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Review

New Conotoxin SO-3 Targeting N-type Voltage-Sensitive Calcium Channels

Lei Wen [1, 2], Sheng Yang [1], Wenxia Zhou [1], Yongxiang Zhang [1,*] and Peitang Huang [3]

[1] Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China
[2] School of Traditional Chinese Medicine, Southern Medical University, Guangzhou 510515, China
[3] Beijing Institute of Biotechnology, Beijing 100071, China

* Author to whom correspondence should be addressed: Tel. (86)-10-66931625; Fax (86)-10-68211656; E-mail: zhangyx@nic.bmi.ac.cn

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Abstract: Selective blockers of the N-type voltage-sensitive calcium (Ca_V) channels are useful in the management of severe chronic pain. Here, the structure and function characteristics of a novel N-type Ca_V channel blocker, SO-3, are reviewed. SO-3 is a 25-amino acid conopeptide originally derived from the venom of *Conus striatus*, and contains the same 4-loop, 6-cysteine framework (C-C-CC-C-C) as O-superfamily conotoxins. The synthetic SO-3 has high analgesic activity similar to ω-conotoxin MVIIA (MVIIA), a selective N-type Ca_V channel blocker approved in the USA and Europe for the alleviation of persistent pain states. In electrophysiological studies, SO-3 shows more selectivity towards the N-type Ca_V channels than MVIIA. The dissimilarity between SO-3 and MVIIA in the primary and tertiary structures is further discussed in an attempt to illustrate the difference in selectivity of SO-3 and MVIIA towards N-type Ca_V channels.

Keywords: ω-conotoxins, SO-3, MVIIA, voltage-sensitive calcium channels, N-type calcium channel blockers, pain.

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1. Introduction

Conotoxins are a diverse set of peptide neurotoxins secreted by the venomous marine snails of the genus *Conus* for prey capture and other biological purposes. It is estimated that more than 50,000 distinct active peptides are present in *Conus* venoms, most of which can selectively target a specific subtype of voltage-gated ion channel, ligand-gated ion channel, or G-protein-coupled receptor. Due to their highly pharmacological potency and target selectivity, *Conus* peptides have attracted extensive attention with their potentials to be developed as new research tools in the field of neuroscience and novel medications in clinic for neurological diseases [1-3]. Several conopeptides and their related compounds with potential clinical applications are in the early stage of development. Recently, a synthetic conopeptide, *ω*-conotoxin MVIIA (MVIIA), has been approved in the USA and Europe for the management of severe resistant pain. This peptide has been given a generic name, Ziconotide, and a commercial name, Prialt, by its developer, Elan Pharmaceuticals [4,5]. MVIIA is a selective blocker of the N-type voltage-sensitive calcium (Ca_V) channels, with a 25-amino acid sequence identical to that of the parent venom constituent in *C. magus*. A new 25-residue conopeptide, SO-3, was identified by us from the venom of *C. striatus* (Figure 1), a fish-eating snail inhabiting the South China Sea [6,7]. The amino acid sequence of SO-3 shows 72% identity to MVIIA, and 56% to another conopeptide derived from *C. striatus*, *ω*-conotoxin SVIB, which is a selective P/Q-type Ca_V channel blocker [8]. Further bioactivity evaluation on the synthetic SO-3 showed that SO-3 is also a potent analgesic agent with lesser side effects than that of MVIIA [9-11]. Electrophysiological studies on voltage-sensitive ion channels indicated that SO-3 is an N-type Ca_V channel blocker and more selective towards N-type Ca_V channels than MVIIA [12].

Figure 1. *Conus striatus* inhabiting the South China Sea.

2. Discovery and Synthesis of SO-3

The South China Sea is in a temperate zone and provides an optimal environment for *Conus* proliferation. To date, approximately 100 species of cone snails from this habitat have been identified, but *C. striatus* is the only available fish-eating snail near the coast. Conopeptide content in *C. striatus* venoms were examined by rapid amplification of 3’ cDNA ends of O-superfamily conopeptide cDNA [6,7]. Four new O-superfamily conopeptide sequences (SO-3~SO-6), and four previously biochemically characterized conopeptide sequences [*ω*-conotoxin SVIA, SVIA(m1), SVIA(m2) and SVIB] from *C. striatus* were identified. Conopeptide SO-3 is highly homologous to MVIIA from *C.*

magus in sequence and has the same positive charges as MVIIA (see Table 1). SO-4~SO-6 have no homologous to suggest for their function [6,7].

The synthesis of the linear peptide SO-3 was performed using the 9-fluorenylmethoxycarbonyl (F-moc) strategy [9,13-15]. Because SO-3 contains six cysteine residues maintained in three disulfide bridges, linear peptide SO-3 was subsequently folded in ammonium acetate buffer (pH 7.9) containing oxidized glutathione (GSSG) and glutathione (GSH) or cysteine at 4˚C. Under these conditions, a 20% overall yield of oxidized peptide was obtained. After two steps of HPLC purification, the final product was greater than 98% purity. Using Matrix Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) mass spectrometry, the molecular mass (single isotope) of SO-3 was 2560.1 Da, in excellent agreement with the calculated molecular mass of 2561.1 Da [9,13-15].

Table 1. Selected ω-conotoxins targeting different Ca_V channel subtypes.

Peptide	*Conus* Specie	Sequence [a]	Subtype Targeted	Reference
		C------C------CC---C------C $^{\Delta}$		
		loop 1 loop 2 loop 3 loop 4		
ω-GVIA	*C. geographus*	CKSOGSSCSOTSYNCCR-SCNOYTKRCY* (4)	N-type	[16]
ω-MVIIA	*C. magus*	CKGKGAKCSRLMYDCCTGSC--RSGKC* (5)	N-type	[17]
ω-MVIIC	*C. magus*	CKGKGAPCRKTMYDCCSGSCGRR-GKC* (6)	P/Q/N-type	[18]
ω-CVIA	*C. catus*	CKSTGASCRRTSYDCCTGSC--RSGRC* (4)	N-type	[19]
ω-CVIB	*C. catus*	CKGKGASCRKTMYDCCRGSC--RSGRC* (6)	N/P/Q-type	[19]
ω-CVIC	*C. catus*	CKGKGQSCSKLMYDCCTGSCSRR-GKC* (5)	N/P/Q-type	[19]
ω-CVID	*C. catus*	CKSKGAKCSKLMYDCCSGSCSGTVGRC* (4)	N-type	[19]
ω-TVIA	*C. tulipa*	CLSOGSSCSOTSYNCCR-SCNOYSRKCR* (4)	N-type	[20]
ω-TxVII	*C. textile*	CKQADEPCDVFSLDCCTGIC---LGVCMW* (−3)	L-type	[21,22]
ω-SVIA	*C. striatus*	CRSSGSPCGVTS-ICC-GRC--YRGKCT* (4)	poor activity	[8]
ω-SVIB	*C. striatus*	CKLKGQSCRKTSYDCCSGSCG-RSGKC* (5)	P/Q-type	[8]
SO-3	*C. striatus*	CKAAGKPCSRIAYNCCTGSC--RSGKC* (5)	N-type	[12]

[a] Almost all ω-conotoxins are C-terminally amidated (*), each net positive/negative charge is indicated in the braces after the sequence of the corresponding ω-conotoxin. The disulfide bridges motif of ω-conotoxins and its 4 loops ($^{\Delta}$) are also displayed above the sequences. Hydroxyproline residues are denoted by the letter O.

3. Biological Activity and Analgesic Effects of SO-3

Using a range of pain models, including the standard hot-plate, light radiation, and acetic acid stimulus in mice, and heat-elicited tail-flick latency and mechanical tail tests in rats, SO-3 demonstrated analgesic effects that were comparable to or slightly better than those observed for identical doses of MVIIA following either intracerebral or intrathecal injections [9-11]. The toxicity of SO-3 and MVIIA after intramuscular injection into red common goldfish (*Carassius carassius*) showed that no lethality was associated with SO-3 at doses as high as 8.5 μg/per goldfish. However, severe lethality was observed after MVIIA treatment at the same dose [9]. In mice, when higher doses of SO-3 and MVIIA (>3.2 μg/kg) were intracerebrally delivered, some trembling or shaking activity was noted. Overall, high doses of SO-3 resulted in slightly lower adverse effects compared with MVIIA at the same doses as SO-3. The median lethal dose (LD_{50}) of SO-3 was 13.5 mg/kg after intracerebral administration in mice, which is 18,000 times higher than the median effective dose

(ED_{50} = 0.75 μg/kg; derived from the hot plate test). This difference suggests that SO-3 has a favorable safety index [9-11].

4. Ion Channel Target of SO-3

To ascertain the ion channel target of SO-3 is necessary and important for its further development as drug candidate. Conopeptides in the same superfamily share a characteristic arrangement of cysteine residues and a highly conserved signal sequence in their precursors [1-3]. Amino acid residue sequence analyses indicate that SO-3 belongs to O-superfamily conotoxins [6,7]. O-superfamily conotoxins mainly target voltage-sensitive ion channels. According to their different pharmacological targets, these O-superfamily conotoxins can be further divided into several functional families. For example, δ-conotoxins delay the inactivation of sodium channels, while μO-, κ-, ω- and χ-conotoxins block sodium, potassium, calcium and pacemaker channels, respectively [1-3]. In order to identify the ion channel target of SO-3, the characteristics of the effects of SO-3 on several voltage-sensitive ion channels were observed by using the whole-cell patch clamping techniques. Within the concentration of 100 μM, SO-3 had no effect on voltage-sensitive sodium currents, delayed rectifier potassium currents and transient outward potassium currents in primary cultured neonatal rat hippocampal neurons. However, similar to the selective N-type Ca_V channel blocker MVIIA, SO-3 blocks high voltage-activated (HVA) calcium currents in a concentration-dependent fashion. For both SO-3 and MVIIA, there was a plateau of inhibition at 1-3 μM. The calculated drug concentration to produce 50% channel inhibition (IC_{50}) for SO-3 and MVIIA were 0.16 μM and 0.20 μM, respectively [12]. These properties of SO-3 indicated that it is a novel Ca_V channel blocker and a member that belongs to a new ω-conotoxin family.

Based on different physiological and pharmacological properties, Ca_V channels have been categorized into several subtypes. At least four distinct types of HVA Ca_V channels, including the L-, N-, P/Q- and R-type channels, are expressed in cultured hippocampal neurons and are sensitive to different blockers [23-26]. We further studied whether specific types of calcium channels were inhibited by SO-3. As shown in Figure 2, four components of HVA calcium currents in hippocampal neurons were isolated by using the classical pharmacological protocol, only the N-type calcium currents were selectively blocked by 3 μM SO-3 [12]. Considering the high expression of N-type Ca_V channels in dorsal root ganglia (DRG) neurons and the significance of N-type channels in DRG neurons for pain transduction [27], the effects of SO-3 on HVA calcium currents in DRG neurons were also observed and a similar conclusion was derived: 1 μM SO-3 selectively inhibited the pharmacologically isolated N-type calcium currents in DRG neurons (unpublished data).

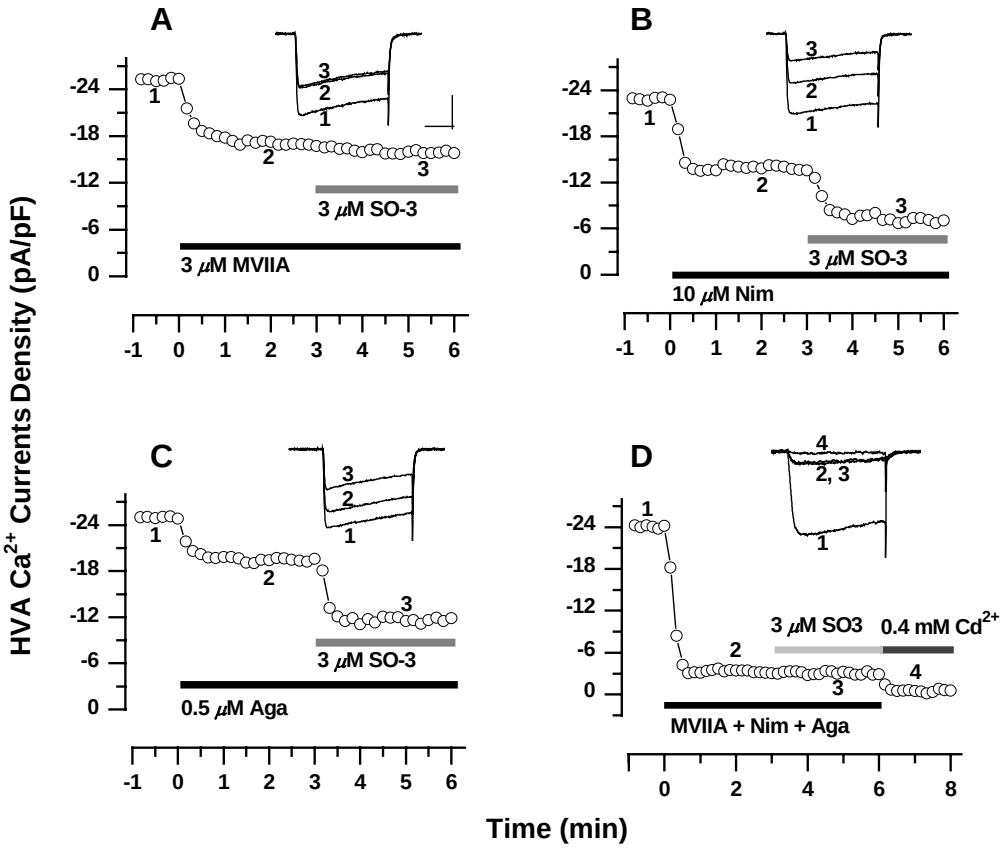

Figure 2. SO-3 selectively inhibiting the N-type calcium currents in cultured hippocampal neurons. Distinct Ca_V channel subtype blockers were applied to the cells as indicated by the *horizontal bars* to pharmacologically isolate the whole-cell HVA calcium currents. Additional application of SO-3 did not further inhibit the HVA currents after N-Ca_V channel blocker MVIIA application, which indicated they inhibited the overlapping components of HVA currents (A). No overlapping component of HVA currents was inhibited by SO-3 and L-Ca_V blocker nimodipine (Nim) (B), or by SO-3 and P/Q-Ca_V channel blocker ω-agatoxin (Aga) (C). 3 μM SO-3 also had no effect on R-type currents, which were completely blocked by 0.4 mM Cd^{2+} (D). Currents were evoked by 150-ms depolarizing voltage step commands from −80 to 0 mV at 10 s intervals. Upper insets showed the current traces at each time point. Scale bars: 10 pA/pF, 50 ms. (Modified from Wen et al. [12]).

In general, neurons co-express several types of Ca_V channels and the pharmacological isolation of Ca_V channel subtypes in neurons is not absolutely distinct. Recently, the single foreign ion channel gene expression was proved to giving a pure channel protein, which is very suitable for research on ion channel targeting. Thus, we further studied the selectivity of SO-3 on L-, N-, P/Q- or R-type calcium channels transiently expressed in Human Embryonic Kidney (HEK) 293 cells, respectively. SO-3 selectively blocked the expressed N-type calcium channel currents in a concentration-dependent manner, at 0.01-0.1 μM, and its effects on N-type currents were more obvious than the effects of MVIIA. A kinetic analysis of the SO-3 effects on the expressed N-type calcium channels showed that SO-3 blocked resting, open, and inactivated channels (unpublished data). The block effects of SO-3 and MVIIA on the expressed N-type calcium channels were both reversible and the recovery from block by SO-3 was slower than the recovery from block by MVIIA, which is consistent with the

results in cultured hippocampal neurons [12]. However, at higher concentrations (30 μM and 100 μM), SO-3 had inhibitory effects on non-N-type HVA currents recorded from both cultured hippocampal neurons [12] and HEK 293 cells expressing L-, P/Q- or R-type calcium channels (unpublished data), but its inhibitory effects on non-N-type HVA currents were less than those of MVIIA at the same higher concentrations. These results indicate that SO-3 is a new N-type Ca_V channel blocker and it possibly possesses more selectivity towards the N-type channels than MVIIA. However, how the pharmacological effect and clinical application will be influenced because of these differences between SO-3 and MVIIA should be studied.

5. Structure–activity Relationships of SO-3

The ω-, κ-, μO-, δ- and χ-conotoxins, which all belong to the O-superfamily conotoxins, contain the same cysteine framework C-C-CC-C-C, but ω-conotoxins have some structural characteristics different from κ-, μO-, δ- and χ-conotoxins. To date, more than ten ω-conotoxins have been identified from the venoms of fish-, worm- or mollusc-hunting *Conus* species. Almost all ω-conotoxins are C-terminally post-translationally modified and amidated (Table 1) [1-3]. Besides the conserved cysteine framework, there are three conserved residues Gly5, Tyr13, and Ser19 (numbering of MVIIA) throughout this set of peptides except ω-conotoxins SVIA and TxVII (L-type Ca_V channel blockers derived from *C. textile*; Table 1). It is known that the Tyr13 residue is important for binding to Ca_V channels [28-30]. Apart from these characteristics, all ω-conotoxins possess 4-6 positive charges (except ω-conotoxin TxVII) due to the high content of basic amino acid residues (Table 1), which are also known to play an important role in the blocking of Ca_V channels [29,31]. Among these three conotoxins derived from *C. striatus*, SVIA is the smallest ω-conotoxin without the Tyr13, Ser19 residues, and has relatively poor activity on most Ca_V channels found in mammalian systems [8]; whereas SO-3 has similar primary structural characteristics to SVIB and other derived ω-conotoxins [6,7], which may contribute to its selectivity on Ca_V channels (Table 1).

The Ca_V channels are complex proteins composed of four or five distinct subunits. The α1-subunit is the main subunit and it incorporates the conduction pore, the voltage sensor and gating apparatus, and the known sites of channel regulation by drugs and toxins. The α1-subunit is organized in four homologous domains (I-IV) with six transmembrane segments (S1-S6) in each domain. The S4 segment serves as the voltage sensor. The pore loop (P region) between transmembrane segments S5 and S6 in each domain determines ion conductance and selectivity. Although the auxiliary β-, $\alpha 2\delta$- and γ-subunits modulate the properties of the channel complex, the pharmacological and electrophysiological diversity of Ca_V channels arises primarily from the existence of multiple α1-subunits. The P region of domain III (III P region) has been shown to be the main binding region for ω-conotoxins [32-35]. The model of the III P region was preliminarily simulated using the graphic molecular modeling program and the results showed that the interaction between the III P region and SO-3 was similar as the interaction between the III P region and MVIIA or other ω-conotoxins [36].

Most ω-conotoxins specifically target N- and/or P/Q-type Ca_V channels, expect ω-conotoxin TxVII, which selectively blocks the L-type subtype. TxVII contains high number of hydrophobic amino acid residues and possesses three negative charges (Table 1) [21,22]. Both ω-conotoxin SVIB and SO-3 were isolated from *C. striatus*. SVIB is selective for the P/Q-type Ca_V channels [8], whereas SO-3 selectively targets the N-type Ca_V channels [12]. SO-3 has a 56% sequence identity to SVIB and a

higher (72%) sequence identity to MVIIA. However, the identity in primary sequences is insufficient to define Ca_V channel selectivity. Sequence hypervariability has been observed between functionally homologous *ω*-conotoxins. *ω*-Conotoxins with high sequence identity may also have different selectivity (Table 1). Therefore, studies of secondary and tertiary structures of SO-3 and other *ω*-conotoxins might help to understand the subtype selectivity in blocking Ca_V channels. The molecular structure of *ω*-conotoxins is stable and this stability depends on three disulfide bridges and a short triple-stranded antiparallel *β*-sheet with four turns. In addition, the backbone conformation of *ω*-conotoxins was quite conserved [30,31,37,38]. The three-dimensional solution structure of SO-3 (Figure 3) was determined by ^{1}H NMR and showed that it contains a short antiparallel *β*-sheet involving residues 6-9, 19-21, and 24-25, and the disulfide bridge pattern of SO-3 was identical to the cystine framework found in MVIIA and other *ω*-conotoxins [39,40]. These results indicate that the three-dimensional structure of SO-3 and MVIIA contributes to their selectively targeting the same N-type Ca_V channels. Additionally, the previous structure-activity analysis of *ω*-conotoxins also indicate that loops 1 and 3 have little effect on selectivity, whereas loops 2 and 4 are critical determinants in controlling the selectivity of *ω*-conotoxins for N-, P/Q-, or R-type Ca_V channels [19,31,37,38]. Hence, the assumption that SO-3 and SVIB target different Ca_V channel subtypes due to the difference in their three-dimensional structure requires further studies.

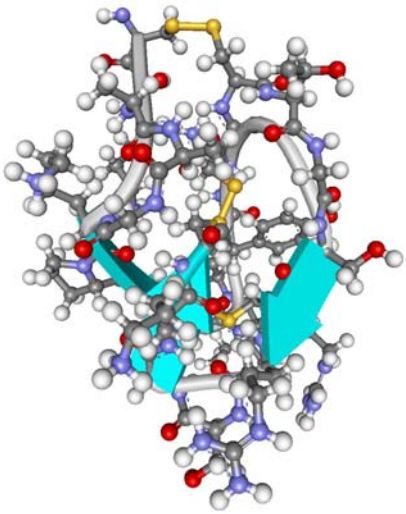

Figure 3. The three-dimensional structure of SO-3. The three-dimensional structure of SO-3 was determined by ^{1}H NMR (PDB ID 1FYG). The figure shows the disulphide bridges (yellow balls and sticks) and the *β*-sheet region (cyan arrows). Photo was provided by Dr. Yongbin Yan from Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing, China.

Although both SO-3 and MVIIA are N-type Ca_V channel blockers, they demonstrate some different selectivity towards N-type Ca_V channels. Such a difference in function might be caused by the subtle difference in the tertiary structure. The most identified regions between SO-3 and MVIIA are the first three *β*-turns (residues 3-6, 9-12, 15-18) and the first strand of *β*-sheets (residues 6-8), indicating that these regions may be important for stabilizing the structures. The loop regions and the sheet from residues 19-25 have the lowest identity except for residue 24, indicating that these regions may be

related to the difference in the function [39,40]. In addition, Tyr13 was found to be crucial for the calcium channel binding activity of *ω*-conotoxins, suggesting that minor structural changes in the region around Tyr13 may be responsible for the selectivity. Comparison of the tertiary structure indicated that for SO-3 and MVIIA, the region including Tyr13 is the most flexible region around the dihedral angles or the most disordered stretch of backbone [39,40]. This dispersion property suggests that the backbone conformation of *ω*-conotoxins blocking the N-type Ca_V channel is flexible.

Previously, some researchers have reported and discussed the reversibility of the blocking effects of several *ω*-conotoxins on the transiently expressed N-type calcium channels and these results were not consistent with each other. Taken together, the blocking effect of *ω*-conotoxin GVIA is poorly reversible, while that of both MVIIA and *ω*-conotoxin CVID are readily reversible [41-48]. Since *ω*-conotoxin GVIA dissociates very slowly from Ca_V channels, it may be difficult to control in a clinical setting, and is therefore not an ideal drug candidate. In our study, the block effects of SO-3 and MVIIA on Ca_V channels were both almost completely reversed, and the recovery from block by MVIIA was more rapid than the recovery from block by SO-3 [12]. The recovery from block by *ω*-conotoxins is partly dependent on the divalent cation in extracellular solution [46], the holding potential [44,47], and the *α2δ* auxiliary subunit of Ca_V channels [48]. Apart from these factors, the amino acid residue of *ω*-conotoxins at position 10 has a significant impact on the extent of the reversibility of these toxins [48]. SO-3 and MVIIA, both with Arg at position 10 (Table 1), can reversibly block the Ca_V channels, whereas GVIA demonstrates different reversibility because of the different residue (Hydroxyproline) at position 10 (Table 1). The different extent of recovery from the block by SO-3 and MVIIA may due to the different sequences of the amino acid residues near this position or other unknown mechanisms.

6. Potential Therapeutic Implications of SO-3

N-type Ca_V channels are critical for pain transduction and modulation. Although they are located on pre-synaptic nerve terminals in both central and peripheral nervous systems, N-type channels are highly present at the pre-synaptic terminals of nociceptive neurons in dorsal horn of the spinal cord where they regulate the release of the key pro-nociceptive neurotransmitters such as glutamate, substance P, neurokinin A and/or calcitoningene-related peptide [33,49]. The crucial role of the N-type channels in nociception is also supported by the evidence that mice lacking the N-type channel gene have higher pain thresholds compared to wild-type mice [50-53]. It is reasonable to consider that N-type Ca_V channel blockers have the therapeutic potential as a new class of analgesic agents [54,55]. However, based on the fact that N-type Ca_V channels are also located on numerous other synapses in non-pain pathways, including the pre-synaptic nerve terminals in sympathetic neurons, it is not surprising that N-type Ca_V channel blockers may result in adverse effects in analgesia [54,55]. These adverse effects include increased dizziness, blurred vision, nystagmus, sedation, anxiety, hallucinations, hypotension, etc. [4,5]. A similar pathological syndrome was observed in N-type channel α1-subunit knockout mice [56,57]. In contrast with opioids, which always give rise to dependence and tolerance, N-type Ca_V channel blockers do not seem to have these clinical limits and are thereby considered as the alternative for the alleviation of severe chronic pain states [54,55].

The side effects of the N-type Ca_V channel blockers in pain control may also arise from their activities at non-N-type Ca_V channels. Some non-N-type Ca_V channels are also involved in neurotransmitter release in most central synapses. P/Q-type Ca_V channels exist primarily in

neuromuscular junction; selective P/Q-type Ca_V channel blockers (such as *ω*-conotoxin MVIIC and SVIB) are likely to be lethal at relatively low dose and are considered useless in pain treatment [58-60]. Consequently, *ω*-conotoxins, more selective for N-type Ca_V channels than P/Q- and other Ca_V channel types, can largely minimize the side effects in analgesic therapy. Recently, another *ω*-conotoxin (CVID, also known as AM336 and developed by Amrad Corporation) was reported that it is more selective towards the N-type Ca_V channels than MVIIA, and it may have more advantages relative to MVIIA for the alleviation of persistent pain states [61,62]. Our results mentioned previously also indicate that, SO-3, which shows a higher selectivity for N- *vs.* L-, P/Q- and R-type Ca_V channels in electrophysiological experiments, may have superior clinical utility in the management of severe chronic pain.

Acknowledgments

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