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Conantokins Derived from the *Asprella* Clade Impart con*Rl*-B, an *N*-Methyl D-Aspartate Receptor Antagonist with a Unique Selectivity Profile for NR2B Subunits

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Supporting Information

ABSTRACT: Using molecular phylogeny has accelerated the discovery of peptidic ligands targeted to ion channels and receptors. One clade of venomous cone snails, *Asprella*, appears to be significantly enriched in conantokins, antagonists of *N*-methyl D-aspartate receptors (NMDARs). Here, we describe the characterization of two novel conantokins from *Conus rolani*, including conantokin con*Rl*-B that has shown an unprecedented selectivity for blocking NMDARs that contain NR2B subunits. Con*Rl*-B shares only some sequence similarity with the most studied NR2B selective conantokin, con*G*. The divergence between con*Rl*-B and con*G* in the second inter-Gla loop was used to design analogues for structure−activity studies; the presence of Pro10 was found to be key to the high potency of con*Rl*-B for NR2B, whereas the ε-amino group of Lys8 contributed to discrimination in blocking NR2B- and NR2A-containing NMDARs. In contrast to previous findings for Tyr5 substitutions in other conantokins, con*Rl*-B[L5Y] showed potencies on the four NR2 NMDA receptor subtypes that were similar to those of the native con*Rl*-B. When delivered into the brain, con*Rl*-B was active in suppressing seizures in the model of epilepsy in mice, consistent with NR2B-containing NMDA receptors being potential targets for antiepileptic drugs. Circular dichroism experiments confirmed that the helical conformation of con*Rl*-B is stabilized by divalent metal ions. Given the clinical applications of NMDA antagonists, con*Rl*-B provides a potentially important pharmacological tool for understanding the differential roles of NMDA receptor subtypes in the nervous system. This work shows the effectiveness of coupling molecular phylogeny, chemical synthesis, and pharmacology for discovering new bioactive natural products.

N-Methyl D-aspartate (NMDA) receptors make up a major class of glutamate receptors that play critical roles in excitatory neurotransmission. These receptors have been clinically validated therapeutic drug targets and are implicated in the synaptic plasticity in neuropathic pain, learning, mood disorders, and addiction. Functional NMDARs are hetero-tetrameric complexes comprising two NR1 subunits and two NR2 subunits. Four genes, namely, NR2A, NR2B, NR2C, and NR2D, encode the NR2 subunits. NR2B targeting antagonists are being developed for the treatment of pain, epilepsy, stroke, and Parkinson's disease.[1,2] Small molecule NMDA antagonists, summarized in Table S1 of the Supporting Information, that preferentially or selectively block NMDARs containing various NR2 subunits have been developed.[3−14] Given the molecular complexity and importance of NMDARs, there is a constant need for novel NMDA antagonists that selectively discriminate with a wide separation in affinities among the four individual NR2 subunits. Such compounds should be useful pharmacological tools for defining the role of individual NMDA receptor subtypes in the nervous system.

Conantokins make up a diverse group of *Conus* peptides that target NMDA receptors.[15,16] Characterization using heterologous expression assays showed that conantokins act competitively at the glutamate-binding site on the NR2 subunit.[17] Most conantokins have been found to preferentially target NMDA receptors containing the NR2B subunit, although the affinity for the other NR2 subunits of the NMDA receptor varies substantially.[18−22] Table 1 depicts the amino acid sequences of all conantokins characterized thus far. Among the conantokins characterized, con*G* has demonstrated the greatest selectivity for the NR2B subunit. Con*G* has shown efficacy in a number of preclinical studies, including models of pain, epilepsy, and neuroprotection following ischemia.[15,21,23−26] On the basis of favorable preclinical studies, con*G* has reached phase I clinical trials for the treatment of epilepsy.[21,27−29]

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Table 1. Amino Acid Sequences of Previously Characterized Conantokins

Conus species	conantokin	amino acid sequence	ref
Conus geographus	con*G*	GE$\gamma\gamma$LQγNQγLIRγKSN[a]	51
Conus tulipa	con*T*	GE$\gamma\gamma$YQKMLγNLRγAEVKKNA[a]	52
Conus radiatus	con*R*	GE$\gamma\gamma$VAKMAAγLARγNIAKGCKVNCYP[b]	53
Conus lynceus	con*L*	GE$\gamma\gamma$VAKMAAγLARγDAVN[a]	54
Conus parius	con*Pr*-A	GEDγYAγGIRγYQLIHGKI[b]	34
C. parius	con*Pr*-B	DEOγYAγAIRγYQLKYGKI[b]	34
C. parius	con*Pr*-C	GEOγVAKWAγGLRγKASSN[a]	34
Conus purpurascens	con*P*	GE$\gamma\gamma$HSKYQγCLRγIRVNKVQQγC[b]	55
Conus brettinghami	con*Br*	GD$\gamma\gamma$YSKFIγRERγAGRLDLSKFP[b]	22
Conus rolani	con*Rl*-A	AD$\gamma\gamma$YLKFIγEQRKQGKLDPTKFP[b]	36

[a]Amidated C-terminus, $CONH_2$. [b]Free carboxyl group at the C-terminus.

Our research group has recently been using molecular phylogeny-guided discovery to facilitate the identification of novel *Conus* peptides targeting sodium channels, nAChRs, and NMDA receptors.[30−33] Several new conantokins have been discovered using this approach, each with a unique pharmacological profile.[22,34−36] Particularly noteworthy is the *Asprella* clade of *Conus* spp. that contains *Conus brettinghami*, *Conus sulcatus*, *Conus bocki*, *Conus rolani*, and *Conus samiae*, which appears to be a rich source of NMDA receptor-targeted peptides. Recently, two new conantokins, con*Br* and con*Rl*-A, from *C. brettinghami* and *C. rolani*, respectively, were described; despite having divergent sequences, these peptides exhibited similar pharmacological properties.[35,36] Here, we describe characterization of two new conantokins from *C. rolani*; one of these, con*Rl*-B, has a subtype specificity more pronounced than that of any conantokin previously reported.

MATERIALS AND METHODS

Preparation of Genomic DNA and Characterization of Clones Encoding con*Rl*-B. Genomic DNA was prepared from 20 mg of *C. rolani* tissue using the Gentra PUREGENE DNA Isolation Kit (GentraSystems, Minneapolis, MN) according to the manufacturer's standard protocol. Ten nanograms of *C. rolani* genomic DNA was used as a template for polymerase chain reaction (PCR) with oligonucleotides corresponding to conserved regions of the signal sequence and 3′ UTR sequences of conantokin prepropeptides, as described previously.[22,34−36] The resulting PCR product was purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN) following the manufacturer's suggested protocol. The eluted DNA fragment was ligated to the pNEB206A vector using the cloning kit (New England BioLabs, Inc., Beverly, MA) following the manufacturer's suggested protocol and the resulting product transformed into DH5α competent *Escherichia coli* cells. The nucleic acid sequences of the resulting conantokin toxin-encoding clones were determined according to the standard protocol for DNA sequencing.

Peptide Synthesis. Native peptide con*Rl*-B and its analogues were synthesized using an Apex 396 automated peptide synthesizer (AAPPTec, Louisville, KY) and a standard solid-phase Fmoc (9-fluorenylmethyloxycarbonyl) protocol. The peptides were assembled on preloaded Fmoc-L-Asn (Trt)-Rink Amide MBHA resin purchased from Peptides International, Inc. (Louisville, KY) (substitution, 0.38 mmol/g). All standard amino acids were purchased form AAPPTec; Fmoc-γ-carboxy-γ-(di-*tert*-butyl ester)-L-glutamic acid (γ-carboxyglutamic acid) was from Advanced ChemTech (Louisville, KY), *N*-α-Fmoc-*O*-*tert*-butyl-L-*trans*-4-hydroxyproline (Hyp) from NovaBiochem/EMD Chemicals (Gibbstown, NJ), and Fmoc-L-norleucine from ChemImpex Int. (Wood Dale, IL). Side chain protection was as follows for the following amino acids: *O*-*tert*-butyl (OtBu) for Gla and Glu, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg, *tert*-butyloxycarbonyl (Boc) for Lys, *tert*-butyl (tBu) for Hyp and Tyr, and trityl (Trt) for Asn and Gln. Peptides were synthesized on a 30 μmol scale. Coupling activation was achieved with 1 equiv of 0.22 M benzotriazol-1-yl-oxy-tripyrrolidinophosphonium hexafluorophosphate and 2 equiv of 2 M *N*,*N*-diisopropylethylamine in *N*-methyl-2-pyrrolidone. A 10-fold excess of amino acid was used except for γ-carboxyglutamic acid, for which a 3-fold excess was applied. Each coupling reaction was conducted for 60 min except for γ-carboxyglutamic acid, for which the reaction time was 90 min. Fmoc deprotection was conducted for 20 min with a 20% solution of piperidine in DMF. Each peptide was cleaved from 25−50 mg of resin by a 3 h treatment with 0.5 mL of Reagent K [trifluoroacetic acid (TFA)/water/phenol/thioanisole/ethanedithiol mixture, 82.5/5/5/5/2.5 by volume] and subsequently filtered and precipitated with cold methyl *tert*-butyl ether (MTBE). The crude peptides were then collected by centrifugation at 5000*g* for 8 min and washed two times with cold MTBE. The washed peptide pellet was dissolved in 20% acetonitrile in 0.1% TFA and purified by reversed-phase HPLC using a preparative C_{18} Vydac column (218TP510, 250 mm × 10 mm, 5 μm particle size) eluted with a linear gradient from 20 to 60% solvent B over 40 min at a flow rate 4 mL/min. The HPLC solvents were 0.1% (v/v) TFA in water (solvent A) and 0.1% TFA (v/v) in 90% aqueous acetonitrile (solvent B). The eluent was monitored by measuring the absorbance at 220 nm. The purity of the peptides was assessed by an analytical C_{18} Vydac reversed-phase HPLC system (218TP54, 250 mm × 4.6 mm, 5 μm particle size) using a linear gradient from 20 to 55% solvent B over 30 min (retention times and gradients specified in Table S1 of the Supporting Information) with a flow rate of 1 mL/min. Peptides were quantified against a reference peptide using the same HPLC separation conditions. Molecular masses of all analogues were confirmed by ESI-MS (Table S2 of the Supporting Information).

Heterologous Expression of NMDA Receptors. Rat NMDA receptor clones NR2A, NR2B, NR2C, NR2D, NR3A, NR3B, NR1-2a, NR1-2b, and NR1-4b were used (GenBank entries AF001423, U11419, U08259, U08260, NM_001198583, NM_133308, U08262, U08264, and U08268, respectively). Splice variant NR1-2b was used for all concentration−response assays, as it is widely expressed in the

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central nervous system.[37,38] To control for the possibility that exon 5 may affect the sensitivity of the NMDA receptor to conantokins (i.e., refs 39 and 40), NR1-2a was separately coexpressed with all NR2 subtypes. We observed low expression levels of NR3A and NR3B when they were coexpressed with NR1-2b or NR1-2a; thus, the NR1-4b splice variant was coexpressed with these subunits. All of the expression clones, except NR3B, were under control of a T7 promoter. A T3 promoter controlled expression of NR3B. For each clone, Ambion RNA transcription kits (Ambion, Inc.) were used to make capped RNA (cRNA) for injection into *Xenopus* oocytes. To express NMDA receptors, 2−5 ng of RNA encoding each subunit was injected into each oocyte. Oocytes were maintained in ND96 solution [96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.2−7.5)] with antibiotics (Septra, Amikacin, and Pen/Strep). All voltage-clamp electrophysiology was performed prior to 7 days postinjection.

Two-Electrode Voltage-Clamp Electrophysiology. All oocytes were voltage clamped at −70 mV and room temperature. Oocytes were gravity-perfused with Mg^{2+}-free ND96 buffer [96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES (pH 7.2−7.5)]. Mg^{2+} was omitted from the ND96 buffer to prevent the voltage-dependent blockade of NMDA receptors at −70 mV. BSA (0.1 mg/mL) was added to reduce the rate of nonspecific absorption of peptide. In an additional set of experiments, con*Rl*-B was also assessed on oocytes in the presence of Ca^{2+}-free, Mg^{2+}-free ND96 buffer substituted with barium chloride [96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $BaCl_2$, and 5 mM HEPES (pH 7.2−7.5)]; no difference in the effect of peptide was seen between oocytes tested in the presence of calcium-containing buffer or barium-containing buffer. The NMDA receptor-mediated current was elicited by the administration of 1 s pulses of agonist (200 μM glutamate and 20 μM glycine, in Mg^{2+}-free ND96 for NR1−NR2 subunit combinations; 20 μM glycine, in Mg^{2+}-free ND96 buffer for NR1−NR3 subunit combinations). To measure the effect of conantokins and analogues on oocytes expressing NMDA receptors, the buffer flow was halted, and the peptides were applied in a static bath for a duration sufficient to reach equilibrium, or a minimum of 5 min. A blockade of NMDA receptor-mediated current by peptides was measured by normalizing the response of the first agonist pulse following a static bath to the baseline response (current in response to agonist prior to peptide application). A virtual instrument made by D. Yoshikami at the University of Utah was used for data acquisition, and concentration−response curves were generated using Prism (GraphPad Software, Inc.). The equation % response = $100/[1 + ([\text{peptide}]/IC_{50})^{n_H}]$, where n_H is the Hill coefficient and IC_{50} is the concentration required to achieve half-maximal block, was used to fit concentration−response curves.

Anticonvulsant Assay of con*Rl*-B. The 6 Hz partial psychomotor seizure test was performed to assess the anticonvulsant potential of con*Rl*-B as described previously.[41] Adult male CF No 1 albino mice (30−35 g) obtained from Charles River (Portage, MI) were utilized for behavioral seizure testing in the 6 Hz model of partial psychomotor seizure activity following intracerebroventricular (icv) administration of con*Rl*-B. Stock solutions of the peptide were prepared in 0.9% saline and were diluted to the required concentration prior to icv injections. For icv administration, the test solution was administered in a volume of 5 μL, using a Hamilton syringe (size number 701), directly through the skull into a lateral ventricle of the brain at a depth of 3 mm. A 6 Hz current of 32 mA was administered via corneal electrodes for 3 s to elicit a partial psychomotor seizure. Animals not displaying behavioral seizure activity, characterized by an initial momentary stun followed immediately by forelimb clonus, twitching of the vibrissae, and Straub tail, were considered "protected".

Circular Dichroism Spectroscopy. Circular dichroism spectra were recorded on an AVIV model 62D spectropolarimeter, using the method and parameters described in the CD studies of con*Rl*-A.[35,36] Briefly, peptides were dissolved at a final concentration of 100 μM in 10 mM HEPES buffer (pH 7.0) with or without 2 mM $CaCl_2$, and measurements were taken at room temperature. Subtracting the peptide CD signal from that of the buffer alone eliminated the contribution of buffer to the peptide CD signal. The spectral intensities were expressed as mean residue ellipticities using the equation reported elsewhere,[34] and a molar ellipticity of −33530.78 deg cm^2 $dmol^{-1}$ was estimated to be a perfect α-helix (100% α-helix). The percent of helical conformation was calculated by assuming a linear relationship in comparison with 100% α-helix. The estimate of the percent of helical conformation induced by divalent calcium in con*Rl*-B was calculated by subtracting the percent of peptide helical content with calcium from that of peptide helical content in the absence of calcium.

RESULTS AND DISCUSSION

Molecular Cloning, Sequence Prediction, and Synthesis. Two *C. rolani* gene sequences encoding peptide precursors with a high degree of homology to other members of the conantokin family were cloned and designated con*Rl*-B and con*Rl*-C. The predicted peptide precursor and mature toxin sequences corresponding to the open reading frames of con*Rl*-B and con*Rl*-C are shown in Figure 1 and compared to the previously elucidated sequences for con*Rl*-A and con*G*. As predicted, the propeptide regions of con*Rl*-B and con*Rl*-C are highly conserved with respect to other conantokin sequences (Figure 1A).

Remarkably, when the sequences were optimally aligned, there was a high degree of similarity between the predicted

Figure 1. Predicted amino acid sequences of con*Rl*-B and con*Rl*-C. Predicted translated sequences from genomic DNA are shown for the prepeptide (A), propeptide (B) and mature toxin regions (C) of con*Rl*-B and con*Rl*-C, aligned with the sequences of con*Rl*-A and con*G* for comparison. Shading indicates residues conserved among the four sequences. Two potential mature sequences predicted for con*Rl*-B (C). The proline that may undergo post-translational modification to hydroxyproline is highlighted in bold. O denotes 4-trans-hydroxyproline; γ denotes γ-carboxyglutamate, and # denotes C-terminal amidation.

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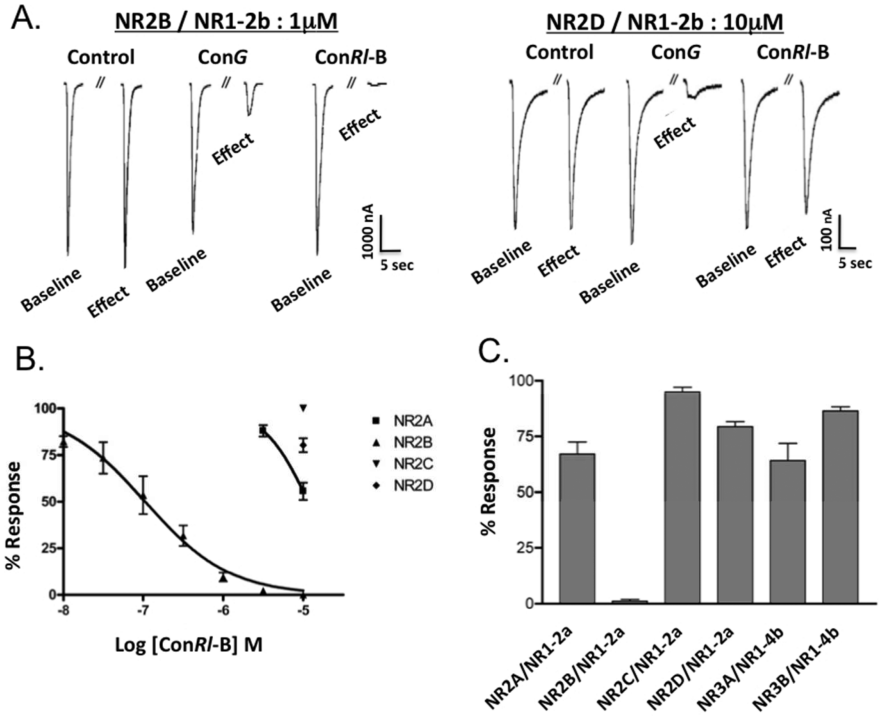

Figure 2. NMDA receptor subtype selectivity of con*Rl*-B. (A) Current traces from *Xenopus* oocytes expressing heterologous NR1-2b–NR2B and NR1-2b–NR2D combinations. con*Rl*-B blocks most of the agonist-elicited current in oocytes expressing NR1-2b–NR2B (left) but only weakly blocks NR1-2b–NR2D (right). (B) Concentration–response curves for con*Rl*-B tested against the four NR2 NMDA receptor subtypes. Data points represent normalized peak currents ± the standard error of the mean (SEM) from a minimum of three oocytes. (C) Normalized current responses of NR1-2a–NR2 and NR1-4b–NR3 subunit combinations, in response to 10 μM con*Rl*-B.

mature peptide sequences of con*Rl*-B or con*Rl*-C and con*G* (65% of the amino acids of con*G* are identical); this was in striking contrast to a comparison of con*Rl*-B or con*Rl*-C to con*Rl*-A[36] from the same species (only 17% of the amino acids of con*Rl*-B and con*Rl*-C are identical, the majority of these being Gla residues) (Figure 1B). Because of the high degree of similarity to venom-purified con*G*, con*Rl*-B and con*Rl*-C were predicted to have a similar pattern of post-translational modification: a Gla at positions 3 and 4 and a Gla every three to four amino acids after, in addition to an amidated C-terminus. Interestingly, the presence of proline at position 10 in con*Rl*-B was a novel structural feature, but given the high degree of post-translational modifications in *Conus* peptides including 4-trans-hydroxyproline (Hyp), we predicted that this proline is likely hydroxylated; conantokins from *C. parius* contain Hyp residues, though not at the homologous position.[34]

Chemical synthesis of the predicted mature sequences of both peptides from *C. rolani* was performed on a solid support as described in Materials and Methods. γ-Carboxyglutamate residues were coupled in all positions where there was a Glu codon in the corresponding mature toxin derived from the cDNA clone (except for Glu2, which is never post-translationally modified). Given that the presence of Hyp was based on a less secure prediction, we also synthesized the con*Rl*-B analogue containing Pro10 instead of Hyp10. The HPLC elution of purified con*Rl*-B is shown in Figure S1 of the Supporting Information. Mass spectrometry results, summarized in Table S2 of the Supporting Information, were consistent with the predicted sequence of the synthetic peptides.

Electrophysiological Characterization. Con*Rl*-B and con*Rl*-C were assessed for antagonist activity on the heterologous expression of an array of NMDA receptor subtypes in *Xenopus* oocytes, using two-electrode voltage-clamp electrophysiology (see Materials and Methods). Figure 2A depicts agonist-elicited current traces from NMDA receptors expressing the NR2B and NR2D subunits for con*Rl*-B. Con*Rl*-B blocked the current in NR2B-containing NMDA receptors at 1 μM more completely than did con*G* (left panel). Dose–response experiments for con*Rl*-B (Figure 2B) yielded an IC_{50} of 0.1 μM for blocking NR2B.[34] Strikingly, at the highest concentration tested (10 μM), con*Rl*-B had little or no antagonist activity on three of the four NR2 subunits when coexpressed with NR1-2b, including NR2C and NR2D for which con*G* has an IC_{50} of 1 μM (NR2D traces shown, Figure 2A, right panel). Thus, con*Rl*-B discriminated at least 100-fold between NR2B and all other NR2 subunits. As shown in Figure S2 of the Supporting Information and summarized in Table 2, con*Rl*-C was significantly less selective than con*Rl*-B in blocking NMDARs containing NR2B subunits.

As the potency of conantokins has been reported to vary as a function of the presence or absence of the N-terminal exon (exon 5) in the NR1 subunit,[39,40] the potency of con*Rl*-B was also assessed using oocytes expressing the NR1-2a splice variant in combination with each of the four NR2 subunits (Figure 2C). Similar to the effects seen on NMDA receptor subtypes expressing the exon 5-containing splice variant, NR1-2b, 10 μM con*Rl*-B had little or no potency on any NR1-2a-containing subtypes, with the exception of the NR1-2a–NR2B combination.

NR3 subunits have been reported to form a functional glycine receptor when expressed in *Xenopus* oocytes in combination with NR1 subunits,[42] con*Rl*-B was also assessed for potency on the NR1/NR3A and NR1/NR3B subtypes. As

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Table 2. IC_{50} Values for con*Rl*-B and Its Analogues Determined Using Heterologous Expression of Four NMDA Receptor Subtypes Expressed in *Xenopus* Oocytes

peptide	IC_{50} (μM)			
	NR2A	NR2B	NR2C	NR2D
con*Rl*-B	~10	0.1	>10	>10
con*Rl*-B[L5Y]	>10	0.12	>10	>10
con*Rl*-B[O10A]	>10	0.94	>10	>10
con*Rl*-B[desO10]	>10	2.17	>10	>10
con*Rl*-B[K8Nle]	0.55	0.04	>10	>10
con*Rl*-B[desKAO;N8Q9]	>10	>10	>10	>10
con*Rl*-C	2.9	1.4	>10	>10
con*G*[a]	>10	0.1	1	1

[a]Values from ref 34.

shown in Figure 2c, 10 μM con*Rl*-B showed little or no potency on subtype NR1 or NR3. Thus, con*Rl*-B is the most selective conantokin for NR2B-containing NMDA receptors characterized to date.

Anticonvulsant Assay of con*Rl*-B. Conantokins have anticonvulsant activity (reviewed in ref 15); given the high subtype selectivity of con*Rl*-B for NR2B, this peptide was assessed for activity using the 6 Hz partial psychomotor seizure test in mice. At a dose of 0.1 nmol following icv injection, 50% of mice were protected (n = 8) from seizures at a time to peak effect (TPE) of 1 h, whereas no control mice (n = 8) were protected (5 μL of saline, icv). The rectal body temperature measured at 1 h (TPE) did not differ between groups.

Determinants of NR2B Selectivity. Comparing sequences of con*Rl*-B and con*G* points to striking structural differences in the second inter-Gla fragments (Figure 3). Indeed, the presence

Con*G*	G E γ γ L Q γ N Q - γ L I R γ K S N#[a,b]
Con*Rl*-B	G E γ γ L A γ K A O γ F A R γ L A N#[c]
Con*Rl*-B[K8X]	G E γ γ L A γ X A O γ F A R γ L A N#[d]
Con*Rl*-B[O10P]	G E γ γ L A γ K A P γ F A R γ L A N#
Con*Rl*-B[O10A]	G E γ γ L A γ K A A γ F A R γ L A N#
Con*Rl*-B[desO10]	G E γ γ L A γ K A - γ F A R γ L A N#
Con*Rl*-B[L5Y]	G E γ γ Y A γ K A O γ F A R γ L A N#
Con*Rl*-B[desKAO;N8Q9]	G E γ γ L A γ N Q - γ F A R γ L A N#

Figure 3. Sequences of native con*Rl*-B and its analogues. The shaded region indicates the region of the peptide that primary sequence analysis suggests is important for the selectivity profile of con*Rl*-B. [a]γ denotes γ-carboxyglutamic acid. [b]# denotes C-terminal amidation. [c]O denotes 4-*trans*-hydroxyproline. [d]X denotes L-norleucine.

of either Pro10 (Hyp10) or a positively charged residue at position 8 (Lys8) is a sequence feature not reported for any of the conantokins characterized so far. This prompted us to examine whether the second inter-Gla loop might contain key determinants of the high subtype selectivity of con*Rl*-B. We designed and synthesized SAR analogues in which Pro10 was either deleted (resulting in making the size of the inter-Gla loop similar to that of con*G*) or replaced with Ala (Figure 3). In addition, we assessed the role of the positively charged Lys8 adjacent to Gla7 with an analogue containing norleucine at this position (K8Nle). To examine the effect of Pro10 hydroxylation, we synthesized the Hyp10Pro analogue. Lastly, we substituted the residues found in the second inter-Gla loop of con*G* for those in con*Rl*-B (desKAO;N8Q9). All analogues were chemically synthesized and tested on NMDARs containing different NR2 subunits.

Dose–response studies for SAR analogues are summarized in Figure 4 and Table 2. The potencies of both con*Rl*-B[O10A]

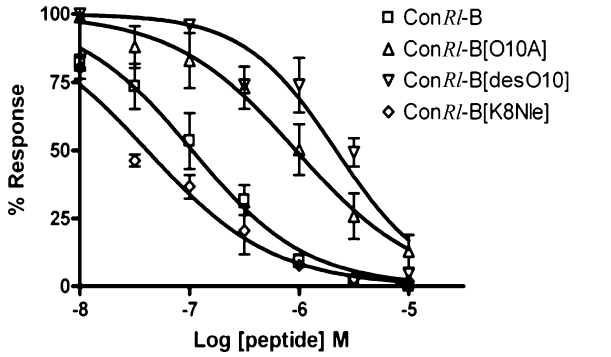

Figure 4. Concentration–response curves of con*Rl*-B analogues on NR2B–NR1-2b combinations, compared to that of native con*Rl*-B. Potency is decreased by O10A and desO10, but not by K8Nle. Sequences of con*Rl*-B and variants are shown in Figure 3. Each data point represents the average peak current, normalized to baseline from a minimum of three oocytes. Error bars represent the SEM.

and con*Rl*-B[desO10] in blocking NR2B decreased by more than 20-fold, suggesting that this residue is an important determinant for activity. Interestingly, the Lys8Nle replacement did not affect the peptide's ability to block NR2B but increased the potency for NR2A-containing NMDA receptors, indicating that the ε-amino group of Lys8 is important for selectivity. No significant difference from con*Rl*-B was observed for con*Rl*-B containing Pro10 instead of Hyp10 (Figure S4 of the Supporting Information). Surprisingly, con*Rl*-B[desKAO;N8Q9] showed little or no activity on any of the NMDA receptor subtypes tested, further indicating that the residues found in the second inter-Gla loop are highly important for the activity of con*Rl*-B. In addition, we also evaluated how the naturally occurring Gla-to-Lys replacements in conantokins (see Table 1) may affect the potency of con*Rl*-B in blocking NR2B-containing NMDA receptors. The potencies of con*Rl*-B[γ7K] and con*Rl*-B[γ15K] (IC_{50}) were 0.12 and 0.68 μM, respectively, suggesting that this replacement has little effect. Lastly, we tested whether Leu5 is an important determinant of selectivity in con*Rl*-B; to this end, we synthesized and tested a Tyr5 variant of con*Rl*-B[L5Y]. Interestingly, and in contrast with data from Tyr5 substitutions in other conantokins,[20,22] con*Rl*-B[L5Y] showed potencies on the four NR2 NMDA receptor subtypes that were very similar to those of native con*Rl*-B.

Structural Characterization of con*Rl*-B. The characteristic structural feature of conantokins is their helical conformation. Most conantokins adopt a helical conformation in the presence of divalent cations, which aligns the Gla residues to stabilize the helical conformation. A few conantokins, such as con*Pr*-C, con*P*, and con*Rl*-A, are inherently helical peptides.[22,34−36,43−46] For example, con*G* is unstructured in the absence of divalent cations (i.e., calcium) and adopts a helical conformation in the presence of divalent cations representing a characteristic metal-dependent helical transition in many conantokin peptides. The metal-dependent helical transition in con*G* is attributed to Gla residues chelating

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calcium by tetravalent interaction, thereby restricting the conformation of the peptide and favoring helix formation.[47]

Given the similarities in sequence and the presence of an identical number and distribution of Gla residues in con*Rl*-B compared to those in con*G*, we hypothesized that con*Rl*-B was structurally similar to con*G*. We employed circular dichroism spectroscopy to study the effect of divalent cations in inducing the helical conformation of con*Rl*-B. Figure 5 shows CD spectra of con*Rl*-B in the presence and absence of Ca^{2+}. Con*Rl*-B is unstructured in the absence of calcium and adopts a helical conformation in the presence of calcium, a feature similar to that of con*G*. The estimated helical content of con*Rl*-B in the presence of calcium is 59% (that of con*G* is 57%).[34] The percent of helical transition induced by calcium in con*Rl*-B is 49%, and that of con*G* is 44%.[34] CD spectra of con*Rl*-B[O10P] in the presence and absence of calcium (Figure S3 of the Supporting Information) show that this analogue is unstructured in the absence of calcium and adopts a helical conformation in the presence of calcium, similar to that of con*Rl*-B. The estimated helical content of con*Rl*-B[O10P] in the presence of calcium is 51%, and the percent of helical transition induced by calcium is 40%. Comparison of the CD spectra of con*Rl*-B and con*Rl*-B[O10P] suggests that they have similar helical content in the absence of calcium.

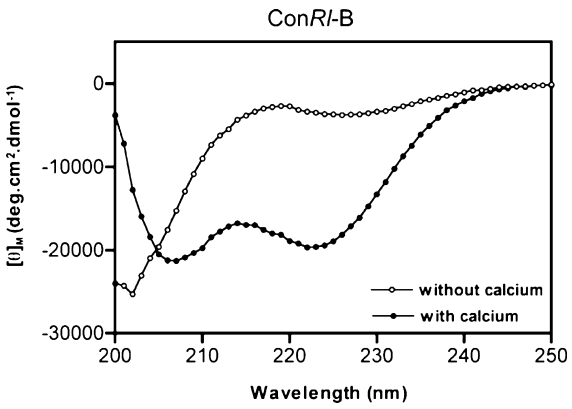

Figure 5. CD spectra of con*Rl*-B. Spectra were recorded with or without 2 mM $CaCl_2$ containing 10 mM HEPES buffer (pH 7.0), and shown are average spectra obtained from five independent scans (n = 5). The dual minima at 208 and 222 nm, in the presence of calcium, suggest that con*Rl*-B adopts a helical conformation. The estimated percentage of helicity of the peptide in the absence of calcium is 10% and in the presence of calcium is 59%.

CONCLUSION

We describe the characterization of a novel NMDA antagonist that is highly selective for NMDA receptors containing NR2B subunits and exhibits anticonvulsant activity. Two novel sequence features, the presence of a positively charged Lys residue at position 8 and a Hyp at position 10, contribute to the potency and selectivity of this peptide. Prior to this report, con*G* has been regarded as the most NR2B selective member of the conantokin superfamily;[17,34] however, some reports suggest that con*G* is more broadly selective.[48,49] con*G* is reported to have biphasic effects and at least two binding sites on NR2A receptor subtypes.[50] Some differences in con*G* pharmacology have also been attributed to variations in NR1 splicing, in particular exon 5 (i.e., ref 40). In this work, we have assessed con*Rl*-B for potency toward all four NR2 subunits in combination with either NR1a or NR1b. In all cases, con*Rl*-B maintains a high degree of selectivity for NR2B. Thus, con*Rl*-B is an important subtype pharmacological tool for dissecting the role of NMDARs in the nervous system.

ASSOCIATED CONTENT

Supporting Information

Selected examples of antagonists for NMDARs containing the NR2B subunit (Table S1), purity, HPLC retention times, and mass spectrometry results for conantokins studied in this work (Table S2), concentration−response curves of con*Rl*-C (Figure S2) and con*Rl*-B[O10P] (Figure S4) on the four different NR2 subunits of the NMDA receptor separately coexpressed with NR1-2b in *Xenopus* oocytes, and CD spectroscopy of con*Rl*-B[O10P] (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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