequences differ by only two amino acids at position 10 and 11, yet PnIA is selective for α3β2, whereas PnIB binds preferentially to α7 nAChR. It was later determined that position 10 alone was responsible for the shift in nAChR selectivity (Hogg et al., 1999; Luo et al., 1999). In addition, sequential truncation of the second loop was shown to influence potency and ultimately significantly alter the structure of PnIA (Jin et al., 2008). On the receptor side, mutation of three residues on the α3 nAChR subunit (Pro182, Ile188, and Gly198) affected the high affinity of PnIA (Everhart et al., 2003). These structure-activity relationship studies allowed the construction of a model of the interaction between PnIA and nAChR (Dutertre et al., 2004), which was consistent with the subsequent cocrystal structure of the acetylcholine binding protein (AChBP) and a variant of PnIA (Celie et al., 2005).

ImI is another α-conotoxin for which structure-activity relationship studies have identified determinants that influence potency. Modification of Asp5, Pro6, Arg7, and Trp10 altered the potency of ImI at the α7 nAChR (Quiram and Sine, 1998a, b). Later, thermodynamic mutant cycle analysis identified pairwise interactions between ImI and α7 nAChR (Quiram et al., 1999). The results revealed a major interaction between Arg7 of ImI and Tyr195 in α7, accompanied by smaller contributions between Asp5 of ImI and Trp149, Tyr151, and Gly153 of α7, and between Trp10 of ImI and Thr77 and Asn111 of α7. Again, most of these pairwise interactions were confirmed in the struc-

TABLE 8

*α-Conotoxins inhibiting nAChRs (loop formula **C0C3–4C3–7C**, excluding 3 + disulfide bonded and framework XIV α-conotoxins)*

Cysteines are bolded and aligned separately for the different α-conotoxin classes.

Species	Diet	Name	Sequence (Disulfide Bonded 1–3/2–4) 12 3 4	nAChR select	References
C. achatinus	P	α-Ac1.1a	-NGR**CC**-HPA**C**--GKHFN**C***	Muscle nAChR	Liu et al., 2007b
		α-Ac1.1b	-NGR**CC**-HPA**C**--GKHFS**C***	Muscle nAChR	Liu et al., 2007b
C. anemone	V	α-AnIA	----**CC**SHPA**C**AANNQDy**C***	α3β2	Loughnan et al., 2004
		α-AnIB	--GG**CC**SHPA**C**AANNQDy**C***	α3β2 > α7	Loughnan et al., 2004
C. arenatus	V	α-ArIA	IRDE**CC**SNPA**C**RVNNPHV**C**RRR	α7 ~ α3β2	Whiteaker et al., 2007
		α-ArIB	--DE**CC**SNPA**C**RVNNPHV**C**RRR	α7 > α3β2	Whiteaker et al., 2007
C. aulicus	M	α-AuIA	---G**CC**SYPP**C**FATNSDY**C***	α3β4	Luo et al., 1998
		α-AuIB	---G**CC**SYPP**C**-FATNPD**C***	α3β4 > α7	Luo et al., 1998
		α-AuIC	---G**CC**SYPP**C**FATNSGY**C***	α3β4	Luo et al., 1998
C. bullatus	P	α-BuIA	---G**CC**STPP**C**---AVLY**C***	α6α3β2 > α6α3β4 > α3β2	Azam et al., 2005
C. consors	P	α-CnIA	--GR**CC**-HPA**C**--GKYYS**C***	Muscle nAChR	Favreau et al., 1999
C. ermineus	P	α-EI	-RDO**CC**YHPT**C**NMSNPQI**C***	Muscle nAChR (α/δ > α/γ)	Martinez et al., 1995
C. episcopatus	M	α-EpI	---G**CC**SDPR**C**NMNNPDy**C***	α7	Loughnan et al., 1998; Nicke et al., 2003b
C. geographus	P	α-GI	---E**CC**-NPA**C**--GRHYS**C***	Muscle nAChR	Gray et al., 1981
		α-GIA	---E**CC**-NPA**C**--GRHYS**C**GK	Muscle nAChR	Gray et al., 1981
		α-GIC	---G**CC**SHPA**C**AGNNQHI**C***	α3β2 > α4β2	McIntosh et al., 2002
		α-GID	IRDγ**CC**SNPA**C**RVNNOHV**C**	α7 ~ α3β2 > α4β2	Nicke et al., 2003a
		α-GII	---E**CC**-HPA**C**G--KHFS**C***	Muscle nAChR	Gray et al., 1981
C. imperialis	V	α-ImI	---G**CC**SDPR**C**-AWR---**C***	α3β2 > α7 > α9	McIntosh et al., 1994; Ellison et al., 2004
		α-ImII	---A**CC**SDRR**C**-RWR---**C***	α7, noncompetitive	Ellison et al., 2004
C. leopardus	V	α-Lp1.1	---G**CC**ARAA**C**AGIHQEL**C***	α3β2 > α6α3β2	Peng et al., 2008
C. litteratus	V	α-LtIA	---G**CC**ARAA**C**AGIHQEL**C***	α3β2 > α6α3β2β3	Luo et al., 2010
C. magus	P	α-MI	--GR**CC**-HPA**C**--GKNYS**C***	Muscle nAChR	McIntosh et al., 1982
		α-MII	---G**CC**SNPV**C**HLEHSNL**C***	α6α3β2β3 > α3β2 > α7	Cartier et al., 1996
C. omaria	M	α-OmIA	---G**CC**SHPA**C**NVNNPHI**C**G*	α3β2 > α7	Talley et al., 2006
C. pergrandis	V	α-PeIA	---G**CC**SHPA**C**SVNHPEL**C***	α9/α10 > α3β2 > α3β4	McIntosh et al., 2005
C. purpurascens	P	α-PIA	-RDP**CC**SNPV**C**TVHNPQI**C***	α6α3β2	Dowell et al., 2003
		α-PIB	ZSOG**CC**WNPA**C**VKNR---**C***	Muscle	López-Vera et al., 2007b
C. pennaceus	M	α-PnIA	---G**CC**SLPP**C**AANNPDy**C***	α3β2 > α7	Fainzilber et al., 1994a
		α-PnIB	---G**CC**SLPP**C**ALSNPDy**C***	α7 > α3β2	Fainzilber et al., 1994a
C. quercinus	V	α-Qc1.2	---Q**CC**ANPP**C**KHVN---**C***	α3β2, α3β4	Peng et al., 2009
C. regius	V	α-Reg1b/c	---G**CC**SDOR**C**KHQ----**C***	N.D.	Franco et al., 2006
		α-Reg1d	---G**CC**SDPR**C**KHE----**C***	N.D.	Franco et al., 2006
		α-Reg1e	---G**CC**SDOR**C**RYR----**C***	N.D.	Franco et al., 2006
		α-Reg1f	--DY**CC**RROO**C**TLI----**C***	N.D.	Franco et al., 2006
		α-Reg2a	---G**CC**SHPA**C**NVNNPHI**C***	N.D.	Franco et al., 2006
		α-RgIA	---G**CC**SDPR**C**RYR----**C**R	α9/α10	Ellison et al., 2006
C. striatus	P	α-SI	---I**CC**-NPA**C**-GPKYS-**C***	Muscle nAChR	Zafaralla et al., 1988
		α-SIA	---Y**CC**-HPA**C**-GKNFD-**C***	Muscle nAChR	Myers et al., 1991
C. spurius	V	α-SrIA	--RT**CC**SROT**C**RMγYPγL**C**G*	Muscle/α4β2	López-Vera et al., 2007a
		α-SrIB	--RT**CC**SROT**C**RMEYPγL**C**G*	Muscle/α4β2	López-Vera et al., 2007a
C. tinianus	V	α-TiIA	--GG**CC**SHPA**C**-QNNPDy**C***	Neuronal nAChR	Kauferstein et al., 2011
C. textile	M	α-TxIA	---G**CC**SRPP**C**IANNPDL**C**	α3β2 > α7	Dutertre et al., 2007
C. victoriae	M	α-VcIA	---G**CC**SDPR**C**NYDHPEI**C***		Sandall et al., 2003; Vincler et al., 2006
C. striatus	P	α-SII	---G**CCC**NPA**C**GPNYG--**C**GTS**C**S	Muscle nAChR	Ramilo et al., 1992
C. ermineus	P	αA-EIVA	---G**CC**GPYONAA**C**HO**C**GC**K**VGROOY**C**DROSGG*	Muscle nAChR	Jacobsen et al., 1997
		αA-EIVB	---G**CC**GKYONAA**C**HO**C**GC**T**VGROOY**C**DROSGG*	Muscle nAChR	Jacobsen et al., 1997
C. obscurus	P	αA-OIVA	----**CC**G-VONAA**C**HO**C**V**C**KNT----**C***	Muscle nAChR	Teichert et al., 2004
		αA-OIVB	----**CC**G-VONAA**C**PO**C**V**C**NKT----**C**G*	Fetal > adult muscle nAChR	Teichert et al., 2005b
C. pergrandis	V	αA-PeIVA	----**CC**G-VONAA**C**HO**C**V**C**TGK----**C**	Fetal > adult muscle	Teichert et al., 2006
		αA-PeIVB	----**CC**G-IONAA**C**HO**C**V**C**TGK----**C**	Fetal > adult muscle	Teichert et al., 2006
C. purpurascens	P	αA-PIVA	---G**CC**GSYONAA**C**HO**C**S**C**KDROS-Y**C**GQ*	Muscle nAChR	Hopkins et al., 1995
		ψ-PIIIE	-HOO**CC**LYGK**C**RRYOG**C**SSAS-----**CC**QR*	Muscle, noncompetitive	Shon et al., 1997
		ψ-PIIIF	-GOO**CC**LYGS**C**ROFOG**C**YNAL-----**CC**RK*	Muscle, noncompetitive	Van Wagoner et al., 2003
C. litteratus	V	αL-LtXIVA	-----M**C**PPL**C**KPS-------**C**TN**C***	Neuronal nAChR	Peng et al., 2006
C. planorbis	V	αJ-PlXIVA	FPRPRI**C**NLA**C**RAGIGHKYPF**C**H**C**R*	Muscle > α3β2	Imperial et al., 2006
C. radiatus	P	αS-RVIIIA	K**C**NFDK**C**KGTGVYN**C**Gγ**C**S**C**γGLHS **C**RC**T**YNIGSMKSG**C**A**C**I**C**TYY	Muscle and neuronal nAChR	Teichert et al., 2005a
C. parius	P	ψ-PrIIIE	-AAR**CC**TYHGS**C**LKEK**C**RRKY**CC***	Adult > fetal muscle nAChR	Lluisma et al., 2008
C. vexillum	V	αD-VxXXA	DVQD**C**QVSTOGSKWGR**CC**LNRV**C**GPM**CC**PASH **C**Y**C**VYHRGRGHG**C**S**C** (dimer)	N.D.	Loughnan et al., 2006
		αD-VxXXB	DDγSγ**C**IINTRDSPWGR**CC**RTRM**C**GSM**CC**PRNG**C**T **C**VYHWRRGHG**C**S**C**PG (dimer)	α7 > α3β2	Loughnan et al., 2006
		αD-VxXXC	DLRQ**C**TRNAPGSTWGR**CC**LNPM**C**GNF**CC**PRSG **C**T**C**AYNWRRGIY**C**S**C** (dimer)	N.D.	Loughnan et al., 2006

P, fish; M, molluscs; V, worms; *, C-terminal amidation; N.D., not determined.

ture of ImI bound to AChBP (Hansen et al., 2005; Ulens et al., 2006).

A third α-conotoxin of particular interest is MII, which blocks α3β2- and α6-containing nAChRs (Cartier et al., 1996). A series of analogs were designed to selectively target the α6α3β2β3 combination (McIntosh et al., 2004) and later used to determine the contribution of α6-containing nAChR in dopamine release in the striatum and to reveal a down-regulation of this subtype upon long-term nicotine exposure (McCallum et al., 2005; Perry et al., 2007). Radiolabeled and flurorescently labeled MII/analogs have also been used to demonstrate down-regulation of these α-conotoxin binding sites in nigrostriatal damaged animal models as well as in post mortem brain from humans with Parkinson's disease (Quik et al., 2004). Residues involved in MII binding on the α3β2 nAChR have been identified, and these include Lys185 and Ile188 on α3, and Thr59, Val109, Phe117, and Leu119 on the β subunit, allowing a model of the interaction to be built (Harvey et al., 1997; Dutertre et al., 2005). More recently, ArIB, which blocks both α7 and α3β2 nAChR, was compared with other α-conotoxin sequences and rationally modified to increase α7 nAChR selectivity (Whiteaker et al., 2007). This structure-function analysis yielded two analogs, V11L,V16A-ArIB and V11L,V16D-ArIB, which had low affinity for α3β2 but retained α7 nAChR activity as predicted, with an iodinated form developed as a pharmacological tool (Whiteaker et al., 2008).

GID is the only α-conotoxin with relatively high affinity for the α4β2 nAChR subtype (Nicke et al., 2003a). This α-conotoxin is unusual in that it possesses an extended N terminus of four residues, as well as two post-translational modifications. A complete alanine scan of all noncysteine residues revealed that most analogs had at least a 10-fold reduced activity at the α4β2 subtype, implying a highly specific interaction (Millard et al., 2009). Docking of GID to α4β2 or its cocrystallization with AChBP could reveal the specific interactions responsible for its α4β2 affinity and provide a rationale basis for the design of more selective analogs.

Newly discovered α-conotoxins Vc1.1, RgIA, and PeIA target the α9α10 nAChR subtype (McIntosh et al., 2005; Clark et al., 2006; Ellison et al., 2006), which may potentially be involved in immune responses and pain (Satkunanathan et al., 2005; McIntosh et al., 2009). Scanning mutagenesis of α-conotoxin Vc1.1 revealed that Ser4 and Asn9 are important for α9α10 nAChR potency (Halai et al., 2009), whereas for RgIA, Asp5, Pro6, and Arg7 were shown to be critical for both α9α10 and α7 nAChR blockade (Ellison et al., 2008). It is noteworthy that these α9α10-selective α-conotoxins are analgesic in different animal models of chronic pain, but there is an uncertainty about the true molecular target responsible for this effect. Indeed, whereas α9α10 nAChR-selective small molecules were recently shown to also provide pain relief, N-type calcium channel inactivation through a novel interaction with $GABA_B$ receptor has also been proposed (Callaghan and Adams, 2010; Zheng et al., 2011).

The determination of several X-ray crystal structures of AChBP/α-conotoxin complexes significantly advanced our understanding of the structural basis for the nAChR subtype selectivity of α-conotoxins (Celie et al., 2005; Hansen et al., 2005; Ulens et al., 2006; Dutertre et al., 2007). We were surprised to find that the three conotoxins (α-ImI, and variants of α-PnIA and α-TxIA) that have been cocrystallized with AChBP showed a similar orientation within the ACh binding pocket despite divergent primary sequences. All three structures show a strong contribution of hydrophobic contacts between a conserved proline and several hydrophobic residues of the α-conotoxins and several residues in the aromatic cage of AChBP (Fig. 7). Therefore, the different nAChR selectivity profiles arise from specific electrostatic interactions and hydrogen bonds formed between α-conotoxin and nAChR subunits. For instance, α-conotoxin TxIA uses a unique electrostatic pairing between Arg5 and AChBP-Asp195 to achieve its high-affinity binding (Dutertre et al., 2007). In addition, a tilt in the orientation of the α-conotoxin TxIA structure within the ACh binding pocket was found to correlate with nAChR subtype selectivity. Clearly, additional α-conotoxin-AChBP crystal structures and new models of α-conotoxin-nAChR interactions are expected to emerge, which should further expand our understanding of subtype-selectivity at nAChR. The structures of the 4/7 α-conotoxin A10L-TxIA and the smaller 4/3 α-conotoxin ImI and their interactions with the AChBP are shown in Fig. 7.

Compared with the α-conotoxins, the other conotoxin families targeting nAChRs are less well understood, both in their mode of action and pharmacophore. These nontraditional α-conotoxins are often named with a letter to designate their superfamily origins. For example, the first member of the αC-conotoxin family, PrXA, was isolated from the venom of *Conus spurius* (Jimenez et al., 2007). This 32-amino acid peptide induced paralysis when injected into mice and fish and was shown to potently inhibit the muscle nAChR in vitro in a competitive manner. The primary structure of this peptide seems very different from that of α-conotoxins, with only one disulfide bond and three hydroxyprolines. A second family comprises αS-Conotoxin RVIIIA, which is also unique, with a broad selectivity profile against nAChR subtypes and the presence of γ-carboxyl glutamates, a post-translational modification not otherwise found in conotoxins targeting nAChRs (Teichert et al., 2005a). RVIIIA is also paralytic to mice and fish but elicits seizures in mice upon intracranial injection. Electrophysiological recordings of recombinantly expressed nAChR in oocytes revealed it competitively inhibited several neuronal subtypes in addition to the muscle nAChR. Within the same family is σ-GVIIIA, a peptide that targets another ligand-gated ion channel, the serotonin receptor (see section IV.B). Conotoxin lt14a belongs to yet another family αL-conotoxin and was discovered using a cDNA library from the venom gland of *C. litteratus* (Peng et al.,

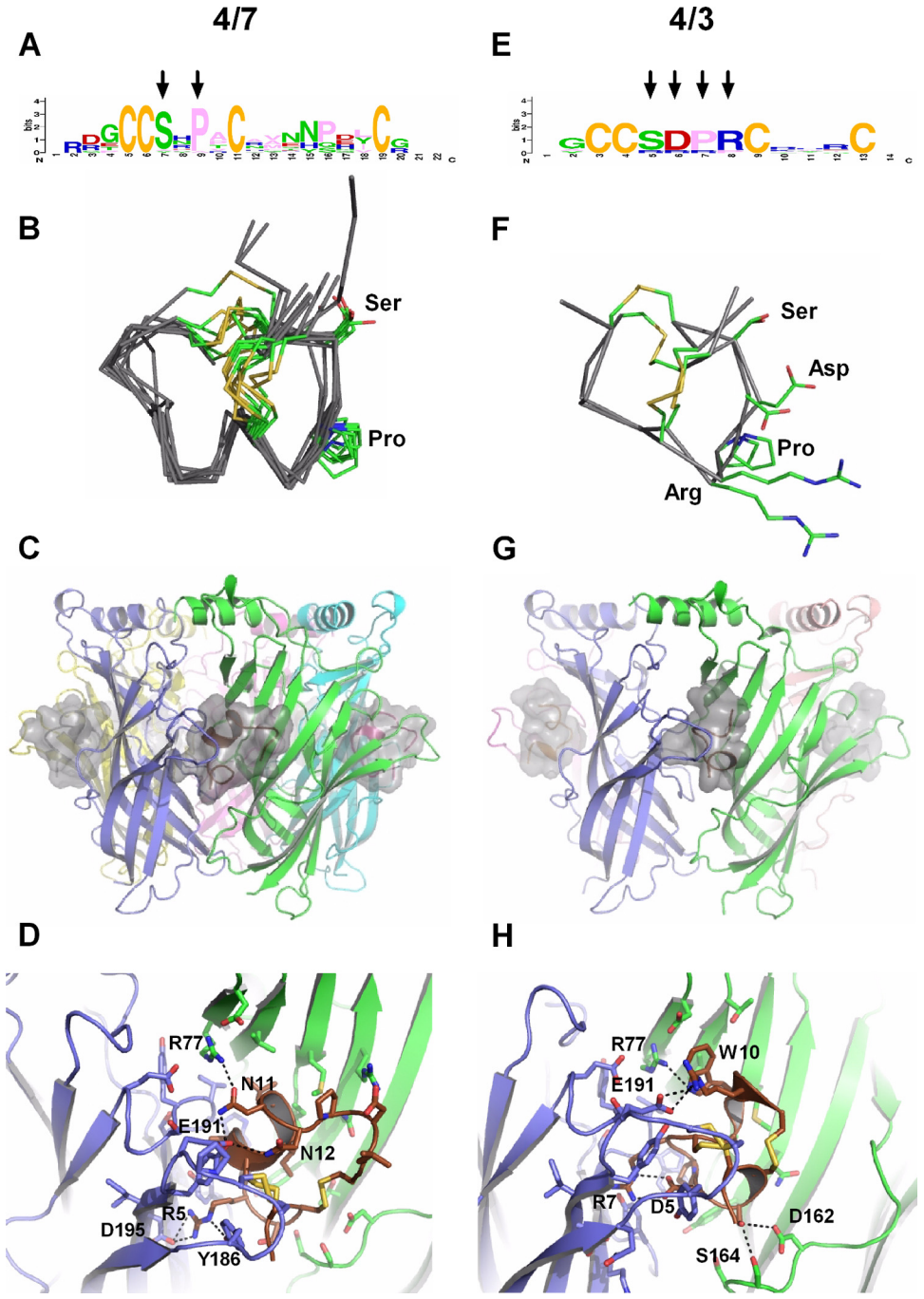

FIG. 7. Structure activity relationship of 4/7 (A, B, C, and D) and 4/3 (E, F, G, and H) α-conotoxins. A, the consensus sequence for 4/7 α-conotoxins. B, overlay of the structures of α-conotoxins OmIA (2GCZ), EI (1K64), GIC (1UL2), GID (1MTQ), MII (1MII), PIA (1ZLC), PnIA (1PEN), PnIB (1AKG), and Vc1.1 (2H8S). C, crystal structure of α-A10L-TxIA bound to AChBP. D, details of the interactions of α-A10L-TxIA with AChBP. E, consensus sequence for 4/3 α-conotoxins. F, overlay of the structures of α-ImI (1CNL) and RgIA (2JUT). G, crystal structure of ImI bound to AChBP. H, details of the interactions of α-ImI with AChBP.

2006). The sequence showed no homology to other families, but the cysteine arrangement is reminiscent of that of the α-conotoxins. A synthetic version was tested on frog nerve preparations and PC12 cells, assuming the α-conotoxin cysteine connectivity (1–3; 2–4), and it was found to block the response of ACh-evoked currents. It is noteworthy that although this peptide has not been tested across the various subtypes of nAChRs, lt14a produced dose-dependent analgesia in a hot-plate assay in mice, suggesting that it may target therapeutically relevant nAChRs. Further atypical α-conotoxins include the αJ-pl14a, which was recently identified with an unusual dual activity at two different classes of ion channels (Imperial et al., 2006). Indeed, this 25-amino acid peptide inhibits both $K_v1.6$ and muscle and $\alpha3\beta2$ nAChRs (see section III.D). Its well defined structure, comprising an α-helix and two short 3_{10}-helices, may allow future struc-

ture-activity relationship studies to reveal whether this peptide uses the same or different pharmacophores to interact with both receptors.

An unusually large family of conopeptides found only in one clade of worm-hunting cone snails also inhibit nAChRs (Loughnan et al., 2006, 2009; Kauferstein et al., 2009). These αD-conotoxins have a molecular mass of ~11 kDa, with Edman degradation, mass spectrometric, and NMR data revealing they assemble as a pseudo-homodimer comprising two 47- to 50-residue peptides. So far, αD-conotoxins have been isolated only from worm-hunting species of cone snails where traditional α-conotoxins seem to be absent. Therefore, they may represent another example of convergent evolution to a common prey target. Binding assays and two-electrode voltage-clamp analyses showed that αD-conotoxins are potent inhibitors of several nAChRs, including α7, α3β2, and α4β2 subtypes. Finally, several noncompetitive conotoxins have also been discovered (Shon et al., 1997). These ψ-conotoxins show homology to the μ-conotoxins, which are Na_v channel pore blockers. PIIIE was originally shown to block muscle nAChR but targets a different binding site compared with α-bungarotoxin. PrIIIE was shown to also potently inhibit muscle nAChR, with a preference for the adult over the fetal subtype (Lluisma et al., 2008). Thus, it seems that *Conus* spp. have devised many original solutions to target this important physiological target, providing us with as many templates for nAChR subtype-selective tools and drugs.

B. σ-Conotoxin Inhibitors of Serotonin 5-HT$_3$ Receptors

One family of conotoxins targets the 5-HT_3 receptor, pentameric, cation-selective channels that open in response to the binding of the neurotransmitter serotonin (Hoyer et al., 1994) and share overall topology with nicotinic receptors. Five human subtypes (5-HT_{3A}, 5-HT_{3B}, 5-HT_{3C}, 5-HT_{3D}, and 5-HT_{3E}) have been cloned (Karnovsky et al., 2003; Niesler et al., 2003) but only homomeric 5-HT_{3A} and heteromeric combinations of 5-HT_{3A} and 5-HT_{3B} have been pharmacologically characterized in detail. To date, only the large σ-conotoxin GVIIIA has been identified to target 5-HT_3 receptors (England et al., 1998). GVIIIA inhibits 5-HT_3 receptors expressed in *Xenopus laevis* oocytes in a competitive manner. This 41-amino acid peptide contains 10 cysteines (five disulfide bonds), an amidated C terminus, and the unusual post-translational modification 6-bromoptryptophan. Unfortunately, this complicated structure precluded chemical synthesis and any structure-activity relationship studies at the receptor.

C. Ikot-Ikot Conopeptide Enhancers of α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid Receptors

A novel polypeptide isolated from *C. striatus* venom has been shown to selectively target AMPA receptors (Walker et al., 2009). This toxin, named con-ikot-ikot (referring to the Filipino word for "spinning around" in reference to the swimming phenotype observed in fish injected with this toxin), is 86 amino acids long with 13 cysteines, corresponding to a molecular mass of 9432 Da. It is noteworthy that the active form of con-ikot-ikot is a dimer of dimers, as evidenced by a band corresponding to a molecular mass of 38 kDa on an electrophoresis gel. This peptide achieves its biological effect through block of AMPA desensitization, greatly increasing the current magnitude. AMPA receptors belong to the ionotropic glutamate receptors, and together with NMDA and kainate receptors, mediate excitatory neurotransmission in the central nervous system (Dingledine et al., 1999). Native AMPA receptors assemble as heterotetramers (dimer of dimers) to form a functional channel. Each subunit (GluA1–4) consists of four domains: the N-terminal domain, the ligand binding domain, the pore forming transmembrane domain, and finally a small C-terminal domain (Chen and Wyllie, 2006). The structure of intact AMPA receptors was solved by Sobolevsky et al. (2009), revealing a dimer of dimers arrangement of four subunits. This structure is expected to help identify the binding site of con-ikot-ikot and guide further structure-activity studies for this novel class of conotoxin.

TABLE 9
Conantokins inhibiting NMDA glutamate receptors

Species	Diet	Name	Sequence	NMDA Selectivity	References
C. caracteristicus	V	Con-Ca2	GYγγRγIAγTVRγLEEA	N.D.	Franco et al., 2006
C. ermineus	P	Con-E	GEγγHSKYQγCLRγIRVNNVQQγC	N.D.	Gowd et al., 2008
C. geographus	P	Con-G	GEγγLQγNQγLIRγKSN*	NR2B > NR2C, NR2D	McIntosh et al., 1984; Gowd et al., 2008
C. gloriamaris	M	Con-Gm	GAKγRNNAγAVRγRLEEI	N.D.	Franco et al., 2006
C. lynceus	V	Con-L	GEγγVAKMAAγLARγDAVN*	N.D.	Jimenez et al., 2002
C. purpurascens	P	Con-P	GEγγHSKYQγCLRγIRVNKVQQγC	NR2B, NR2A	Gowd et al., 2008
C. orchroleucus	V	Con-Oc	GEγγRKAMAγLEAKKAQγALKA	N.D.	Franco et al., 2006
C. parius	P	Con-Pr1	GEDγYAγGIRγYQLIHGKI	NR2B, NR2D	Teichert et al., 2007b
		Con-Pr2	DEPγYAγAIRγYQLKYGKI	NR2B > NR2D	Teichert et al., 2007b
		Con-Pr3	GEPγVAKWAγGLRγKAASN*	NR2B, NR2D	Teichert et al., 2007b
C. quercinus	V	Con-Qu	GYγγRγVAγTVRγLDAA	N.D.	Franco et al., 2006
C. radiatus	P	Con-R	GEγγVAKMAAγLARγNIAKGCKVNCYP	NR2B, NR2A	White et al., 2000
C. sulcatus (brettinghami)	P	Con-Br	GDγγYSKFIγRERγAGRLDLSKFP	NR2B, NR2D, NR2A > NR2C	Twede et al., 2009
C. tulipa	P	Con-T	GEγγYQKMLγNLRγAEVKKNA*	NR2B, NR2A	Haack et al., 1990; Klein et al., 2001

P, fish; M, molluscs; V, worms; γ, γ-carboxyglutamate; *, C-terminal amidation; N.D., not determined.

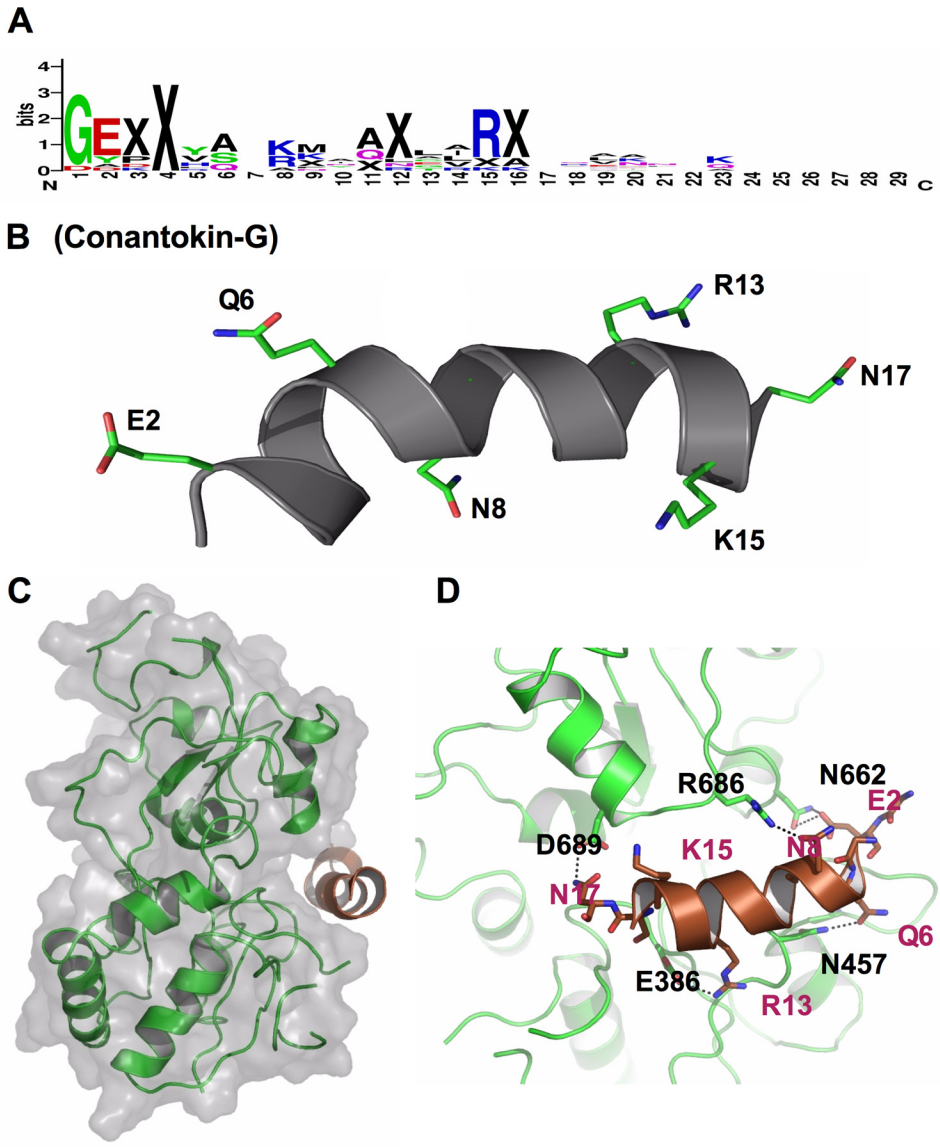

FIG. 8. Structure activity relationship of conantokins. A, consensus sequence for conantokins. B, structure of conantokin-G. C, model of likely interactions between conantokin-G and extracellular domain of the NMDA receptor NR2 subunit. D, details of the interactions between conantokin-G and NR2.

D. Conantokin Inhibitors of N-Methyl-D-aspartate Receptors

Conantokins are small peptides (17–22 amino acids) isolated from the venom of various *Conus* species that selectively and potently inhibit NMDA receptors. The first conantokin isolated was Con-G; more recent members have been isolated from another 11 species (see Table 9). Conantokins have therapeutic potential as analgesics (Malmberg et al., 2003) and anticonvulsants (Jimenez et al., 2002). As opposed to the vast majority of conopeptides, conantokins do not contain cysteines but instead have a high content of γ-carboxyglutamic acid residues that when correctly spaced, induce α-helicity in the presence of divalent cations. Structure-activity studies of conantokins (Nielsen et al., 1999b) have revealed a number of important residues for binding, and a docking model showed that Con-G can fit into the agonist binding cleft of the NR2 subunit (see Fig. 8) (Wittekindt et al., 2001), revealing interactions between Glu386/Asp689 and Asn662 from NR2 subunit and Asn17/Lys15 and Glu2 of Con-G, respectively.

NMDA receptors are tetrameric ligand-gated ion channels with high Ca^{2+} permeability that mediate fast excitatory neurotransmission in the CNS (Dingledine et al., 1999). They are composed mainly of two NR1 and two NR2 subunits, with an NR3 subunit potentially coassembling with NR1 and NR2 to modify NMDA receptor-mediated responses, reducing calcium permeability and single-channel conductance (Ciabarra et al., 1995). The topology of each subunit comprises an extracellular N-terminal domain, an agonist binding core, three transmembrane domains (domains 1 and 2 are separated by a "P-loop"), and a cytoplasmic tail of a variable length (Mayer and Armstrong, 2004). For the proper functioning and gating of NMDA receptors, the binding of both the agonist gluta-

mate (on the NR2 subunit) and the coagonist glycine (on the NR1 subunit) is required at the same time. Differential expression of isoforms (i.e., NR2A–D) occurs in the central nervous system, where they mediate fast excitatory neurotransmission. NMDA receptors have also been implicated in a number of chronic and acute neurological disorders, and significant research efforts are currently directed toward the development of specific drugs that can inhibit these targets (Loftis and Janowsky, 2003). Atomic resolution structures of the NMDA extracellular binding domains bound to agonists, partial agonists, and antagonists have been determined that might open the way to the rational development of NMDA receptor antagonists (Furukawa and Gouaux, 2003; Furukawa et al., 2005). Based on the docking model shown in Fig. 8, it is conceivable that conantokin analogs might be rationally designed to have selectivity for subunit combinations of clinical interest.

E. Ligand-Gated Ion Channels Inhibitors in Pain Management

A number of ligand-gated ion channels have been implicated in pain modulation and chronic pain states but this discussion will focus on the two families of channels with evidence for application of conopeptides. Conopeptides targeting subtypes of nAChR subtypes (α-conotoxins) and NMDA receptors (several conantokins) have shown good efficacy in preclinical pain models and have reached different stages of development for use in humans. Their pharmacological characteristics underlying their analgesic effects are discussed below.

The involvement of nAChR subtypes in pain modulation is complex, and the mechanisms of action of α-conotoxins in pain relief remains controversial. Much of the difficulty in resolving the actual targets of these toxins arises from the complexity of physiologically and pathologically relevant nAChR subtypes found in vivo, together with limited knowledge of expression and role of subunits in pain pathways (Olivera et al., 2008). For example, nicotinic agonists selective for $\alpha4\beta2$ subtypes of nAChRs produce analgesic actions in neuropathic pain models (Bannon et al., 1998; Rashid and Ueda, 2002), probably as a result of stimulation $\alpha4\beta2$ nAChRs on inhibitory interneurons in the dorsal horn of the spinal cord (Genzen and McGehee, 2005). By contrast, analgesic α-conotoxins are selective antagonists of different neuronal nAChR subtypes (and agonists at $GABA_b$; see below) but avoid muscle or $\alpha4\beta2$ nAChRs.

Synthetic Vc1.1 (ACV1) identified from cDNA was the first α-conotoxin shown to exhibit efficacy in pain models (Sandall et al., 2003; Satkunanathan et al., 2005). The peptide was potent and peripherally active after intramuscular injection, producing partial reversal of mechanical allodynia in several neuropathic pain models. The findings that the effectiveness of the peptide escalated with repeated daily injections and seemed to allow functional recovery of injured nerves (Satkunanathan et al., 2005) suggested that it had clinical potential in treatment of neuropathic pain. Vc1.1 was initially selected for testing in pain models on the basis of its potent block of neuronal (bovine adrenal chromaffin cells) versus muscle nAChRs (Livett et al., 2006; see Table 10). Bovine adrenal chromaffin cells express $\alpha3$-, $\alpha5$-, $\alpha7$-, and $\beta4$-nAChR subunits (Sala et al., 2008). Accordingly, the main nAChR subtypes expressed on nociceptive peripheral fibers and their central terminations are composed of $\alpha3$, $\alpha5$, $\beta4$, and $\beta2$ subunits (Khan et al., 2003; Lang et al., 2003; Rau et al., 2005). Vc1.1 also inhibited peripheral nerve-mediated vascular responses in rats (Satkunanathan et al., 2005) and, at low concentrations (100 nM), inhibited excitatory responses of

TABLE 10
Target selectivity and efficacy of analgesic α-conotoxins administered systemically

α-Conotoxins	Efficacy	nAChR selectivity	Nanomolar $GABA_B$-Ca_v Activity	References
Vc1.1	Partially reverses mechanical allodynia in several neuropathic models after intramuscular dosing. Escalating actions and recovery of nerve function.	$\alpha9\alpha10 \gg \alpha6\alpha3\beta2\beta3 > \alpha6\alpha3\beta4 > \alpha3\beta4 \sim \alpha3\beta2$	Yes	Sandall et al., 2003; Satkunanathan et al., 2005; Vincler et al., 2006; Klimis et al., 2011
CyclizedVc1.1	Partially reverses mechanical allodynia in several neuropathic models after oral dosing.	Very weak $\alpha9\alpha10$	Yes	Clark et al., 2010
Vc1a	No reversal of allodynia in a neuropathic pain model. Recovery of nerve function retained	$\alpha9\alpha10 \gg \alpha3\beta4 \sim \alpha3\beta2$	No	Nevin et al., 2007
Rg1A	Partially reverses mechanical allodynia in several neuropathic models after intramuscular dosing.	$\alpha9\alpha10 \gg \alpha7 \gg \alpha3\beta4 \sim \alpha3\beta2$	Yes	Vincler et al., 2006
AuIB	Partially reverses mechanical allodynia in several neuropathic models after intramuscular dosing.	$\alpha3\beta4 > \alpha3\beta2 \gg \alpha9\alpha10$	Yes	Klimis et al., 2011
MII	Partially reverses mechanical allodynia in several neuropathic models after intramuscular dosing.	$\alpha3\beta2 > \alpha3\beta4 \gg \alpha9\alpha10$	No	Klimis et al., 2011
Lt14a	Acute thermal antinociception (hotplate test) after intraperitoneal administration.	Unkown but inhibits PC12 cell nAChR ($\alpha3$- $\alpha5$-, $\alpha7$-, $\beta2$-, $\beta3$-, and $\beta4$-subunits)	Unknown	Sun et al., 2011

human sural nerve to nicotine (Lang et al., 2005). Vc1.1 has relatively low affinity for $\alpha3\beta2$ and $\alpha3\beta4$ nAChRs (Clark et al., 2006; Vincler and McIntosh, 2007) but may have higher affinity for more complex subunit combinations; e.g., its affinity for $\alpha6/\alpha3\alpha2\beta2\beta3$ nAChRs is 140 nM (Vincler and McIntosh, 2007). It was therefore suggested that Vc1.1 might produce pain relief via inhibition of $\alpha3$ and/or $\alpha5$ nAChR channels on sensory nerves. It is noteworthy that the analgesic α-AuIB has relatively low affinity for $\alpha3\beta4$ but MII has very high affinity for $\alpha3\beta2$ nAChRs (~1 nM). It therefore remains possible that pain relief may be achieved in the periphery by antagonism of nAChRs comprising $\alpha3$, $\alpha5$, $\beta4$, and $\beta2$ subunits, perhaps in combination with other less common subunits. The novel α-conotoxin, lt14a, potently inhibits nAChR responses in rat PC12 cells and produces analgesia in a hotplate assay after systemic administration (Peng et al., 2006). Because PC12 cells express $\alpha3$- $\alpha5$-, $\alpha7$-, $\beta2$-, $\beta3$-, and $\beta4$-nAChR subunits (Henderson et al., 1994), the analgesic target likely involves subunit combinations that comprise one or more of these subunits. Other potential targets of Vc1.1 and other analgesic α-conotoxins are summarized in Table 10 and discussed below.

Vc1.1 was also shown to be a potent antagonist at $\alpha9\alpha10$ nAChRs, potentially contributing to its analgesic effects (Vincler et al., 2006). Another α-conotoxin, RgIA, displayed similar activity at $\alpha9\alpha10$ nAChRs and was also efficacious in neuropathic pain models (Vincler et al., 2006). However, $\alpha9\alpha10$ nAChRs show very limited tissue distribution, being expressed predominantly in the olivocochlear system (Vetter et al., 2007), and their role in sensory nerve function is unclear. A more plausible hypothesis was forwarded by Vincler and McIntosh (2007), who suggested the analgesic efficacy of α-conotoxins that inhibit $\alpha9\alpha10$ nAChRs was due to inhibition of immune cell migration to injured nerves, although it remains to be determined whether this process is modulated by $\alpha9\alpha10$ nAChRs. However, two analogs of Vc1.1, Vc1a and [P6O]Vc1.1, retain full activity $\alpha9\alpha10$ nAChRs but lose activity at other targets (see below) and fail to reverse allodynia in a neuropathic pain model (Nevin et al., 2007). Conversely, α-AuIB and α-MII are efficacious in a neuropathic pain model but do not inhibit $\alpha9\alpha10$ nAChRs (Klimis et al., 2011). These findings cast doubt on the role of $\alpha9\alpha10$ nAChRs in acute analgesia in neuropathic pain models, although immune cell interactions may contribute to the longer term functional recovery of nerves. In support, Livett et al. (2008) claimed that Vc1a retained activity at $\alpha9\alpha10$ nAChRs and lost analgesic activity as reported by Nevin et al. (2007) but retained an apparent ability to induce functional recovery in injured nerves as measured by the peripheral vascular response to substance P.

Another potential target for the pain-relieving actions of α-conotoxins was identified by Callaghan et al. (2008). This group reported that the analgesic α-conotoxins Vc.1.1, RgIA, and AuIB, but not MII, potently inhibit calcium currents mediated by N-type ($Ca_v2.2$) channels in mouse sensory neurons (Callaghan et al., 2008; Klimis et al., 2011). After extensive pharmacological characterization, they identified that this effect was blocked by $GABA_B$ receptor antagonists and mediated by nonclassical G-protein-coupled modulation of $Ca_v2.2$ channels (Callaghan et al., 2008; Klimis et al., 2011). To date, all α-conotoxins tested that potently inhibit $Ca_v2.2$ are also analgesic when administered systemically (Callaghan et al., 2008; Clark et al., 2010; Klimis et al., 2011) and analogs of Vc1.1 (Vc1a and [P6O]Vc1.1) that retain activity at $\alpha9\alpha10$ nAChRs but lose analgesic activity (Nevin et al., 2007) also lose their ability to modulate N-type currents in sensory neurons (Callaghan et al., 2008). Furthermore, modulation of N-type calcium channels persists in DRG neurons from $\alpha9\alpha10$ knockout mice (Callaghan and Adams, 2010). However, the mechanism of $GABA_B$ receptor-dependent α-conotoxin modulation of N-type calcium channels remains controversial. $Ca_v2.2$ inhibition by classic $GABA_B$ agonists is due to direct association of G-protein $\beta\gamma$ subunits with the $Ca_v2.2$ α-subunit. This effect is rapid, involves slowing of the activation of the channel, and is relieved by strong depolarizations (Herlitze et al., 1996; Ikeda, 1996). None of these properties were observed for inhibition by Vc1.1, strongly suggesting another mechanism is involved. Another form of G-protein-coupled inhibition of N-type channels in sensory neurons is voltage-independent and mediated by tyrosine phosphorylation of the e37a splice variant (Raingo et al., 2007). However, this mechanism is unlikely to account for the actions of α-conotoxins on N-type calcium channels, because direct $\beta\gamma$-subunit-mediated inhibition also occurs on both the e37a and 37b variants expressed by sensory neurons (Raingo et al., 2007). This interpretation is consistent with the failure of Vc1.1 and RgIA to modulate GIRK channels via coexpressed $GABA_B$ receptors, which is also G-protein $\beta\gamma$-mediated (McIntosh et al., 2009).

In summary, several α-conotoxins seem to be potential candidates for development of treatments for neuropathic pain. Although the mechanism(s) of action remain controversial, they produce partial relief from the signs of neuropathic pain and seem to accelerate functional recovery of sensory nerve function. The α-conotoxins are also relatively small peptides, and the recent development of a cyclized analog of Vc1.1 (Clark et al., 2010) revealed that the peptide could relieve signs of neuropathic pain when administered orally. ACV1 (Vc1.1) was taken through a phase I clinical trials by Metabolic Pharmaceuticals (Melbourne, VIC, Australia) but subsequently dropped after completion of a phase 2A trial over potential concerns over efficacy and its reduced affinity at human versus rat $\alpha9/\alpha10$ nAChRs.

Finally, NMDA receptors, particularly those in the dorsal spinal cord that include the NR2B subunit, have long been recognized as candidates for development of pain therapeutics (Parsons, 2001), with small-molecule NR2B-selective antagonists selectively inhibiting signs of chronic pain in animal models (Boyce et al., 1999).

TABLE 11
Conopeptides targeting GPCRs and monoamine transporters

Species	Diet	Name	Sequence 12 3 4	Target	Reference
C. tulipa	F	ρ-TIA	FNWR**CC**LIPA**C**RRNHKKF**C***	α1-Adrenoceptor	Sharpe et al., 2001
C. marmoreus	M	χ-MrIA	-NGV**CC**GYKL**C**HO-----**C**	NET	Sharpe et al., 2001
		χ-MrIB	-VGV**CC**GYKL**C**HO-----**C**	NET	Sharpe et al., 2001
		χ-CMrVIA	---V**CC**GYKL**C**HO-----**C**	N.D.	Balaji et al., 2000
		χ-CMrX	--GI**CC**GVSF**C**YO-----**C**	N.D.	Balaji et al., 2000
C. geographus	P	Conopressin G	**C**FIRN**C**PKG*	Vasopressin R	Cruz et al., 1987; Nielsen et al., 1994
C. imperialis	V		**C**IIRN**C**PRG*		
C. striatus	P	Conopressin S	**C**FIRN**C**OP	$V_{1b}R$, OTR > $V_{1a}R$	Cruz et al., 1987; Dutertre et al., 2008
C. textile	M	Conopressin Tx	**C**YIQN**C**LRV*	N.D.	Ueberheide et al., 2009
C. tulipa	P	Conopressin-T	**C**LIQD**C**PγG*	OT, $V_{1a}R$	Dutertre et al., 2008
C. villepini	V	Conopressin-vil	ZSEEGGSNAtKKPYIIL	N.D.	Möller and Marí, 2007
C. geographus	F	Contulakin-G	ZSEEGGSNAtKKPYIIL	Neurotensin R	Craig et al., 1999

P, fish; M, molluscs; V, worms; γ, γ-carboxyglutamate; *, C-terminal amidation; N.D., not determined.

Several conantokins, including conantokin-G, selectively inhibit NMDA receptors composed of NR1 and NR2B subunits (Layer et al., 2004) and inhibit signs of neuropathic pain intrathecally. Unfortunately, Con-G produced motor side effects in a similar dose range in nerve injury models (Malmberg et al., 2003), and the utility of intrathecal conantokins targeting NR2B for pain management remains to be established.

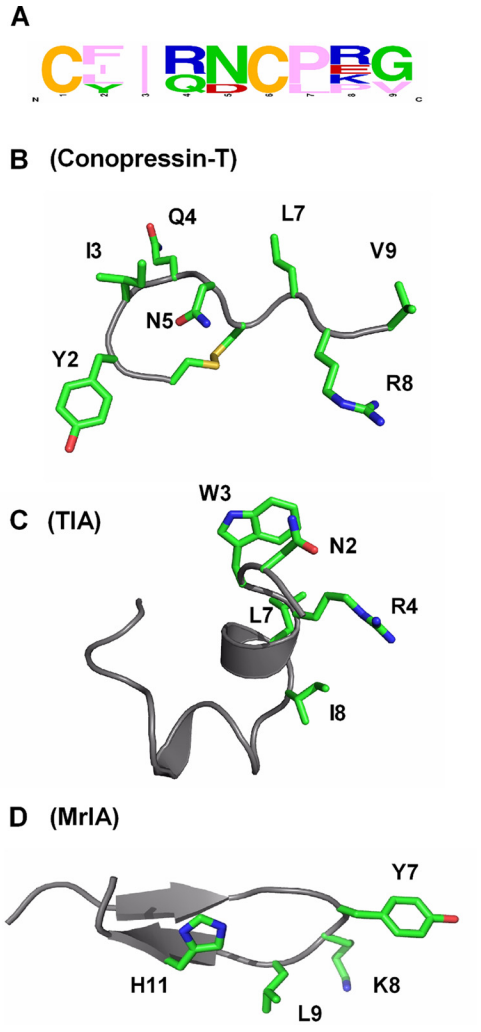

FIG. 9. Structure activity relationship of conopeptides interacting with GPCRs and transporters. A, consensus sequence for conopressins. B, structure of conopressin-T, a $V_{1A}R$ selective antagonist. C, structure of ρ-TIA, a selective, noncompetitive antagonist of the α1B-adrenergic receptor. D, structure of χ-MrIA, a selective and noncompetitive antagonist of the noradrenaline transporter.

V. Conotoxins Interacting with G Protein-Coupled Receptors and Neurotransmitter Transporters

Two novel classes of conopeptide (ρ- and χ-conopeptides) were discovered that inhibit noradrenaline activation of α1-adrenoceptors or its transport via the noradrenaline transporter (Sharpe et al., 2001). It is noteworthy that both noncompetitively (allosterically) inhibited noradrenaline, making them useful new probes of allosteric sites on these physiologically and pathologically important membrane proteins. In contrast, competitive (isosteric) agonists of the vasopressin and neurotensin receptors have also been discovered that are closely related to the endogenous peptides for these targets. Sequences of known conopeptides belonging to these four classes are shown in Table 11, and their structures are compared in Fig. 9.

A. ρ-Conopeptide Inhibitors of α1-Adrenoceptors

The conopeptide ρ-TIA is a 19-amino acid peptide with two disulfide bonds isolated from the venom of *Conus tulipa* (Sharpe et al., 2001). This peptide was originally discovered after reduction of the noradrenergic component of the electrically evoked contraction of the rat vas deferens. Competition experiments with radiolabeled ligands revealed TIA was a selective and negative allosteric modulator of hamster α_{1B} (Sharpe et al., 2003b). Additional radioligand displacement studies showed that TIA is also 10-fold selective for human α_{1B} over α_{1A} and α_{1D} (Chen et al., 2004). An alanine walk identified Asn2, Trp3, Arg4, Leu7, and Ile8 as the major binding determinants on TIA (Sharpe et al., 2003b). It is noteworthy that the first four residues in the N-terminal "tail" are key determinants of binding, despite being outside the disulfide bonds and relatively unconstrained in solution (Sharpe et al., 2003b). F18A (and F18N) analogs of TIA had increased selectivity for α_{1B}, whereas the I8A analog had slightly reduced af-

finity for α_{1B}, and its mode of action was switched to become a competitive antagonist (Chen et al., 2004).

High-resolution crystal structures of the closely related β-ARs have been determined in both the resting and activated states, as well as in complex with various ligands (Cherezov et al., 2007; Rasmussen et al., 2007, 2011a, 2011b; Warne et al., 2008; Rosenbaum et al., 2011; Warne et al., 2011). Although these structures confirmed the location of the orthosteric binding site for competitive ligands that had been mapped previously using affinity labeling, site-directed mutagenesis, and substituted-cysteine accessibility methods, the site of allosteric compounds remains rather elusive. Allosteric ligands are of particular interest to both fundamental science and the pharmaceutical industry, because they can achieve greater selectivity and act only where the endogenous agonist exerts its physiological effect (Conn et al., 2009). Using the recent crystal structure of β-adrenoceptors, modeling of the interaction between TIA and α_{1B}-AR should provide a clearer view of this allosteric binding site and potentially allow the development of more selective inhibitors.

It is worthwhile to consider the therapeutic potential of peptide inhibitors of α1-adrenoceptors, a traditional small-molecule GPCR drug target. These receptors belong to the family A of GPCRs, and therefore share the common topology comprising a core composed of seven transmembrane helices connected by three extracellular and three intracellular loops. Adrenoceptors regulate critical aspects of blood pressure control, myocardial contractility, pulmonary function, metabolism, and activities in the CNS (Koch et al., 1995). They are broadly divided in α_1-, α_2-, and β-adrenoceptors based on anatomical localization; then, each group is further separated into three subtypes based on pharmacological and molecular strategies (α_{1A}, α_{1B}, and α_{1D}; α_{2A}, α_{2B}, and α_{2C}; β_1, β_2, and β_3) (Bylund et al., 1994; Graham et al., 1996). The α_1-ARs are located on postsynaptic membranes, whereas α_2-ARs are found on presynaptic nerve terminals (Langer, 1974). In particular, α_1-ARs are crucial for the stimulation of smooth muscle contraction (Bylund et al., 1994). Agonists at the α_1-ARs may be associated with an alerting or antidepressant action, whereas antagonists are being used and refined for the treatment of benign prostatic hypertrophy (Hieble and Ruffolo, 1996). Peptides with improved selectivity and restricted to peripheral compartments have the potential to treat diseases such as benign prostatic hypertrophy.

B. χ-Conopeptide Inhibitors of the Noradrenaline Transporter

χ-Conopeptides are small hydrophobic peptides that selectively inhibit the transport of noradrenaline by NET, an important target for drugs treating a range of neurological diseases, including depression, anxiety, obsessive-compulsive disorder, and attention deficit hyperactivity disorder (Goddard et al., 2010). χ-MrIA and -MrIB noncompetitively inhibit NET (Sharpe et al., 2001), providing opportunities to regulate NET efficiently, irrespective of the concentration of noradrenaline present. χ-MrIA seems to be specific for NET, with no effect on the function of a diverse range of targets (Sharpe et al., 2001), including other monoamine neurotransmitter transporters (Sharpe et al., 2001). The solution structure of MrIA revealed that χ-conopeptides have a rigid framework stabilized by two disulfide bonds and two antiparallel strands connected by an inverse γ-turn (Sharpe et al., 2001; Nilsson et al., 2005). Although the cysteine spacings in the linear sequence are reminiscent of small α-conotoxins, χ-conopeptides have an alternative cysteine-stabilized scaffold, with cysteines bonded 1 to 4 and 2 to 3 (see Table 11), that allows a γ-turn to form in an optimized conformation for high-affinity interactions with NET. Residues in this γ turn (Gly6, Tyr7, Lys8, and Leu9), plus His11 in loop 2, have been identified as critical for high-affinity interactions at NET through alanine-scanning mutagenesis, but only mutations to Gly6 affect structure (Sharpe et al., 2003a). To define the χ-conopeptide pharmacophore, an expanded series of analogs was synthesized, revealing that χ-conopeptides interact with NET mainly through interaction of Tyr7 with NET via aromatic and/or H-bond acceptors, Lys8 contributing an essential basic residue for NET binding, Leu9 interacting with a large hydrophobic patch on NET, and His11 and hydroxyproline 12 contributing weaker interactions through aromatic and basic interactions (Brust et al., 2009).

Based on its mode of action to inhibit NET, intrathecal administration of MrIA was evaluated in rodent models of acute, inflammatory, and neuropathic pain, where it was able to produce significant reversal of the signs of acute and neuropathic pain in mice and rats, respectively, without significant side effects (McIntosh et al., 2000; Nielsen et al., 2005). To consider χ-MrIA as a therapeutic lead, its chemically unstable N-terminal asparagine needed to be replaced or removed to allow long-term human use in implanted pumps. Extensive replacements and truncations/extensions at the N-termini revealed that replacing asparagine with a pyroglutamate significantly improved chemical stability without compromising the side-effect profile observed for MrIA (Brust et al., 2009). Pyroglutamate1-MrIA (Xen2174), produced few side effects even at high doses, and an extended duration of action after a single bolus intrathecal dose in the rat model of neuropathic pain (Nielsen et al., 2005). Xen2174 was also found to accelerate recovery from pain when given pre-emptively in a rat-paw-incision model of postsurgical pain (Obata et al., 2005). Xen2174 was progressed to a phase I intravenous study, where it was determined to be safe and well tolerated in healthy volunteers, and most recently a phase IIa intrathecal study in patients with cancer pain. This open label, dose-escalating study was conducted in 37 cancer patients suffering severe chronic pain that was poorly controlled using conventional therapies. It is noteworthy that Xen2174 reportedly provided pain relief rapidly and for a sustained period across a wide range of tolerated intrathecal bolus doses from 0.1 to 30

mg. Xen2174 is presently being prepared for a phase IIb double-blind study to establish whether Xen2174 is safe and efficacious in a bunionectomy setting of postsurgical pain. Further details on the discovery and development of Xen2174 are reported in a review by Lewis (2011).

C. Conopeptides Modulating Vasopressin/Oxytocin Receptors

Conopressins were first discovered in the venom of fish-hunting cone snails based on the "scratching effect" induced in mice upon intracerebral injection (Cruz et al., 1987). The sequences of both conopressin-G and conopressin-T were very similar to vasopressin itself, with only an additional positive charge in position 4. It is noteworthy that conopressin-G was later isolated from a worm-hunting cone snail species, *Conus imperialis*, as well as from the nonvenomous snails *Lymnea stagnalis* and *Aplysia californica* and the leech *Erpobdella octoculata* (McMaster et al., 1992; Salzet et al., 1993; Nielsen et al., 1994; Van Kesteren et al., 1995). Therefore, conopressin-G was proposed to represent the endogenous vasopressin homolog in these invertebrates. However, novel conopressins have since been discovered from other cone snail species, supporting a role in envenomation (Möller and Marí, 2007; Dutertre et al., 2008; Ueberheide et al., 2009). We were surprised that conopressin-T acted as a V_1-vasopressin receptor-selective antagonist, with partial agonist activity at the oxytocin receptor and replacing Gly9 with Val9 also switched the activity of vasopressin and oxytocin from agonist to antagonist (Dutertre et al., 2008). Therefore, new conopressins may serve as templates for the design of novel ligands with tailored pharmacological profiles.

Vasopressin and oxytocin receptors belong to the GPCR family A and apparently bind vasopressins and conopressins in a pocket formed by the arrangement of the seven transmembrane domains (Mouillac et al., 1995; Dutertre et al., 2008). On the basis of extracellular domain exchanges between and oxytocin receptor, it has been proposed that the three extracellular loops are also important for agonist binding (Postina et al., 1996). Models of the interaction of vasopressin with V_1 and V_3 developed using a rhodopsin structural template were consistent with most experimental data and supported by follow-up site-directed mutagenesis (Rodrigo et al., 2007). At least three different subpopulations of vasopressin receptors have been recognized: V_1 (or V_{1a}), V_2, and V_3 (or V_{1b}) (Birnbaumer, 2000). V_1 regulates blood pressure and vasoconstriction, whereas activation of V_2 produces an antidiuretic effect on the kidney, and stimulation of V_3 is responsible for corticotrophin release from the pituitary gland. Only one oxytocin receptor has been characterized, and its activation stimulates the contraction of uterine and mammary myocytes during parturition and lactation, respectively (Petersson, 2002). Using such models, it may be possible to rationally develop vasopressin/oxytocin receptor antagonists with tailored selectivity.

D. Neurotensin Receptor Agonists

Contulakin-G (also known as CGX-1160) was isolated from the venom of *Conus geographus* and was shown to possess agonist activity at neurotensin receptors 1 (NTSR1) and 2 (NTSR2) (Craig et al., 1999). The primary sequence of contulakin-G (ZSEEGGSNATKKPYIL) closely resembles neurotensin (ZLYENKPRRPYIL), yet contulakin-G is approximately 150-fold less potent at NTSR2 and NTSR1 receptors compared with the endogenous ligand, which contains a glycosylation at Thr10 (Craig et al., 1999, 2001). It is noteworthy that the native peptide was more biologically active than the synthetic nonglycosylated peptide (Craig et al., 1999).

Neurotensin plays important roles in neurotransmission and neuromodulation in the nervous system (Hermans and Maloteaux, 1998) by activating NTSR1 and NTSR2, which belong to G protein-coupled receptor family A (Tanaka et al., 1990; Vita et al., 1993). Both subtypes exhibit distinct pharmacological properties, NTSR1 showing high affinity for levocabastine, whereas NTSR2 has much lower affinity (Vita et al., 1998). Docking of neurotensin in the NTSR1 binding pocket was modeled based on rhodopsin structure (Härterich et al., 2008). Neurotensin has since been shown also to interact with D2 dopamine receptor (Fuxe et al., 1992); the recent crystal structure of D3 dopamine receptor may provide a more appropriate template to model these interactions (Chien et al., 2010). Because neurotensin is analgesic, contulakin-G was assessed for its antinociceptive activity in formalin models of acute and inflammatory pain in rats and dogs (Allen et al., 2007). Contulakin-G showed an antinociceptive effect greater than morphine, without motor deficits, prompting preclinical development and progression to phase I clinical trials. However, the mechanism by which contulakin-G induces analgesia remains unclear, because the antinociceptive effect is realized at a concentration 100-fold lower than its NTSR1 binding affinity.

VI. Conclusions and Outlook

In addition to their well recognized value as pharmacological tools to dissect the physiological and pathophysiological roles of ion channels, receptors, and transporters in a vast range of disease pathways, cone snail venom peptides are attracting growing interest as potential therapeutic leads because of their high potency and specificity at mammalian targets. With only a fraction of the conopeptides identified and characterized to date, the natural product space of cone snail venom peptides represents a vast untapped biological resource. Given this potential, accelerated discovery platforms are becoming increasingly important to unravel conotoxin diversity. In particular, high-throughput and high-content approaches promise to accelerate the discovery of novel bioactives as well as the potential to provide unique insight into the effects and mechanisms of action of conopeptides even on heterogeneous cell populations such as DRG neurons. In addition,

recent technological advances and improved bioinformatic tools have led to the successful application of transcriptomic and proteomic approaches to *Conus* spp. venom peptide discovery. These approaches are likely to lead to the rapid identification of novel conotoxins belonging to known pharmacological classes and to accelerate the identification and characterization of conopeptides with new sequences and potential novel biological activities.

Naturally occurring conotoxins are ideal scaffolds for the interrogation of specific pharmacophores using alanine-scanning mutagenesis approaches, and these structure-activity relationships can help guide molecular docking studies to define new allosteric sites on membrane proteins. Detailed studies of the interaction of conotoxins with their respective pharmacological targets have already provided invaluable insight into the pharmacology of ion channels, receptors and transporters. Exemplifying this are Na_v interactions with naturally derived compounds, including μ-, μO-, and δ-conotoxins, which have contributed significantly to our understanding of this channel's structure and function. As crystal structures of these membrane protein targets become available, such as the recently reported sodium channel structure (Payandeh et al., 2011), further refinement of our understanding of toxin-target interactions will without doubt lead to improved conotoxin-derived compounds with exceptional affinity and subtype selectivity. It is noteworthy that non-native disulfide bond isomers of conopeptides can also provide useful membrane protein probes, further expanding on the pharmacology of conopeptides. For example, the two isomers of α-AuIB differ in their inhibitory mechanisms, with the AuIB (ribbon) form competitively inhibiting only $\alpha3\beta4$ nAChRs containing an $\alpha3$ subunit in the fifth position, an effect distinct from the native AuIB (globular) form, which inhibited $\alpha3\beta4$ nAChRs independent of the fifth subunit, primarily through a noncompetitive mechanism (Grishin et al., 2010). Furthermore, a structural isomer of ρ-TIA named NMB-1 was found to preferentially inhibit the sustained component of mechanically evoked current in DRG neurons, providing a novel diagnostic tool for the molecular definition of channels involved in hearing and pressure-evoked pain (Drew et al., 2007).

In recent years, an increasing emphasis has been placed on genetic ablation or knockdown of pharmacological targets to dissect their role in physiology and the pathophysiology of disease. However, an inherent flaw of this approach is that heteromultimeric ion channels composed of several subunits, such as nAChR or K_v channels, cannot be targeted selectively. This has profound implications for our understanding of the composition and identity of receptor subtypes expressed in native tissues, as well as their physiological roles. The high selectivity and subtype specificity of conopeptides can yield novel insights especially for such targets and can help unravel the physiologic and pathophysiological roles of specific subunit combinations of these ion channels. Here, the α-conotoxins have proved especially valuable for chemically dissecting the role of a wide range of physiologically and pathologically relevant nAChR subunit combinations.

In addition, the importance of conotoxins as therapeutics in their own right is becoming increasingly apparent. Both naturally occurring conotoxins such as synthetic ω-MVIIA (ziconotide) or the sequence-optimized χ-conopeptide Xen2174 have become either novel pharmaceutics or promising drug leads. Although conotoxins acting at peripheral targets can be delivered by systemic routes, central targets still require direct delivery to the site of action (e.g., intrathecal injection) because of the limited permeability of the blood-brain barrier to peptides. However, our understanding of conotoxin structure-activity relationships is now being applied to the design of smaller, orally active conotoxin peptidomimetics, with the hope of engineering novel therapeutic molecules based on the unique pharmacological properties of conotoxins. The growing realization of the many limitations inherent to small molecules, as well as improved peptide dosage form design and drug delivery strategies, such as liposomal packaging, pro-drug approaches, conjugation to carrier molecules, and microinjection devices (Malik et al., 2007), promise to refocus drug development efforts beyond traditional approaches to more fully embrace the potential of peptide therapeutics. The favorable characteristics of conotoxins as peptide therapeutics in particular arise from their small size, relative ease of synthesis and high structural stability as a result of their disulfide connectivity and compact, relatively rigid structures. These properties of conotoxins also impart a low immunogenic potential and perhaps advantageous pharmacodynamic and pharmacokinetic behavior allowing compartmentalized delivery that can reduce side effects. For example, delivery of conotoxins to the intrathecal space could, in principle (on the basis of limited redistribution because of their peptidic nature), avoid side effects resulting from on-target effects at different sites. In addition, once delivered to these compartments, it seems that many conotoxins are surprisingly stable, with half-lives of hours or more, rather than minutes (Wermeling et al., 2003; Kern et al., 2007). For example, a relatively long half-life in cerebrospinal fluid may also account for the extended duration of action observed after intrathecal dosing of Xen2174 (Lewis, 2011). In addition, it is now possible to engineer potentially more stable N-C-cyclized forms as exemplified by using α-conotoxin Vc1.1, which retained analgesic activity even after oral dosing (Clark et al., 2010). Thus, it is now possible to extend the broadly evolved bioactivity of cone snail venom-derived peptides to novel uses that will further expand the potential and unique source of new research tools and potential therapeutic agents.

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Dr. Anderson Wang prepared the homology models of $Na_v1.2$ and $Ca_v2.2$ and performed the docking simulations shown in Fig. 3.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Lewis, Dutertre, Vetter, and Christie.

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