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Original Article

Chemical synthesis and characterization of two α4/7-conotoxins

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α-Conotoxins are small disulfide-constrained peptides that act as potent and selective antagonists on specific subtypes of nicotinic acetylcholine receptors (nAChRs). We previously cloned two α-conotoxins, Mr1.1 from the molluscivorous *Conus marmoreus* and Lp1.4 from the vermivorous *Conus leopardus*. Both of them have the typical 4/7-type framework of the subfamily of α-conotoxins that act on neuronal nAChRs. In this work, we chemically synthesized these two toxins and characterized their functional properties. The synthetic Mr1.1 could primarily inhibit acetylcholine (ACh)-evoked currents reversibly in the oocyte-expressed rat α7 nAChR, whereas Lp1.4 was an unexpected specific blocker of the mouse fetal muscle α1β1γδ receptor. Although their inhibition affinities were relatively low, their unique receptor recognition profiles make them valuable tools for toxin-receptor interaction studies. Mr1.1 could also suppress the inflammatory response to pain *in vivo*, suggesting that it should be further investigated with respect to its molecular role in analgesia and its mechanism or therapeutic target for the treatment of pain.

Keywords *Conus*; α4/7-conotoxin; nicotinic acetylcholine receptor; analgesic

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Introduction

The hunting marine gastropods (genus *Conus*) capture their preys using venoms that are rich in neuropharmacologically active peptides. Most of them are disulfide-rich peptides, namely conotoxins, which potently and selectively target a range of ion channels, receptors, and transporters [1]. Conotoxins are synthesized *in vivo* as precursors that undergo a series of post-translational modifications before they are released as mature toxins [2]. Conotoxins are classified into several superfamilies based on their conserved signal sequence or distinct cysteine frameworks according to the arrangement of cysteines along their mature peptide sequence, as well as different pharmacological families that have a particular molecular target [3].

Nicotinic acetylcholine receptors (nAChRs) are transmembrane pentamers that form a central ion-conducting channel. Many different nAChR subunits have been identified, with α1–α10, β1–β4, γ, δ, and ε in vertebrates [4]. Different combinations of these subunits can form diverse receptor subtypes that have distinct tissue localizations, physiological effects, and pharmacological properties. For example, the nAChRs at the neuromuscular endplate can cause muscle contraction, while the nAChRs in the central and peripheral nervous system can mediate neurotransmission [5]. Since nAChRs are now implicated in analgesia, nicotine addiction, and some neurological disorders such as Parkinson's and Alzheimer's diseases, the development of selective nAChR modulators is urgently needed for dissecting the roles of the combinatorial diversity of subtypes [6].

In the A-superfamily of conotoxins, three different cysteine frameworks have been found, framework I (CC-C-C), framework II (CCC-C-C-C), and framework IV (CC-C-C-C-C). α-Conotoxins of A-superfamily generally contain 12–19 residues, including four cysteines (CC-C-C) that form two disulfide bonds with a connectivity of I–III and II–IV [7]. α-Conotoxins have a three-dimensional structure dominated by an α-helical segment that presents the amino acid side chains in the correct orientations for receptor binding [8]. They can be grouped into different subfamilies according to their loop length. The 3/5-subfamily α-conotoxins exclusively antagonize the fetal muscular nAChR subtype (α1β1γδ) except for GI that targets the adult subtype (α1β1εδ) [9]. The 4/3-subfamily α-conotoxins are potent antagonists of nAChRs containing only α subunits [10,11], except that ImII has subtype specificity of both α7 and α1β1εδ nAChRs [12,13]. The 4/4-subfamily α-conotoxins have a wide spectrum of activities on both the muscular and neuronal nAChRs [14].

No functional characterization has been reported for 4/5-subfamily α-conotoxins. Only one 4/6-subfamily α-conotoxin, AuIB, has been functionally characterized: it blocks oocyte-expressed rat α3β4 nAChR with a low affinity [15]. The 4/7-subfamily α-conotoxins show different antagonistic effects on diverse neuronal nAChRs with the exception of EI, SrIA, and SrIB that target both muscle and neuronal nAChRs [16,17]. These subtype-specific inhibitors of nAChRs are valuable neurochemical tools and may be developed as therapeutic agents [4]. However, with more α-conotoxins investigated, some are found to have highly variable structures and hit additional molecular targets. For instance, we identified and characterized a novel conotoxin named mr1e from *Conus marmoreus* [18]. Although its intercysteine spacing pattern is identical to that of α4/3-conotoxin, the native mr1e peptide coeluted using the regioselectively synthesized ribbon disulfide isomer (C1−C4, C2−C3) but not the globular isomer (C1−C3, C2−C4). Sequences of mr1e and other α4/3-conotoxins do not share significant homology. Furthermore, the recent finding that α4/7-Vc1.1 and α4/3-Rg1A, which inhibit the α9α10 nAChRs, act as agonists via G-protein-coupled $GABA_B$ receptors and subsequently inhibit $Ca_v2.2$ channel currents in rat DRG neurons to mediate their analgesic properties is largely unexpected [19].

Two novel α4/7-conotoxins, Mr1.1 and Lp1.4, have recently been identified by PCR amplification of venom duct cDNA and muscle tissue genomic DNA from *C. marmoreus* and *Conus leopardus*, respectively [20]. Here, we carried out the chemical synthesis, oxidative folding, and preliminary function characterization of these two toxins.

Materials and Methods

Peptide synthesis and site-directed disulfide bond formation

Linear Mr1.1 (GCCSHPACSVNNPDIC-amide) and Lp1.4 (GCCSHPACSGNHQELCD-amide) were chemically synthesized on an automated ABI 433A peptide synthesizer (ABI, Foster City, USA) using Fmoc chemistry and standard side-chain protection by GL Biochem Ltd (Shanghai, China). In both peptides, Cys2 and Cys8 were protected by the stable *S*-acetamidomethyl group; while Cys3 and Cys16 were protected by the acid-labile *S*-trityl moiety. The synthesized peptide was cleaved form resin and purified by reverse-phase (RP)-HPLC using a ZORBAX 300SB-C18 semi-preparative column (9.4 mm × 250 mm; Agilent Technologies, Santa Clara, USA) with a flow rate of 2 ml/min. The peptide was eluted from the column by an acetonitrile gradient composed of solvent A and solvent B. Solvent A was 0.1% aqueous trifluoroacetic acid, and solvent B was acetonitrile containing 0.1% trifluoroacetic acid. The elution gradient was listed as follows: 0 min, 0% solvent B; 5 min, 0% solvent B; 7 min, 20% solvent B; and 17 min, 30% solvent B. The molecular mass of the purified linear peptide was characterized by a Q-trap mass spectrometer (Applied Biosystems, Foster City, USA). Thereafter, a two-step oxidation was used to prepare folded Mr1.1 and Lp1.4 with site-direct disulfide formation [21]. The first disulfide bond between Cys3 and Cys16 whose protection groups were simultaneously removed during peptide cleavage from resin was formed by air oxidation. The linear peptide (20 μM) was dissolved in 50 mM Tris−HCl buffer (pH 8.7) and the solution was stirred at 4°C for 72 h. The intermediate with one disulfide was then purified by HPLC on an analytical PepMap-C18 column (4.6 mm × 250 mm; LC Packings, Bannockburn, USA) with a flow rate of 1 ml/min using the following gradient: 0−10 min, solvent A; 10−60 min, 0−50% solvent B. The second disulfide bond between Cys2 and Cys8 was formed by iodine oxidation that can remove the acetamidomethyl groups and simultaneously form the second disulfide bond. The lyophilized intermediate (20 μM) was dissolved in the oxidation solvent (86% water, 10% acetonitrile, 4% trifluoroacetic acid, and 5 mM iodine) and the solvent was stirred at room temperature for 1 h. Then 1% (*v*/*v*) ascorbic acid was added to quench the oxidation. The folded peptide was loaded onto a semi-preparative C18 column and eluted by an acetonitrile gradient listed as follows: 0 min, 0% solvent B; 10 min, 0% solvent B; 13 min, 15% solvent B; and 33 min, 35% solvent B. The molecular mass of the folded peptide was analyzed by mass spectrometry.

Peptide synthesis and spontaneous disulfide bond formation

The linear Lp1.4 (GCCSHPACSGNHQELCD-amide) whose cysteines were all protected by acid-labile *S*-trityl moieties was also chemically synthesized by standard Fmoc chemistry. The linear peptide was cleaved from resin (the *S*-trityl groups were simultaneously removed in this process) and purified by RP-HPLC. The lyophilized linear peptide was then dissolved in oxidation buffer (50 mM Tris−HCl, pH 8.0, 1 mM reduced glutathione, and 0.5 mM oxidized glutathione) at the final concentration of 20 μM. The refolding was carried out at room temperature for 2 h. Thereafter, the refolding mixture was subjected to C18 RP-HPLC. The major elution peak was manually collected, lyophilized, and confirmed by mass spectrometry.

Electrophysiological recordings from nAchRs exogenously expressed in *Xenopus laevis* oocytes

Oocytes of *X. laevis* frogs were prepared and injected with capped RNA (cRNA) to elicit expression of mouse fetal skeletal muscle and various rat neuronal nAChR subtypes as described previously [21,22]. cRNAs were prepared using mMESSAGE mMACHINE *in vitro* RNA

transcription kit (ABI) with either T7 or SP6 promoter and recovered by lithium chloride precipitation. The isolated *Xenopus* oocytes were dissociated at room temperature in 2 mg/ml collagenase (type I) solution for 30 min and then in 1 mg/ml collagenase (type I) solution for 30 min. De-folliculated oocytes were injected with 50 nl of cRNA [23]. For expression of muscle nAChR, 1 ng cRNA of each subunit was injected into each oocyte. For expression of neuronal nAChRs, 5 ng of each subunit cRNA was injected per oocyte. The injected oocytes were incubated at 15°C in ND96 buffer (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 5 mM HEPES, pH 7.5) with 1 mM glucose-6-phosphate, 50 mg/L gentamycin, and 5 mM pyruvic acid [24,25]. Oocyte recordings were carried out 1–6 days post-injection.

All two-electrode voltage clamp recordings of *Xenopus* oocyte nAChR currents were conducted at room temperature on an OC-725C amplifier (Warner Instruments, Hamden, USA). Oocytes were placed in a 300 μl Warner RC-3Z recording chamber attached with an OC-725 bath clamp. Glass microelectrodes with the resistance between 0.05 and 0.2 MΩ were filled with 3 M KCl solution. Oocytes were voltage clamped at a membrane potential of −60 mV and gravity perfused with OR2 buffer (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES, pH 7.4) at a rate of 5 ml/min using a Warner BPS-8 controller [26]. Three micromolar of atropine sulfate was added to OR2 buffer to block the endogenous muscarinic AChRs for all recordings, except for α7 nAChR, which is antagonized by atropine [27]. ACh-gated currents were elicited by a 5-s pulse of agonist solution applied at intervals of 2 min to obtain the baseline activity: 10 μM of ACh for the muscle subtype and 100 μM of ACh for the neuronal subtypes. To measure their inhibitory effects, Mr1.1 and Lp1.4 were applied to an AChR-expressing oocyte in the static bath for 10 min prior to restoration of OR2 perfusion and ACh pulse. The current signals were sampled and filtered at 500 and 200 Hz, respectively. The average peak amplitude of three control responses preceding exposure to toxin was used to normalize the amplitude of each test response to obtain '% response' and recovery from toxin application. All data were represented as arithmetic means ± SD from measurements of three to five oocytes for each subtype.

Analgesic activity

Male Sprague–Dawley rats (200−250 g) were supplied by the Experimental Animal Center of Fudan University (Shanghai, China) and raised at 21–23°C on a 12/12 h light/dark cycle with free access to food and water. Each rat was manipulated only once. All experimental procedures were approved by the Institution's Animal Care and Use Committee and conformed to the guidelines of the International Association for the Study of Pain.

The inflammation was induced by intraplantar injection of 100 μl carrageenan (1 mg/100 g body weight) in either hindpaw of each rat. The synthetic Mr1.1 (1 mM, 10 μl, 1.6 μg total/200 g rat) was injected to the same place 3 h after the carrageenan administration. Each group contains eight rats. Control rats were injected with the same volume of normal saline solution. Hargreave's test was used to study the nociceptive behavior by measuring the latency of paw withdraw (PWL) in response to a radiant heat stimuli. The injected rats were caged individually in a plastic chamber on a glass platform. The radiant heat source was applied to the glabrous surface of the hindpaw through the glass plate, and turned off when the rat lifted its paw. The time from the onset of radiant heat application to the withdrawal of the rat's hindpaw was termed as the PWL.

Results

Peptide synthesis

The cloning-based strategies had led to the discovery of two novel α4/7-conotoxins, Mr1.1 and Lp1.4 [20]. Their cDNA sequences and the predicted translation products were shown in **Fig. 1**. As previous studies indicated that conotoxins with the C-terminal sequences of -XG, -XGR, -XGK, and -XGRR were usually processed to -X-NH_2 in their mature forms [2], we deduced the C-terminus of these two conotoxins to be amidated. As all native α-conotoxins characterized thus far have a globular disulfide linkage, it was assumed that Mr1.1 and Lp1.4 also have the disulfide connectivity of I–III and II–IV.

The linear peptides of Mr1.1 and Lp1.4 were chemically synthesized using Fmoc methodology and folded efficiently *in vitro* using two-step or one-step procedures. Mr1.1 was prepared by a two-step procedure with site-directed disulfide formation [**Fig. 2(A)**]; Lp1.4 was prepared by both procedures [**Fig. 2(B–D)**]. Co-elution study confirmed the folded Lp1.4 prepared by different procedures were identical. The synthesized and refolded peptides had expected molecular mass as analyzed by mass spectrometry: for Mr1.1, measured value of 1614.7 (expected value 1614.8); for Lp1.4, measured value of 1755.3 (expected value of 1755.9).

Inhibitory action on oocyte-expressed nAchR subtypes

The selectivity and potency of Mr1.1 and Lp1.4 on various oocytes-expressed nAChRs were investigated as shown in **Fig. 3**. The channel-blocking effects of Mr1.1 and Lp1.4 are both concentration dependent and receptor dependent. Mr1.1 showed a preference for the α7 nAChR *in vitro*, while Lp1.4 showed a preference for the α1β1γδ subtype. Contrasting to previous findings that α-conotoxins specific for neuronal subtypes of nAChR are neutral or negatively charged, whereas α-conotoxins specific for muscular

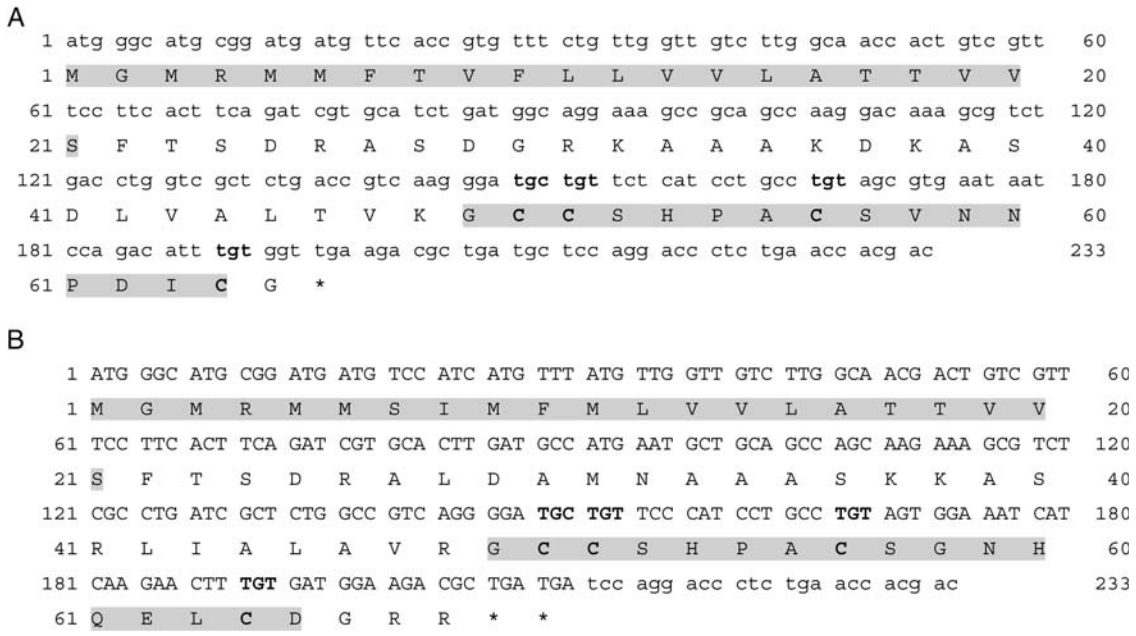

Figure 1 The cDNA sequences and predicted translation products of Mr1.1 (A) and Lp1.4 (B) The signal peptides and mature toxins are shaded. The codons of conserved Cys are shown in bold letter. The nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession numbers DQ311077 and AY580325 for Mr1.1, DQ311056 and AY580324 for Lp1.4.

subtypes are positively charged [28], the negatively charged Lp1.4 was unexpectedly active on both muscular and neuronal types of nAChR.

Typical recordings performed on α7- and α1β1γδ-expressing oocytes with 1 μM of Mr1.1 and Lp1.4 were shown in **Fig. 4**. Mr1.1 could inhibit about 65% of ACh-evoked current, and the application of an additional ACh pulse produced a recovery that was complete after 2 min of washout. Lp1.4 could also reversibly inhibit about 81% of the ACh-evoked current in α1β1γδ nAChR, with a relatively slower dissociation rate. At a higher concentration (10 μM), the blocking effects of Mr1.1 were about 70 and 61% in α3β2 and α6α3β4, respectively; the inhibition effects of Lp1.4 were about 37% and 49% in α3β2 and α6α3β2, respectively. However, the error bars overlapped for the inhibition of α3β2 and α6α3β4 by Mr1.1 might affect us to distinguish its effect on these receptors (**Fig. 3**). This concentration response analysis indicated that Mr1.1 and Lp1.4 had a wide spectrum of activities, with Mr1.1 blocking the homomeric and heteromeric nAChRs and Lp1.4 inhibiting the muscle and neuronal channels.

Analgesic effect of α4/7-mr1.1

Intraplantar injection of carrageenan into the hindpaw of Sprague–Dawley rat can elicit a notable inflammation syndrome characterized with edema and erythema, and significantly decrease the PWL to noxious thermal stimuli. As shown in **Fig. 5**, the PWL decreased quickly and reached the minimal value 3 h after carrageenan administration. Injection of the synthetic Mr1.1 can increase the PWL amplitude 15 min after toxin application. The PWL reached its maximum level 45 min after Mr1.1 application and the increase of PWL 30 min after the injection of Mr1.1 was significant ($P < 0.05$). The analgesic effect of Mr1.1 could last about 45 min and the PWL amplitudes remained the same as those of control rats 60–90 min after Mr1.1 application. Our data suggest that Mr1.1 has potent analgesic effect on the thermal hyperalgesia maintenance in this model. However, it should be noted that the analgesic effect observed in this study may not be the maximum effect of Mr1.1. Its analgesic effect might be improved and last longer if larger amount of peptide is administered.

Discussion

In past years, α4/7-conotoxins have been identified by different strategies. PnIA, PnIB [29], and MII [30] were identified by bioassay approach; EpI was identified by an analytical approach using HPLC in combination with mass spectrometry [31]; AnIB was identified by a combined approach of LC/MS analysis and assay-directed fractionation [32]. Mr1.1 and Lp1.4, were first identified by PCR amplification of α-conotoxins from *C. marmoreus* and *C. leopardus* genomic DNA and cDNA, respectively [20]. This gene cloning approach can identify low-expressed conotoxins that are hardly detected by bioassay or direct peptide isolation. We originally planned to re-name Mr1.1 and Lp1.4 to MrIA and LpID in this study. However, MrIA is already taken by a mislabeled peptide due to a

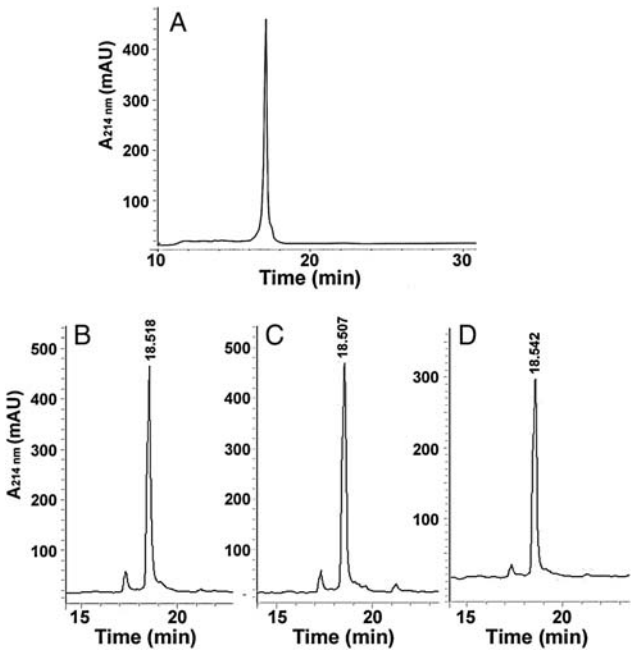

Figure 2 Oxidative folding of α4/7-conotoxins Mr1.1 and Lp1.4 (A) RP-HPLC oxidation profile of Mr1.1 using orthogonal protection on Cys residues. The folded product was loaded onto an analytical PepMap-C18 column in 90% solvent A and eluted using a linear gradient of 20–40% solvent B at a flow rate of 0.5 ml/min for 40 min. (B) The synthetic Lp1.4 prepared by site-directed disulfide formation. (C) The synthetic Lp1.4 prepared by the one-step oxidative folding procedure. (D) Co-elution profile of the two synthetic Lp1.4. Lp1.4 was separated on an analytical PepMap-C18 column and eluted using a flow rate of 1 ml/min using the following gradient: 0–5 min, solvent A; 5–7 min, 0–10% solvent B; 7–22 min, 15–25% solvent B. The molecular mass of the folded Lp1.4 in (B) and (C) was respectively analyzed by mass spectrometry to be identical (for Lp1.4 in B, observed value: 1755.4; for Lp1.4 in C, observed value: 1755.3), further confirming their identity.

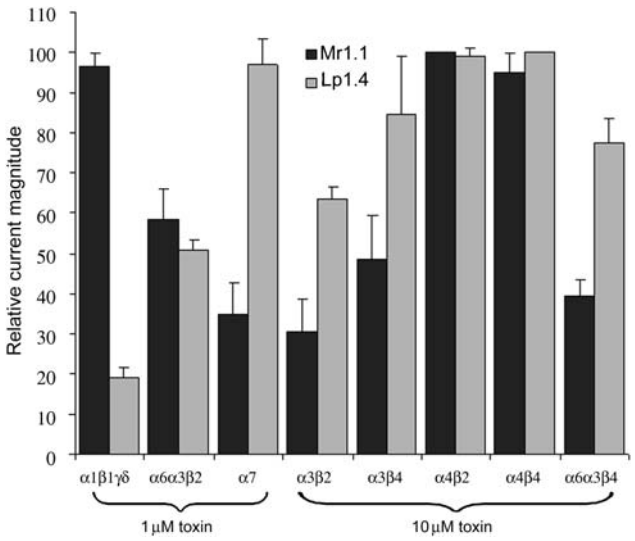

Figure 3 Inhibitory effects of Mr1.1 and Lp1.4 on various mouse and rat nAChRs Each bar indicates the average percent response ± SD after toxin application to *Xenopus* oocytes expressing a variety of nAChRs. Peptides were tested three to five times against each receptor subtype.

historical artifact from early days of conotoxin nomenclature, which probably cannot be changed anymore. Besides, peptides Mr1.1 and Lp1.4 have not been isolated from *Conus* venoms and their physiological mechanisms are still under investigation. We decided to use Mr1.1 and Lp1.4 when describing the two peptides, indicating that they are putative sequences deduced from clones.

Mr1.1 and Lp1.4 were efficiently folded into the globular isomer common to all α-conotoxins. Co-elution studies were conducted to confirm the identity of the synthetic Lp1.4 prepared by different peptide synthesis and disulfide bond formation strategies. As the direct oxidative folding resulted in higher yields of correctly folded α-Lp1.4, compared with the two-step oxidation method, it appears that direct oxidation might be more beneficial for the folding of bicyclic α-conotoxins. However, the efficiency needs to be assessed on a case-by-case basis.

Mr1.1 could specifically inhibit murine neuronal nAChR responses *in vitro*, and showed analgesic effect in *vivo*. Mr1.1 inhibits more than 40% current through the α6α3β2 nAChR at a concentration of 1 μM (**Fig. 4**), indicating that the concentration for effective inhibition at the α7 nAChR also inhibits α6α3β2 to some extent. Thus, the biological effect of Mr1.1 *in vivo* may not come only from the antagonism of α7, but also from other channels including α6α3β2.

The antagonism of nAChRs was originally thought to be the rationale for the analgesic action of α-conotoxins. Previous studies of α4/7-Vc1.1 and α4/3-RgIA attributed their acute analgesia to the inhibition of α9α10 nAChR [11,33]. The native posttranslationally modified peptide of Vc1.1, vc1a, and the partially modified homologs [P6O]Vc1.1 and [E14γ]Vc1.1, were all active at α9α10 nAChRs but not at any other nAChR subtypes studied [34]. However, only Vc1.1, but not vc1a or its analog [P6O]Vc1.1, was able to inhibit a vascular response to pain and reduce chronic pain in several animal models of human neuropathy [35,36]. Hence, it is highly unlikely that α9α10 nAChRs are the molecular mechanism or therapeutic target of Vc1.1 for the treatment of neuropathic pain [37]. As the activation of $GABA_B$ receptors by agonists such as baclofen could produce antinociceptive and antiallodynic actions in chronic pain models [38,39], one mechanism proposed recently indicated that α-conotoxins Vc1.1 and Rg1A also could act as agonists via G-protein-coupled $GABA_B$ receptors and subsequently inhibit $Ca_v2.2$ channel currents in rat DRG neurons to mediate their analgesic properties [19].

One likely mechanism for the analgesic effect of Mr1.1 is by blocking peripheral neuronal type nAChR α3β4, the principal subtype present in human sensory sural nerve [40] and in cutaneous sensory neurons in the rat dorsal horn [41]. At neuronal synapses, nAChRs are electronically

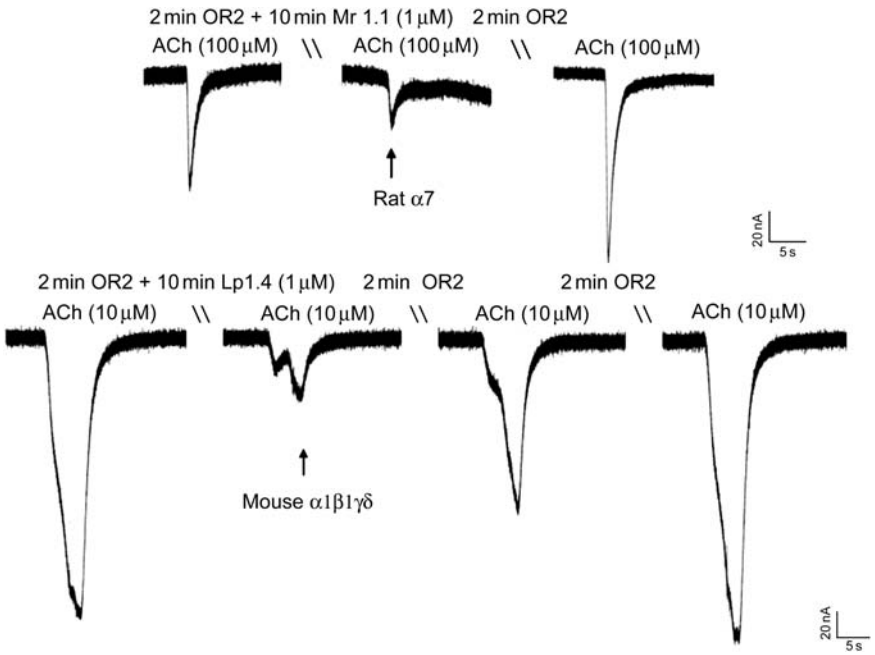

Figure 4 Selectivity of Mr1.1 and Lp1.4 on various nAChRs Representative current traces for Mr1.1 and Lp1.4 at 1 μM on rat α7 and mouse α1β1γδ nAChR, respectively. Control traces are shown prior to peptide application. The arrow marks the first current trace elicited after a 10-min exposure to toxin. Subsequent current traces show peptide dissociation and washout. Two peaks of the ACh-induced response immediately after the 10-min incubation of Lp1.4 are obtained due to the displacement of salt bridges when the high-speed (5 ml/min) gravity-perfusion restored.

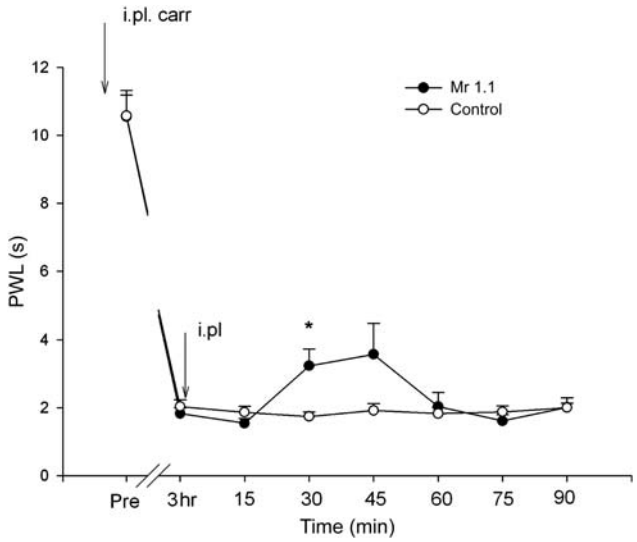

Figure 5 Acute effect of intraplantar injection of Mr1.1 on thermal paw withdrawal threshold in SD rat inflammation pain model Results are expressed as mean ± SEM ($n = 8$). Statistical analyses were performed using two-way repeated measures ANOVA. Asterisks represent significant PWL increase ($P < 0.05$) induced by injection of Mr1.1.

linked to the N-type Ca^{2+} channels which are involved in pain pathways and specifically targeted by *ω*-conotoxin MVIIA and CVID that show analgesic activity in rat and human [42,43]. Inhibition of such neuronal type nAChRs could block the opening of N-type Ca^{2+} channels and reduce the Na^{+} and Ca^{2+} influx. The resulting reduction in sensitization of the voltage-operated Ca^{2+} channels could lead to the block of neurotransmission in sensory pathways [44], and thus bring about the suppression of hyperalgesia by Mr1.1. However, as its modulation of the neuronal nAChRs is relatively low, the analgesic α-conotoxin Mr1.1 might also inhibit native N-type Ca^{2+} channel ($Ca_V2.2$) via G-protein-coupled $GABA_B$ receptor activation. The ~56% sequence similarity between Mr1.1 and Vc1.1 may substantiate this hypothesis. Further detailed experiments will shed light on this interesting point. Therefore, Mr1.1 may represent a valuable tool to further investigate the mechanism of pain neurotransmission and could serve as a template for the development of selective pain blockers.

Among previously studied α4/7-conotoxins, only three have inhibitory action on both muscle and neuronal nAChRS (**Table 1**): [45–56] EI strongly targets the α1β1γδ, α4β2, and α3β4 subtypes, whereas SrIA and SrIB weakly block α4β2 and α1β1γδ receptors at 10 μM [17]. Surprisingly, these peptides showed a remarkable potentiation of α4β2 and α1β1γδ nAChRs if briefly applied (2–15 s) at concentrations several orders of magnitude lower [17], revealing a complex mechanism of toxin-receptor interaction. Since Lp1.4 is also a weak inhibitor of both muscle and neuronal nAChRs at high toxin concentration, it might share the high-affinity potentiating effect on nAChRs as found in EI, SrIA, and SrIB.

As Mr1.1 and Lp1.4 have an identical intercysteine loop I, it is the side-chain modification or structural perturbation of the C-terminal β-turn present within the second disulfide loop which confer their respective α7 and α1β1γδ

Table 1 Properties of characterized α4/7-conotoxins identified from piscivorous (p), molluscivorous (m), and vermivorous (v) Conus **species**

Peptide	Sequence	Species/prey	Selectivity on recombinant nAChRs	Selectivity in native tissues	Reference
AnIA	**CC**SHPA**C**AANNQDY**C**[a]	*Conus anemone*/v	α3β2, α7	N/A	[32]
AnIB	GG**CC**SHPA**C**AANNQDY**C**[a]		α3β2, α7	N/A	
ArIA	IRDE**CC**SNPA**C**RVNNOHV**C**RRR	*C. arenatus*/v	α7, α3β2	N/A	[45]
ArIB	DE**CC**SNPA**C**RVNNPHV**C**RRR		α7, α6α3β2β3, α3β2	N/A	
[V11L,V16D]ArIB	DE**CC**SNPA**C**RLNNPHD**C**RRR		α7	α7	
AuIA	G**CC**SYPP**C**FATNSDY**C**[a]	*C. aulicus*/m	α3β4	α3β4[b]	[46,50]
AuIC	G**CC**SYPP**C**FATNSGY**C**[a]		α3β4	α3β4[b]	
EI	RDO**CC**YHPT**C**NMSNPQI**C**[a]	*Conus ermineus*/p	α1β1γδ, α3β4, α4β2	α1β1γδ	[16,17]
EpI	G**CC**SDPR**C**NMNNPDY**C**[a]	*C. episcopatus*/m	α7	α3β2/α3β4	[31,47]
GIC	G**CC**SHPA**C**AGNNQHI**C**[a]	*Conus geographus*/p	hα3β2	N/A	[48]
GID	IRDγ**CC**SNPA**C**RVNNOHV**C**		α3β2	N/A	[49]
Lp1.1	G**CC**ARAA**C**AGIHQEL**C**[a]	*C. leopardus*/v	α3β2, α6α3β2	N/A	[21]
MII	G**CC**SNPV**C**HLEHSNL**C**[a]	*Conus magus*/p	α6/α3β2β3	α3β2	[50]
OmIA	G**CC**SHPA**C**NVNNPHI**C**G[a]	*Conus omaria*/m	α3β2, α7	N/A	[51]
PeIA	G**CC**SHPA**C**SVNHPEL**C**[a]	*Conus pergrandis*/unknown	α9α10	N/A	[52]
PIA	RDP**CC**SNPV**C**TVHNPQI**C**[a]	*Conus purpurascens*/p	α6/α3β2, α6/α3β2β3	N/A	[53]
PnIA	G**CC**SLPP**C**AANNPDY**C**[a]	*Conus pennaceus*/m	α3β2	α7[b]	[54,55]
[A10L]PnIA	G**CC**SLPP**C**ALNNPDY**C**[a]		α7	α7[b]	
PnIB	G**CC**SLPP**C**ALSNPDY**C**[a]		α7	α3β4	[54,56]
SrIA	RT**CC**SROT**C**RMγYPγL**C**G[a]	*Conus spurius*/v	α4β2, α1β1γδ	N/A	[17]
SrIB	RT**CC**SROT**C**RMEYPγL**C**G[a]		α4β2, α1β1γδ	N/A	
[γ15E]SrIB	RT**CC**SROT**C**RMEYPEL**C**G[a]		α4β2, α1β1γδ	N/A	
Vc1.1	G**CC**SDPR**C**NYDHPEI**C**[a]	*Conus victoriae*/m	α9α10	α3β4[b]	[33,37]
vc1a	G**CC**SDOR**C**NYDHPγI**C**[a]		α9α10	N/A	[37]
Mr1.1	G**CC**SHPA**C**SVNNPDI**C**[a]	*C. marmoreus*/m	α7	N/A	This work
Lp1.4	G**CC**SHPA**C**SGNHQEL**C**D[a]	*C. leopardus*/v	α1β1γδ, α6α3β2	N/A	This work

Conserved cysteine residues are highlighted in bold face. The engineered peptides are underlined to make the distinction from the naturally occurring ones. N/A, not available.
For post-translational modifications: [a]amidated C-terminus; γ, γ-carboxyglutamate; O, hydroxyproline; Y, sulfated tyrosine; [b]additional component.

specificity receptor recognition profile. Given that all known α4/7-conotoxins have a nonpolar residue at position 2 of loop II (**Table 1**), the nonpolar Val and Gly residues at this position might be involved in binding to neuronal nAChR subtypes for Mr1.1 and Lp1.4, respectively. The low-affinity inhibition of Mr1.1 and Lp1.4 suggests that they could act together with other functionally linked groups of peptides through a combination neuropharmacological strategy to capture prey. It is also possible that Mr1.1 and Lp1.4 have alternative molecular targets other than nAChRs, like ρ-conotoxin TIA, which has a cysteine framework I with the 4/7 intercysteine spacing pattern but targets the α1-adrenoreceptor [57].

In conclusion, we describe the chemical synthesis and initial characterization of α4/7-conotoxins Mr1.1 and Lp1.4, which help to understand the molecular mechanism for toxin-receptor interaction. Mr1.1 has analgesic activity and can be developed as a therapeutic agent for the treatment of inflammatory pain.

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