Published in final edited form as:

Toxicon. 2014 April ; 81: 67–79. doi:10.1016/j.toxicon.2014.01.016.

FROM MOLECULAR PHYLOGENY TOWARDS DIFFERENTIATING PHARMACOLOGY FOR NMDA RECEPTOR SUBTYPES

Randall J. Platt^{1,†}, Kigen J. Curtice^{1,†}, Vernon D. Twede¹, Maren Watkins¹, Paweł Gruszczyski^{2,3}, Grzegorz Bulaj², Martin P. Horvath¹, and Baldomero M. Olivera¹

¹Department of Biology, University of Utah, Salt Lake City, Utah 84112 ²Medicinal Chemistry, University of Utah, Salt Lake City, Utah 84112 ³Intercollegiate Faculty of Biotechnology and Chemistry, University of Gdańsk, 80-952 Gdańsk, Poland, and Medical University of Gdańsk, 80-822 Gdańsk, Poland

Abstract

In order to decode the roles that *N*-methyl-D-aspartate (NMDA) receptors play in excitatory neurotransmission, synaptic plasticity, and neuropathologies, there is need for ligands that differ in their subtype selectivity. The conantokin family of *Conus* peptides is the only group of peptidic natural products known to target NMDA receptors. Using a search that was guided by phylogeny, we identified new conantokins from the marine snail *Conus bocki* that complement the current repertoire of NMDA receptor pharmacology. Channel currents measured in *Xenopus* oocytes demonstrate conantokins conBk-A, conBk-B, and conBk-C have highest potencies for NR2D containing receptors, in contrast to previously characterized conantokins that preferentially block NR2B containing NMDA receptors. Conantokins are rich in γ -carboxyglutamate, typically 17–34 residues, and adopt helical structure in a calcium-dependent manner. As judged by CD spectroscopy, conBk-C adopts significant helical structure in a calcium ion-dependent manner, while calcium, on its own, appears insufficient to stabilize helical conformations of conBk-A or conBk-B. Molecular dynamics simulations help explain the differences in calcium-stabilized structures. Two-dimensional NMR spectroscopy shows that the 9-residue conBk-B is relatively unstructured but forms a helix in the presence of TFE and calcium ions that is similar to other conantokin structures. These newly discovered conantokins hold promise that further exploration

Address correspondence to: Kigen J. Curtice, 257 South 1400 East, Rm. 201, Salt Lake City, UT 84112. Tel: 801-585-1868; Fax: 801-585-5010; kigen.curtice@utah.edu.

[†]These authors contributed equally to this manuscript

*The authors declare that there are no conflicts of interest.

The authors acknowledge that they have no conflicts of interest to declare.

Ethical Statement

On behalf of all the contributing authors of this manuscript, Kigen Curtice declares that: (a) The work provided herein is original and described accurately, (b) this work is not being considered for publication elsewhere, (c) all authors listed have made significant contribution to the work described, and (d) the highest ethical standards have been used in any experimentation involving animals.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

of small peptidic antagonists will lead to a set of pharmacological tools that can be used to characterize the role of NMDA receptors in nervous system function and disease.

Keywords

NMDA antagonist; conus peptides; NR2D subunits; NMR structure; molecular modeling; molecular recognition; subtype-selective antagonist; glutamate receptors

1. Introduction

Glutamate receptors are broadly classified into two groups, the metabotropic receptors that transduce ligand binding via G protein-coupled signaling cascades and the ionotropic receptors that report ligand binding by opening an ion channel. Ionotropic glutamate receptors of the mammalian central nervous system are further divided into two subclasses, N-methyl-D-aspartate (NMDA) and non-NMDA receptors. NMDA receptors are heterotetrameric cation channels formed from two obligatory NR1 subunits and two NR2 or NR3 subunits (Schüler et al., 2008). In mammals, seven NMDA receptor subunit genes (NR1, NR2A, NR2B, NR2C, NR2D, NR3A, and NR3B) combined with splice variants for NR1, NR2C, NR2D, and NR3A gene products create multiple molecular isoforms (Cull-Candy et al., 2001; Dingledine et al., 1999; Hollmann and Heinemann, 1994; Simeone et al., 2004). Each isoform likely contributes uniquely to processes such as synaptic plasticity, learning, memory, and nervous system pathologies (Cull-Candy et al., 2001); however, the precise role of each isoform is not well understood.

In combination with gene knockouts, pharmacological reagents provide useful tools for defining the roles of individual NMDA receptor isoforms. To this end, small molecule discovery and design have provided several antagonists that preferentially block NMDA receptors, including N-terminal domain-binding allosteric modulators, such as zinc, which targets NR2A (Choi and Lipton, 1999; Low et al., 2000; Paoletti et al., 1997; Rachline et al., 2005), and ifenprodil derivatives that target NR2B (Acklin et al., 1998; Ametamey et al., 2000; Auberson et al., 1999; Auberson et al., 2002; Kinarsky et al., 2005; Morley et al., 2005; Mosley et al., 2009). In addition, non-competitive quinazolin-4-one derivatives, and competitive piperazine-2,3-dicarboxylate derivatives show moderate selectivity for the NR2C and/or NR2D subunits (Costa et al., 2009; Feng et al., 2004; Mosley et al., 2010). However, a reliable differentiating pharmacology for NMDA receptor subtypes is not currently available.

Conantokins are peptide inhibitors of NMDA receptors that are rich in gamma-carboxyglutamate (Gla) residues and are thought to act competitively at or near the glutamate-binding site on NR2 subunits (Donevan and McCabe, 2000; Hammerland, 1992; Sheng et al., 2009; Tsai et al., 2005; Wittekindt et al., 2001). Post-translationally modified Gla residues in conantokins are generally spaced every three to four amino acid residues and coordinate with divalent cations to stabilize an α -helical conformation (Blandl et al., 2000; Chandler et al., 1993; Rigby et al., 1997; Warder et al., 1998). Some conantokins contain appropriately positioned positive residues that help maintain helicity in the absence of metal ions (Blandl et al., 1999; Lin et al., 1997; Teichert et al., 2007; Warder et al., 1997). All

conantokins characterized to date either mildly or strongly prefer the NR2B subtype (Layer et al., 2004; Twede et al., 2009a). These NR2B-selective peptides, including the well-characterized conG, have shown significant potential in the treatment of epilepsy, chronic pain, ischemic stroke, and probing the mechanisms of drug addiction (Layer et al., 2004; Twede et al., 2009a).

Recently, conBr from *Conus bretinghami* was characterized as a conantokin that, though having highest potency for NR2B-containing receptors, exhibited relatively high potency for NMDA receptors containing NR2D subunits (Twede et al., 2009b). Analogs of conBr highlighted the importance of tyrosine at position five for NR2D subtype inhibition (Twede et al., 2009b). We hypothesized that additional examples of NR2D inhibitors might exist within the venoms of closely related *Conus* species and tested this by searching cDNA libraries. Several conantokins were identified from species in the same clade as *Conus bretinghami*. In this report, we describe our results characterizing conantokins from *Conus bocki* (Fig. 1), particularly focusing on cDNA clones with the tyrosine-at-position-five character.

The phylogeny-directed search yielded eleven conantokin sequences, five of which were chemically synthesized and characterized. While two of these conantokins (conBk-D and conBk-E) were inactive on all NMDA receptors tested, the other three (conBk-A, conBk-B, and conBk-C) blocked NMDA receptor ion currents. Different from previously described conantokins, these *Conus bocki* conantokins are the first identified that show a preference for NR2D-containing NMDA receptors. The NMDA receptor-inhibiting toxins are additionally distinctive in that one (conBk-B) is exceptionally short. These peptides provide proof-of-principle, showing that NR2D-blocking NMDA receptor antagonists exist in cone snail venom and that phylogeny-guided searches can facilitate identification of novel peptides with desirable pharmacological activity.

2. Materials and Methods

2.1 Isolation of conantokin clones from *Conus bocki*

Conus bocki cDNA was used as a template for polymerase chain reactions (PCRs) with oligonucleotides corresponding to conserved regions of the signal sequence and 3' UTR sequences of conantokin prepropeptides. Resulting PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Indianapolis, IN) following the manufacturer's protocol. DNA fragments were annealed to pAMP1 vector DNA and the resulting products were transformed into competent DH5 α cells using the CloneAmp pAMP System for Rapid Cloning of Amplification Products (Life Technologies/Gibco BRL, Grand Island, NY). Nucleic acid sequences of resulting conantokin toxin-encoding clones were determined using ABI (Applied Biosystems) automated sequencing (Core DNA Facility, University of Utah).

2.2 Peptide Synthesis

Peptide sequences encoded by *Conus bocki* cDNA were synthesized using N-(9-fluorenyl) methoxycarbonyl (Fmoc)-protected amino acids. After synthesis, peptides were cleaved from 20 mg of resin by suspension in a 1-ml mixture of TFA/H₂O/1,2-ethanedithiol/phenol/

thioanisole (82.5%/5%/2.5%/5%/5% by volume) for 1.5 hours at room temperature. The resulting mixture was filtered under vacuum into methyl-tert-butyl ether (MTBE) at -20°C . Peptide was collected by centrifugation at $5000 \times g$ for 8 min and washed with MTBE; centrifugation and wash steps were repeated three times. The resulting pellet was dissolved in 0.1% trifluoroacetic acid (TFA)/20% acetonitrile (ACN). The peptide solution was applied to a Vydac C18 semi-preparative column (10 mm $\times$ 250 mm, 5 μm particle size) for purification. Elution was carried out at 4 mL/min with use of 0.1%-TFA/10–40% ACN/ H_2O . Electrospray ionization (ESI) mass spectra were obtained using a Voyager GE STR mass spectrometer at the Mass Spectrometry and Proteomic Core Facility of the University of Utah.

2.3 Heterologous NMDA receptor expression in *Xenopus* oocytes

Rat NMDA receptor cDNA clones of NR1-3b, NR2A, NR2B, NR2C, and NR2D contained in a pSGEM vector were provided by Dr. Michael Hollmann from Ruhr-Universität Bochum (GenBank IDs U08266, AF001423, U11419, U08259, and U08260, respectively). cRNA was prepared and purified using in-vitro RNA transcription kits (Ambion, Inc., St. Louis, MO) according to the manufacturer's protocol. For each NMDA receptor subunit cRNA, 2–5 ng was injected into an oocyte using a nanoinjector. Injected oocytes were incubated at 17°C in ND-96/Pen/Strep/Ami/Septra buffer (96mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2) for 1–6 days containing 100 units/ml penicillin G, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 $\mu\text{g}/\text{ml}$ amikacin sulfate, 160 $\mu\text{g}/\text{ml}$ sulfamethoxazole, and 32 $\mu\text{g}/\text{ml}$ trimethoprim.

2.4 Electrophysiology

Voltage clamp electrophysiology was performed as previously described (36). Briefly, oocytes were voltage-clamped at -70 mV and gravity perfused with magnesium ion-free ND-96 buffer (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl_2 , 5 mM HEPES, pH 7.2–7.5) with 0.1 mg/mL bovine serum albumin (BSA). Currents were elicited by one-second pulses of gravity-perfused agonist solution (200 μM glutamate and 20 μM glycine in magnesium ion-free ND96 with 0.1mg/mL BSA). In order to determine appropriate agonist concentrations, glutamate concentration-response relationships were independently determined for all four NR2 subtypes using saturating glycine (20 μM) (Figure S3), and these showed that 200 μM glutamate elicits greater than 90% maximal response for all subtypes. Calcium ions were used in all ND-96 buffers to establish conditions similar to those thought to be important for the structure of conantokins under physiological conditions (Chandler et al., 1993; Rigby et al., 1997; Warder et al., 1998). To rule out the possibility that Ca^{2+} -activated chloride channels were indirectly affecting current inhibition by peptides, control experiments were performed with 1.8 mM Ba^{2+} , and these demonstrated no difference in inhibition (Gowd et al. unpublished data). During experiments, oocytes were continuously perfused in buffer with the exception of static bath periods during peptide administration. Since on- (and off-) rates of some conantokins are relatively slow (Donevan and McCabe, 2000), static bath durations were a minimum of 5 minutes and were determined empirically by the time point at which maximal inhibition was achieved at a given peptide concentration. Qualitatively, the longer peptides conBk-A and conBk-C had slower kinetics than the ultra-short conBk-B. Analyses of current recovery following removal of toxin provided estimates of dissociation constants (k_{off}) ranging from 0.8 to 2

min^{-1} (see Figure S1), meaning that a 5-minute static bath period would be sufficient to reach at least 97% of equilibrium even at the lowest ligand concentration for the slowest dissociating peptide.

NMDA receptor response at a given peptide concentration was calculated by measuring the first agonist-elicited response after the static bath period and then normalizing to baseline agonist response without peptide. For *conBk-B*, complete inhibition of NR2D-type receptors was not achieved at maximum peptide concentration. This is likely an experimental artifact that arises from fast off-rate kinetics of *conBk-B* paired with slow desensitization of NR2D receptors. Thus, percent response was calculated by comparing equivalent time points in control and test responses. Previous work has shown no significant difference in conantokin inhibition when measured by peak (initial) response compared to plateau response (>10 s agonist exposure) (Wittekindt et al., 2001). Concentration-response data were plotted using Prism software (GraphPad Software, Inc., La Jolla, CA) and model curves were generated by fitting the normalized data to the Hill equation ($\% \text{ response} = 100 / \{1 + ([\text{conantokin}] / \text{IC}_{50})^n\}$). To test whether receptor subtype preferences were statistically significant, we applied a two-tailed, unpaired Student's t-test comparing IC_{50} values determined from multiple trials with no assumption concerning the equivalence of variance.

2.5 Circular dichroism (CD)

Conantokins were dissolved in 10 mM HEPES buffer (pH 7.4) with or without 2 mM CaCl_2 and with or without 60% 2,2,2-trifluoroethanol (TFE) to a final peptide concentration of 100 μM . CD spectra were obtained from an average of five scans measured on an Aviv 62DS (Biomedical Inc., Lakewood, NJ) from 250 to 190 nm at room temperature. The molar ellipticity (θ) was calculated by the following equation: $\theta = [100 * (\text{CD signal at } 222\text{nm})] / [(n-1) * L * (\text{peptide concentration in mM})]$ where n is the number of residues, L is the cuvette path length in cm, and the CD signal is given in millidegrees. Helical content was calculated according to the following equation: $\% \alpha \text{ helix} = 100 * (\theta_{222} / \theta_{\text{max}})$, with the theoretical maximum, $\theta_{\text{max}} = -39500 * (1 - (2.75/n))$.

2.6 Molecular modeling

Models of *conBk-A* and *conBk-C* were built using PyMOL (DeLano, 2002) with a secondary structure as predicted by PSIPRED (Jones, 1999). Initial models were optimized using the LEaP module of the AmberTools package. Topology and coordinates of both models, *conBk-A* and *conBk-C*, were built with and without Ca^{2+} (data not shown). The resulting coordinates were submitted to molecular dynamics (MD) simulation with AMBER 9.0 (Case et al., 2005) applying the ff99SB AMBER force field. Simulations were carried out using the implicit solvent model, namely the Generalized Born (GB) solvation model developed by Onufriev, Bashford and Case (GBOBC) (Bashford and Case, 2000). To monitor conformational changes for all amino acid residues, the DSSP method (Kabsch and Sander, 1983) from the Ptraj program was used. Energy minimization involved 5000 steps of the steepest descent (Arfken, 1985) (SD) method and 15000 steps of the conjugate gradient (CG) method (Fletcher and Reeves, 1964). To heat up the system, a 50-ps MD simulation was carried out using an initial temperature of 250 K and a final temperature of 298 K. Neither restraints nor constraints were applied during the heating step. Next, a 1.25-

ns MD simulation with 2-fs time steps was performed. The temperature was maintained at 298 K with Berendsen coupling (Berendsen et al., 1984) and a 10-Å cutoff was applied.

2.7 NMR spectroscopy

HPLC-purified conBk-B peptide was resuspended in NMR buffer to yield final concentrations of 10 mM MES (13D) pH 6.1, 40% TFE, and 1.13 mM peptide. Peptide concentration was determined by absorbance measurement at 280 nm, using an extinction coefficient for the single tyrosine residue of 1280 cm⁻¹ M⁻¹. Calcium ion titrations were performed by adding small volumes of 0.1 M calcium chloride dissolved in NMR buffer at early titration points and 1M calcium chloride dissolved in NMR buffer at later titration points. A mock titration using identical solutions, but without conBk-B peptide, showed that pH was altered less than 0.1 pH unit from start to end points of the calcium titration, indicating chemical shift changes are a result of Ca²⁺ binding and not changes in pH. Following titration, the sample was used for 2D NMR experiments with final solution conditions: 10 mM MES (13D) pH 6.1, 11 mM CaCl₂, 40% TFE, and 1.10 mM peptide. Additional Ca²⁺ ion titrations were measured in the absence of TFE, but otherwise similar conditions.

One dimensional [¹H] proton spectra and two-dimensional [¹³C, ¹H] HSQC, [¹H, ¹H] COSY, [¹H, ¹H] TOCSY, and [¹H, ¹H] NOESY spectra were recorded at 20 °C on a Varian Inova 600 MHz spectrometer with a cryogenic probe. Data were processed with FELIX2004 and analyzed using SPARKY (T. D. Goddard and D. G. Kneller, University of California, San Francisco). The solution structure of calcium ion-bound conBk-B was determined from 82 NOE distance constraints using NIH-XPLOR (Schwieters and Clore, 2001; Schwieters et al., 2003). Starting with a linear template, simulated annealing generated 100 structures with convergence in backbone architecture and variation in side chain rotamers. To control for potential effects of side chain rotamer in subsequent steps, four low-energy structures that sampled all side chain rotamers were selected to seed refinement in NIH-XPLOR. Each of these four structures seeded a separate refinement protocol in NIH-XPLOR to generate a total of 1000 structures. The five lowest-energy structures from each of the four refinements were combined to create the final 20-structure ensemble. Atomic coordinates and NMR data were deposited with the Protein Data Bank as entry 2m7r.

Mathematical modeling of Ca²⁺ ion titration data was accomplished with *CALCIUM04*, a C language program (M.P.H.) which implements the least-squares Marquardt algorithm (Marquardt, 1963) to fit parameter values. Adjustable parameters describing the system were the intrinsic dissociation constant at each of two binding sites (K_{D1} and K_{D2}), a cooperativity parameter ($K_{\text{cooperativity}}$), and changes in chemical shift values for binding Ca²⁺ at site 1 (δ_1) and at site 2 (δ_2). All data were fit with global values for K_{D1} , K_{D2} , and $K_{\text{cooperativity}}$, and nuclei-specific values for δ_1 and δ_2 . To avoid over-parameterization, changes in chemical shift were assumed to be additive, meaning that the final change in chemical shift observed with both sites occupied was taken as $\delta_1 + \delta_2$. ConBk-B was described as a partition with four possible states: no Ca²⁺ ions bound (p_{00}), site 1 occupied (p_{01}), site 2 occupied (p_{10}), and both sites occupied (p_{11}). Partition values

and fraction of free Ca^{2+} ions ($c = [\text{Ca}^{2+}_{\text{free}}]/[\text{Ca}^{2+}_{\text{total}}]$) were obtained by numerical methods so as to satisfy the following system of equations:

$$n = nc + p_{01} + p_{10} + 2p_{11} \quad (\text{Equation 1.1})$$

$$1 = p_{00} + p_{01} + p_{10} + p_{11} \quad (\text{Equation 1.2})$$

$$K_{D1}/P_{\text{total}} = n c p_{00}/p_{01} \quad (\text{Equation 1.3})$$

$$K_{D2}/P_{\text{total}} = n c p_{00}/p_{10} \quad (\text{Equation 1.4})$$

$$K_{D1} K_{D2} K_{\text{cooperativity}}/P_{\text{total}}^2 = n^2 c^2 p_{00}/p_{11} \quad (\text{Equation 1.5})$$

Where P_{total} is the total peptide concentration, and n is the ratio $[\text{Ca}^{2+}_{\text{total}}]/P_{\text{total}}$, which is a known quantity. Equations 1.1 and 1.2 describe conservation of mass for calcium ions and peptide respectively. Equations 1.3 – 1.5 describe three equilibria: dissociation at site 1, dissociation at site 2, and simultaneous dissociation at both sites. Uncertainties in parameter values were estimated with a bootstrap method that repeated curve-fitting analysis for several simulated data sets, with each simulated data set derived by sampling from the experimental data with replacement.

3. Results

3.1 Identification of *Conus bocki* clones encoding conantokins

Conantokin sequences were identified from cDNA clones of mRNAs isolated from the venom duct of *Conus bocki* (Fig. 1). This species belongs to the clade *Asprella* that also comprises *C. bretinghami*, *C. sulcatus*, *C. rolandi*, and *C. samiae*. Since previous work established that a tyrosine residue at position five was important for high potency towards NMDA receptors with NR2D subunits (Twede et al., 2009b), we focused on such examples found for *Conus bocki* conantokins, although additional sequences not containing tyrosine at position five were also cloned. The nucleotide sequences, predicted translation products, and mature peptide sequences of five *Conus bocki* peptides are shown in Table 1. Post-translational modification of glutamate residues to γ -carboxyglutamate is well established for the conantokin family (Jimenez, 2009). Five glutamate residues in conBk-A, conBk-C, and conBk-D and three of the glutamate residues in conBk-B and conBk-E were predicted to be modified to γ -carboxyglutamate. Predicted mature peptide sequences were synthesized for conBk-A (22 AA), conBk-B (9 AA), conBk-C (18 AA), conBk-D (19 AA) and conBk-E (9 AA). Interestingly, the predicted mature sequences for conBk-B and conBk-E are only 9 residues in length and represent the shortest conantokin sequences identified to date. Of course, it remains to be demonstrated that these peptides are present in injected venom, but this seems likely to be the case as the clones were derived from mRNA isolated from the venom duct.

3.2 Functional characterization of conantokins from *C. bocki*

Peptides were functionally assessed using two-electrode voltage-clamp electrophysiology in *Xenopus oocytes* coexpressing a single NR2 subunit with the NR1-3b subunit. Example recordings from these experiments are provided in the supplementary information, Figure S1. Dose-response curves are shown in Figure 2, and IC₅₀ values are summarized in Table 2. *ConBk-D* and *conBk-E* were inactive at all concentrations tested. *ConBk-A*, *conBk-B*, and *conBk-C* were active and interestingly, exhibited highest potency for NR2D subunits. *ConBk-A* and *conBk-C* blocked NR2D-containing NMDA receptors with IC₅₀ values of 0.46 (±0.04) μM and 0.17 (±0.02) μM, respectively. The shorter peptide, *conBk-B*, was 10-fold less potent on all NMDA receptor subtypes compared to the larger counterparts, yet retained NR2D preference with an IC₅₀ value of 1.9 μM. Though the preference for NR2D over other receptors was modest, differences in IC₅₀ values were statistically significant ($p < 0.05$) for both *conBk-B* and *conBk-C* (Table S1).

3.3 Sequence determinants of NMDA receptor inhibition by *conBk-B*

The short length of *conBk-B* and its preference for NMDA receptor subtypes containing the NR2D subunit motivated experiments to elucidate determinants of subtype selectivity. *ConBk-E* differs from *conBk-B* by substitution of tyrosine 5 with cysteine and is thus a naturally occurring single amino acid substitution variant. Inactivity observed for *conBk-E* demonstrates the importance of position five. We also considered the possibility that *conBk-E* may covalently form a homodimer via cys-5; however, no activity was subsequently observed in a dimerized form of *conBk-E* (not shown). We designed and synthesized additional sequence analogs of *conBk-B* and *conBk-C* to test the importance of other sequence determinants. Table 3 shows amino-acid sequences for *conBk-B*, *conBk-E*, and designed analogs; table 4 shows the corresponding IC₅₀ values. Removing C-terminal amidation from the naturally occurring *conBk-B* sequence (*conBk-B*[COOH]) rendered the peptide inactive, suggesting amidation may stabilize the active conformation or interact with NMDA receptors. The analog constructed with the first nine residues of *conBk-C* (*conBk-C* [1–9]) was not active on any of the NMDA receptor subtypes tested, indicating only certain amino acid combinations are tolerated in a nine residue scaffold.

To test sequence permutations we constructed nine-residue chimeric conantokins with amino acids derived from *conBk-B* and *conBk-C*. The first nine residues of these two conantokins differ at positions 1, 2, 6, and 8. *ChimeraBk-BC* [1–9] contains amino-acid residues derived from *conBk-B* at positions 1–2 and amino-acid residues derived from *conBk-C* at positions 6 and 8, and showed inverted preferences for NR2B and NR2D, meaning that this chimera is more similar to previously characterized conantokins in its preference for NR2B over NR2D. Note that this chimera terminates with a free carboxylic-acid functional group and retains activity, indicating an amide protecting group is not absolutely required for NMDA receptor inhibition. *ChimeraBk-CB* [1–9] swaps *conBk-B* and *conBk-C* derived amino acids in the converse manner, with *conBk-C* residues at positions 1–2 and *conBk-B* at positions 6 and 8, and resulted in an inactive peptide. A point mutation at position eight (*conBk-B* [A8F]) resulted in loss of preference for NR2D containing receptors, suggesting that residue 8 may contact a region that differs between NMDA receptor subtypes. In summary, short conantokin analogs require *conBk-B*-like

sequences in the amino-terminal portion for activity, and changes in carboxy-terminal residues strongly affect either activity or subtype selectivity.

3.4 Circular dichroism

Conantokins are relatively unstructured yet acquire helicity in the presence of divalent metal ions (Blandl et al., 2000; Chandler et al., 1993; Rigby et al., 1997; Warder et al., 1998). Helical content of *C. bocki* conantokins was assessed by circular dichroism spectroscopy in both the presence and in the absence of Ca^{2+} (Fig. 3). In the absence of Ca^{2+} all three peptides had no apparent helical structure, a result consistent with other conantokins. In the presence of Ca^{2+} , however, conBk-A and conBk-B retained low helical content, whereas conBk-C adopted significant helicity.

ConBk-A and conBk-B appear to be the first NMDA receptor-inhibiting conantokins to exhibit little or no helicity in the presence of Ca^{2+} ions. To test if the shortest conantokin, conBk-B, can acquire helical structure in a stabilizing solvent, the effect of 2,2,2-trifluoroethanol (TFE) was examined (Fig. 3). Upon addition of 60% TFE to peptide in a Ca^{2+} -free solution the helical content of conBk-B increased from 2 to 24 percent and upon addition of 60% TFE to peptide in solution containing Ca^{2+} , helical content increased from 5 to 43 percent. The results suggest that conBk-B can coordinate Ca^{2+} ions to adopt a helical structure, albeit more weakly or with faster exchange kinetics than other conantokins.

3.5 Molecular modeling

To explain the differences of Ca^{2+} -stabilized helical content between conBk-A and conBk-C, we applied molecular modeling (see Fig. S2). Molecular dynamics simulations show the secondary structure of conBk-C without Ca^{2+} ions was a short helix between residues 6–9 with a percentage occurrence of 7%. The presence of Ca^{2+} changed this observation and resulted in two helices spanning residues 2–5 and 12–15 with a percentage occurrence of 70%. In the case of the conBk-A, we observed one helical region between residues 13–17, which did not change in location but did increase in percent occurrence from 4% to 50% upon addition of Ca^{2+} in the MD simulations. We quantified helix-stabilizing interactions as Ca^{2+} bridges between negatively charged groups. For conBk-C these are Asp1- Ca^{2+} -Tyr5 (via π -electrons of the Tyr), Asp1- Ca^{2+} -Gla4, Gla4- Ca^{2+} -Tyr5, Gla4- Ca^{2+} -Gla7, Gla3- Ca^{2+} -Gla4, Gla3- Ca^{2+} -Gla7, Asp2- Ca^{2+} -Gla10, Asp2- Ca^{2+} -Gla14, and Gla10- Ca^{2+} -Gla14. And for conBk-A these are Gla3- Ca^{2+} -Gla4, Gla7- Ca^{2+} -Gla14, Gla7- Ca^{2+} -Gla10, and Gla10- Ca^{2+} -Gla14. More numerous calcium ion-bridging interactions observed for conBk-C is consistent with higher helicity observed by CD spectroscopy. The sequence alignment presented in Table 1 shows an Arg-Glu-Arg structural motif (residues 11–13) in conBk-A. In the case of conBk-C, corresponding residues are Gln-Gln-Arg. The MD simulations suggest the presence of positively charged Arg11 situated adjacent to negatively charged Gla10 interrupts helical structure in conBk-A. Presumably, the mutation conBk-A[R11Q] would increase helicity of the peptide in the presence of calcium ions, however this prediction is yet to be tested.

3.6 NMR spectroscopy of conBk-B

To date, conBk-B is the shortest conantokin to be detected in cDNA libraries and also selects for NR2D over NR2B more so than any other known conantokin. For these reasons, we were motivated to characterize the structural behavior of this ultra-short conantokin. Resonances from ^1H and ^{13}C nuclear spins were assigned from 2D NMR spectra obtained with natural abundance samples (Figure 4(A) and Figure 4(B)). To determine the relative contributions of calcium ions and TFE for helical structure in conBk-B, we examined ^{13}Ca chemical shifts in 2D CHSQC spectra. Figure 4(C) shows differences in conBk-B ^{13}Ca chemical shifts measured upon addition of 11 mM calcium chloride (10 peptide equivalents), addition of 40% TFE, and the combination of adding both calcium chloride and TFE. Calcium ion addition moved chemical shifts downfield by 0.07 to 0.48 ppm for all residues except Gla-7, which was essentially unperturbed. Downfield perturbations for ^{13}Ca nuclei are consistent with a shift in structural equilibrium towards an α helix. TFE produced significantly larger downfield shift changes ranging from 3.56 to 5.14 ppm. The combined effect of calcium ions and TFE generally resulted in larger downfield chemical shift changes ranging from 3.54 to 5.6. The data indicate that calcium weakly promotes helical structure and its effects are additive to those of TFE, which strongly promotes helical structure in conBk-B.

Calcium ion binding of conBk-B was assessed by tracking proton chemical shift changes from 1D NMR spectra during a titration. Figure 5 shows proton chemical shift plotted as a function of Ca^{2+} concentration for amide and gamma protons of conBk-B dissolved in 40% TFE. Many protons showed a simple monophasic response to titration with calcium ions, but Gla-4 and Tyr-5 amide protons responded in a biphasic manner, moving upfield with initial increases in calcium ion concentration and moving downfield at later stages of the titration, suggesting there are two Ca^{2+} -binding sites with differing affinities.

We considered several versions of a two-site binding model. One model assumed no interaction between sites, and other models assumed either positive or negative interaction. The independent sites model adequately accounted for all of the data, but had larger residuals than a model with positive cooperativity. A model with negative cooperativity did not fit data well and was not considered further. The independent sites model and the positively interacting sites model yielded the same intrinsic dissociation constant for the high-affinity binding site [$K_{\text{D1}} = 0.31(\pm 0.03)$ mM] and comparable values for the low-affinity binding site [$K_{\text{D2}} = 2.8(\pm 0.5)$ mM for independent sites; $K_{\text{D2}} = 4(\pm 1)$ mM for cooperative sites]. Interaction between Ca^{2+} -binding sites was described by a parameter ($K_{\text{cooperativity}}$) that is the ratio of apparent dissociation constants for a given site with the second site occupied (numerator) and unoccupied (denominator). This parameter refined to a value of $K_{\text{cooperativity}} = 0.6(\pm 0.2)$, indicating the affinity at each binding site appeared to increase 1.67-fold with occupancy at the other site. This corresponds to $-1.4(\pm 0.7)$ kJ mol $^{-1}$ of binding energy that is realized when both sites are occupied. Two homogeneous sites with a similar degree of interaction would exhibit a Hill coefficient of $n = 1.14$. Comparable calcium ion titrations, with similar midpoints and biphasic responses, were also measured without TFE. Curve fitting analyses of these titrations obtained in the absence of TFE could be accomplished with the same dissociation constants defined by data measured with TFE.

3.7 NMR structure determination

The calcium ion-bound structure of con*Bk*-B stabilized with the cosolvent trifluoroethanol was determined by 2D NMR spectroscopy using natural abundance ^1H and ^{13}C nuclear spins. Complete resonance assignments were obtained through examination of NOESY, TOCSY, and CHSQC spectra. The NOESY spectra had 82 NOE resonances with signal to noise greater than 10, and these contained 63 short-range [$i:i$, $i:i+1$] and 19 medium-range [$i:i+2$, $i:i+3$, or $i:i+4$] distance constraints. The fingerprint region of the NOESY spectra (Figure 4(A)) showed sequential HN-H α connectivity and contained $i:i+3$ cross peaks (H α -HN) consistent with an α helix. Additional evidence of α -helical structure was seen in 16 of the 19 medium range NOEs between residue side chains. For example, side chain protons of Tyr-5 and Ile-9 had cross peaks indicating proximity (see Figure 4(B)).

Figure 6(A) shows the ensemble of energy minimized con*Bk*-B structures as short α helices. Ensemble members were aligned with main chain atoms (C α , N, HN, C, O) of residues 3–9, since these adopted more consistent positions than those of residues 1 and 2. The alignment yielded a root-mean-squared deviation of 1.6 Å for all atoms of all residues, 1.1 Å for all main chain atoms of all residues, and 0.5 Å for main chain atoms of residues 3–9. The three Glu residues of con*Bk*-B cluster along one face of the helix, apparently poised to coordinate Ca $^{2+}$ ions (Figure 6(B) and 6(C)). A glutamate residue at position 2 (Glu-2) is on the opposite face of the helix and is therefore unlikely to contribute directly to high-affinity Ca $^{2+}$ binding. Hydrophobic residues Tyr-5 and Ile-9 are grouped at the C-terminal half of the helix and are found on the face opposite to the Glu residues. The hydrophobic contacts involving Tyr-5 probably augment helix stabilization provided by salt bridges between carboxylate groups and calcium ions.

We compared the con*Bk*-B structure to structures of con*G* and con*T*, two longer conantokin toxins previously characterized by NMR structure determination (Skjaerbaek et al., 1997). Representative models of each conantokin are shown in Figure 7. The three structures share identical N-terminal residues 1–4 and the structures align fairly well in this region. The most striking difference between the three structures is overall length, with con*Bk*-B shortest (9 residues), con*T* longest (21 residues), and con*G* in the middle (17 residues). Con*Bk*-B and con*G* have very similar orientations for Glu-3, Glu-4 and Glu-7 (highlighted in Fig. 7 with red dots), suggesting these two peptides coordinate calcium ions in a similar manner. Con*Bk*-B and con*T* have highly comparable orientations for Tyr-5, which makes hydrophobic contacts with residue nine in both structures. In the structural comparison, amino-acid position 8 (highlighted in Fig. 7 with black dots) stands out as having the largest variation in volume and shape while located in an otherwise conserved region. Amino-acid identity at position eight is an important determinant of selectivity in conantokins as shown by the A8F replacement variant (see Table 4) and by previous studies (Sheng et al., 2007; Sheng et al., 2010). The structural differences highlighted here additionally corroborate the idea that this residue position may hold the key for future design of increasingly selective conantokins.

4. Discussion

A discovery strategy incorporating inferences based on phylogenetics and previous structure-function studies led us to the identification of *conBk-A*, *conBk-B*, *conBk-C*, *conBk-D* and *conBk-E* from *Conus bocki*. Molecular phylogeny as a means to targeted drug discovery, also known as exogenomics, has been described and successfully applied to discover *Conus* toxins with novel pharmacological properties (Bulaj, 2008; Olivera, 2006; Olivera and Teichert, 2007; Teichert et al., 2007). In the present case we were searching for conantokins that were potentially active on NR2D-containing NMDA receptors and identified *Conus bocki* as a possible source because of its close phylogenetic relation to *Conus bretinghami*, previously the source of the most NR2D-potent conantokin (*conBr*) (Twede et al., 2009b). As could be predicted from structure-function studies of *conBr* analogs (Twede et al., 2009b), the three peptides derived from *Conus bocki* that contained tyrosine at position five showed inhibition of NMDA receptors with NR2D subunits. Unexpectedly, all three peptides additionally had decreased inhibitory activity for NMDA receptors with NR2B subunits compared to previously described conantokins. Discovery of peptides with decreased potency in block of NR2B containing receptors was, in a sense, a bonus that could not have been predicted *a priori*, and is sure to guide future iterations of the exogenomics approach.

ConBk-D and *conBk-E*, although inactive in our assays, provide insights into sequence determinants for NMDA receptor inhibition that complement previous insights derived from structure activity work. For instance, alanine scanning in *conG*, *conT*, and *conR* highlighted the importance of residues at positions two and five for activity (Blandl et al., 1998; Blandl et al., 2001; Warder et al., 2001). Data obtained in the present study suggests that the positive charge on lysine at position two, of *conBk-D*, abolishes antagonist activity for NMDA receptors tested here. Also, comparison of *conBk-E* with *conBk-B* shows that substitution of a cysteine side chain at position five renders the short peptide scaffold inactive.

Previous work showed that native conantokins can lose biological activity as they are shortened (Blandl et al., 1998; Blandl et al., 2001; Warder et al., 2001). The discovery of *conBk-B* encoding mRNAs in venom ducts strongly suggests that ultra-short conantokin peptides with NMDA receptor inhibitory activity exist in the natural world. Sequence permutations evaluated in this study showed that only certain combinations of amino acids in the nine-residue scaffold retain activity. Surprisingly, some permutations switched peptide preference for receptors between NR2B and NR2D subtypes, suggesting that a few contacts provide distinction between these NMDA receptor homologs. Most critical for selectivity are residues at positions 5, 6, and 8, as previously identified in other conantokins (Sheng et al., 2007; Sheng et al., 2009; Sheng et al., 2010). Thus, this ultra-short peptide likely interacts with receptors utilizing the same mechanism as its larger counterparts. Interestingly, the structural comparison of *conBk-B* to *conG* and *conT* independently highlighted position 8 as a possible determinant of subtype selectivity (see Figure 7).

Our structural analysis suggests that a calcium ion-stabilized helical conformation is not necessary for receptor inhibition, consistent with the observation that *conG* and *conT*

inhibition of MK-801 binding in rat brain is not hindered by the addition of EDTA (Klein and Castellino, 1999). In our analysis, *ConBk-A* and *conBk-B* showed little helical content in the presence of calcium ions, yet had inhibitory activity comparable to other, more helical conantokins. NMR spectroscopy verified the unstructured state of *conBk-B* through absence of mid-range NOEs for peptide in the presence of calcium ions. These mid-range NOE's were, however, present when the co-solvent TFE was included. Conantokins likely adopt a unique conformation upon receptor binding so as to maximize contacts between peptide and receptor. This receptor-bound state may be similar to helical structures of *conG*, *conT*, and *conBk-B* in solution, but is probably a structurally distinct conformation that may or may not require calcium ions. Thus, a major focus of our future research is to determine the receptor interface and conformation of a receptor-bound conantokin.

An important achievement of this work was to demonstrate a measure of NR2D preference for peptide based NMDA receptor antagonists, which makes investigation of the role of these subunits in physiological systems using a pharmacological approach more feasible. The NR2D subunit has a relatively restricted expression pattern that is enriched in midbrain, spinal, and peripheral structures, such as the substantia nigra, striatum, dorsal horn, and dorsal root ganglia (Counihan et al., 1998; Hummel et al., 2008; Karlsson et al., 2002; Kosinski et al., 1998). Thus, NR2D may play a functional role in pathological conditions that affect these regions, such as Parkinson's disease and chronic pain. Although limited work has been done, NR2D knockout studies support this hypothesis (Micu et al., 2006; Minami et al., 2001).

NR2D-containing NMDA receptors additionally have unique physiological properties, including decreased sensitivity to magnesium blockade and prolonged deactivation kinetics (Cull-Candy et al., 2001), which implicates involvement of the NR2D subunit in excitotoxicity. Recent work suggests that NR2D (or NR2C) is involved in excitotoxic damage to myelin sheaths following ischemia (Micu et al., 2006), and may participate in the underlying mechanistic basis for excitotoxic neuronal loss in prion diseases (Khosravani et al., 2008). Subsequently, selective blockage or modulation of NMDA receptor activity should promote neuron survival in these patients if the hypotheses are correct. Motivated by these potential applications, we can now initiate structure-function studies to enhance NR2D selectivity of conantokin peptides through modification of the newly discovered NR2D-preferring *Conus bocki* toxins.

In order to investigate the role of NMDA receptor subtypes, we envision use of peptide combinations that can provide a measure of pharmacological differentiation between pairs of NMDA receptor subtypes. Such a strategy of using pairs of conopeptides has recently been used for differentiating between different voltage-gated sodium channel subtypes (Wilson et al., 2011). To take a particular example in the context of NMDA receptor discrimination, the combination of *conBk-B* and *conR* could, in principle, discriminate between NR2B and NR2D containing receptors. *ConR* is similarly active on NR2B and NR2A containing receptors and inactive on NR2C and NR2D (Table 2). *ConBk-B* is most active on NR2D containing receptors with varying but lower activity on NR2C>NR2A>NR2B. The contrasting effects of these peptides could therefore be compared in experimental paradigms to provide insight into the relative abundance of NR2B and NR2D NMDA receptor subunit

combinations present in various physiological contexts. Similarly, combining conBk-B with conG would be expected to provide more than a two order of magnitude difference in selectivity between the NR2A and NR2D subtypes. Even NR2C versus NR2B containing NMDA receptors might be distinguished by using conBk-B together with conRI-A. Although single pharmacological agents to discriminate between the different NMDA receptor subtypes are preferable, the discovery of conBk-A, conBk-B, and conBk-C provides an initial basis for developing peptide-based differentiating pharmacology for the NMDA receptor family.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of General Medical Sciences, National Institutes of Health [Grant GM48677].

We thank Dr. Doju Yoshikami for his insightful discussions regarding electrophysiology and expert consultation. We also thank K. H. Gowd and Tiffany Han for their assistance with some of the assays, and acknowledge Cognetix for synthesis of peptides for experiments. A special thank you goes out to Jack Skalicky, whose helpfulness with NMR data collection and analysis was invaluable.

Abbreviations

Gla, γ	gamma-carboxylglutamate
NMDA	N-methyl-D-aspartate
Fmoc	N-(9-fluorenyl) methoxycarbonyl
MTBE	methyl-tert-butyl ether
ACN	acetonitrile
TFA	trifluoroacetic acid
ESI	Electrospray ionization
NMR	nuclear magnetic resonance
BSA	bovine serum albumin
TFE	2,2,2-trifluoroethanol
MD	molecular dynamics
DSSP	define secondary structure of protein

References

- Acklin P, Allgeier H, Auberson YP, Bischoff S, Ofner S, Sauer D, Schmutz M. 5-Aminomethylquinoxaline-2,3-diones, Part III: Arylamide derivatives as highly potent and selective glycine-site NMDA receptor antagonists. *Bioorg Med Chem Lett*. 1998; 8:493–498. [PubMed: 9871605]

- Ametamey S, Kokic M, Carrey-Rémy N. Synthesis, radiolabelling and biological characterization of (-)-7-iodo-N-(1-phosphonoethyl)-5-aminomethylquinoxaline-2,3-dione, a glycine-binding site antagonist of. *Bioorganic & medicinal*. 2000
- Arfken G. The method of steepest descents. *Mathematical methods for physicists*. 1985
- Auberson YP, Acklin P, Bischoff S, Moretti R, Ofner S, Schmutz M, Veenstra SJ. N-phosphonoalkyl-5-aminomethylquinoxaline-2,3-diones: in vivo active AMPA and NMDA(glycine) antagonists. *Bioorganic & medicinal chemistry letters*. 1999; 9:249–254. [PubMed: 10021939]
- Auberson YP, Allgeier H, Bischoff S, Lingenhoehl K, Moretti R, Schmutz M. 5-Phosphonomethylquinoxalinediones as competitive NMDA receptor antagonists with a preference for the human 1A/2A, rather than 1A/2B receptor composition. *Bioorg Med Chem Lett*. 2002; 12:1099–1102. [PubMed: 11909726]
- Bashford D, Case DA. Generalized born models of macromolecular solvation effects. *Annual review of physical chemistry*. 2000; 51:129–152.
- Berendsen HJ, Postma JPM, Vangunsteren WF, Dinola A, Haak JR. Molecular dynamics with coupling to an external bath. *Journal of Chemical Physics*. 1984; 81:7.
- Bland T, Prorok M, Castellino FJ. NMDA-receptor antagonist requirements in conantokin-G. *FEBS Lett*. 1998; 435:257–262. [PubMed: 9762921]
- Bland T, Warder SE, Prorok M, Castellino FJ. Binding of cations to individual gamma-carboxylglutamate residues of conantokin-G and conantokin-T. *The journal of peptide research: official journal of the American Peptide Society*. 1999; 53:453–464. [PubMed: 10406223]
- Bland T, Warder SE, Prorok M, Castellino FJ. Structure-function relationships of the NMDA receptor antagonist peptide, conantokin-R. *FEBS Letters*. 2000; 470:139–146. [PubMed: 10734223]
- Bland T, Zajicek J, Prorok M, Castellino FJ. Sequence requirements for the N-methyl-D-aspartate receptor antagonist activity of conantokin-R. *J Biol Chem*. 2001; 276:7391–7396. [PubMed: 11096077]
- Bulaj G. Integrating the discovery pipeline for novel compounds targeting ion channels. *Current opinion in chemical biology*. 2008; 12:441–447. [PubMed: 18678277]
- Case DA, Cheatham TE, Darden T, Gohlke H, Luo R, Merz KM, Onufriev A, Simmerling C, Wang B, Woods RJ. The Amber biomolecular simulation programs. *Journal of computational chemistry*. 2005; 26:1668–1688. [PubMed: 16200636]
- Chandler P, Pennington M, Maccacchini M. Polyamine-like actions of peptides derived from conantokin-G, an N-methyl-D-aspartate (NMDA) antagonist. *Journal of Biological*. 1993
- Choi YB, Lipton SA. Identification and mechanism of action of two histidine residues underlying high-affinity Zn²⁺ inhibition of the NMDA receptor. *Neuron*. 1999; 23:171–180. [PubMed: 10402203]
- Costa BM, Feng B, Tsintsadze TS, Morley RM, Irvine MW, Tsintsadze V, Lozovaya NA, Jane DE, Monaghan DT. N-methyl-D-aspartate (NMDA) receptor NR2 subunit selectivity of a series of novel piperazine-2,3-dicarboxylate derivatives: preferential blockade of extrasynaptic NMDA receptors in the rat hippocampal CA3-CA1 synapse. *The Journal of pharmacology and experimental therapeutics*. 2009; 331:618–626. [PubMed: 19684252]
- Counihan TJ, Landwehrmeyer GB, Standaert DG, Kosinski CM, Scherzer CR, Daggett LP, Veliçelebi G, Young AB, Penney JB. Expression of N-methyl-D-aspartate receptor subunit mRNA in the human brain: mesencephalic dopaminergic neurons. *The Journal of comparative neurology*. 1998; 390:91–101. [PubMed: 9456178]
- Cull-Candy S, Brickley S, Farrant M. NMDA receptor subunits: diversity, development and disease. *Current Opinion in Neurobiology*. 2001; 11:327–335. [PubMed: 11399431]
- DeLano, W. The PyMOL Molecular Graphics System. Palo Alto, CA: DeLano Scientific; 2002.
- Dingledine R, Borges K, Bowie D, Traynelis S. The glutamate receptor ion channels. *Pharmacol Rev*. 1999
- Donevan SD, McCabe RT. Conantokin G is an NR2B-selective competitive antagonist of N-methyl-D-aspartate receptors. *Molecular pharmacology*. 2000; 58:614–623. [PubMed: 10953056]
- Feng B, Tse HW, Skifter DA, Morley R, Jane DE, Monaghan DT. Structure-activity analysis of a novel NR2C/NR2D-preferring NMDA receptor antagonist: 1-(phenanthrene-2-carbonyl)

- piperazine-2,3-dicarboxylic acid. *British journal of pharmacology*. 2004; 141:508–516. [PubMed: 14718249]
- Fletcher R, Reeves CM. Function minimization by conjugate gradients. *The Computer Journa*. 1964; 7:149–154.
- Hammerland L. Conantokin-G selectively inhibits N-methyl-D-aspartate-induced currents in *Xenopus* oocytes injected with mouse brain mRNA. *European Journal of Pharmacology: Molecular Pharmacology*. 1992; 226:239–244.
- Hollmann M, Heinemann S. Cloned glutamate receptors. *Annual review of neuroscience*. 1994; 17:31–108.
- Hummel M, Strassle B, Miller S, Kaftan E, Whiteside G. Anatomical localization and expression pattern for the NMDA-2D receptor subunit in a rat model of neuropathic pain. *Neuroscience*. 2008; 155:492–502. [PubMed: 18585442]
- Jimenez E. Conantokins: from “sleepers” activity to drug development. *Philipp Sci Lett*. 2009
- Jones DT. Protein secondary structure prediction based on position-specific scoring matrices. *Journal of Molecular Biology*. 1999; 292:195–202. [PubMed: 10493868]
- Kabsch W, Sander C. Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers*. 1983; 22:2577–2637. [PubMed: 6667333]
- Karlsson U, Sjödin J, Angeby Möller K, Johansson S, Wikström L, Näsström J. Glutamate-induced currents reveal three functionally distinct NMDA receptor populations in rat dorsal horn - effects of peripheral nerve lesion and inflammation. *Neuroscience*. 2002; 112:861–868. [PubMed: 12088745]
- Khosravani H, Zhang Y, Tsutsui S, Hameed S, Altier C, Hamid J, Chen L, Villemare M, Ali Z, Jirik FR, Zamponi GW. Prion protein attenuates excitotoxicity by inhibiting NMDA receptors. *The Journal of Cell Biology*. 2008; 181:551–565. [PubMed: 18443219]
- Kinarsky L, Feng B, Skifter DA, Morley RM, Sherman S, Jane DE, Monaghan DT. Identification of subunit- and antagonist-specific amino acid residues in the N-Methyl-D-aspartate receptor glutamate-binding pocket. *The Journal of pharmacology and experimental therapeutics*. 2005; 313:1066–1074. [PubMed: 15743930]
- Klein RC, Castellino FJ. Inhibition of MK801 binding in adult rat brain sections by conantokin-G and conantokin-T. *Neuroscience Letters*. 1999; 273:171–174. [PubMed: 10515186]
- Kosinski CM, Standaert DG, Counihan TJ, Scherzer CR, Kerner JA, Daggett LP, Veliçelebi G, Penney JB, Young AB, Landwehrmeyer GB. Expression of N-methyl-D-aspartate receptor subunit mRNAs in the human brain: striatum and globus pallidus. *The Journal of comparative neurology*. 1998; 390:63–74. [PubMed: 9456176]
- Layer RT, Wagstaff JD, White HS. Conantokins: peptide antagonists of NMDA receptors. *Current medicinal chemistry*. 2004; 11:3073–3084. [PubMed: 15579001]
- Lin CH, Chen CS, Hsu KS, King DS, Lyu PC. Role of modified glutamic acid in the helical structure of conantokin-T. *FEBS Letters*. 1997; 407:243–248. [PubMed: 9166908]
- Low CM, Zheng F, Lyuboslavsky P, Traynelis SF. Molecular determinants of coordinated proton and zinc inhibition of N-methyl-D-aspartate NR1/NR2A receptors. *Proc Natl Acad Sci U S A*. 2000; 97:11062–11067. [PubMed: 10984504]
- Marquardt DW. An Algorithm for least-squares estimation of nonlinear parameters. *SIAM J Appl Math*. 1963
- Micu I, Jiang Q, Coderre E, Ridsdale A, Zhang L, Woulfe J, Yin X, Trapp BD, McRory JE, Rehak R, Zamponi GW, Wang W, Stys PK. NMDA receptors mediate calcium accumulation in myelin during chemical ischaemia. *Nature*. 2006; 439:988–992. [PubMed: 16372019]
- Minami T, Matsumura S, Okuda-Ashitaka E, Shimamoto K, Sakimura K, Mishina M, Mori H, Ito S. Characterization of the glutamatergic system for induction and maintenance of allodynia. *Brain research*. 2001; 895:178–185. [PubMed: 11259776]
- Morley RM, Tse HW, Feng B, Miller JC, Monaghan DT, Jane DE. Synthesis and pharmacology of N1-substituted piperazine-2,3-dicarboxylic acid derivatives acting as NMDA receptor antagonists. *Journal of medicinal chemistry*. 2005; 48:2627–2637. [PubMed: 15801853]
- Mosley CA, Acker TM, Hansen KB, Mullasseril P, Andersen KT, Le P, Vellano KM, Bräuner-Osborne H, Liotta DC, Traynelis SF. Quinazolin-4-one derivatives: A novel class of

- noncompetitive NR2C/D subunit-selective N-methyl-D-aspartate receptor antagonists. *Journal of medicinal chemistry*. 2010; 53:5476–5490. [PubMed: 20684595]
- Mosley CA, Myers SJ, Murray EE, Santangelo R, Tahirovic YA, Kurtkaya N, Mullasseril P, Yuan H, Lyuboslavsky P, Le P, Wilson LJ, Yepes M, Dingledine R, Traynelis SF, Liotta DC. Synthesis, structural activity-relationships, and biological evaluation of novel amide-based allosteric binding site antagonists in NR1A/NR2B N-methyl-D-aspartate receptors. *Bioorganic & Medicinal Chemistry*. 2009; 17:6463–6480. [PubMed: 19648014]
- Olivera BM. Conus Peptides: Biodiversity-based Discovery and Exogenomics. *Journal of Biological Chemistry*. 2006; 281:31173–31177. [PubMed: 16905531]
- Olivera BM, Teichert RW. Diversity of the neurotoxic Conus peptides: a model for concerted pharmacological discovery. *Molecular interventions*. 2007; 7:251–260. [PubMed: 17932414]
- Paoletti P, Ascher P, Neyton J. High-affinity zinc inhibition of NMDA NR1-NR2A receptors. *J Neurosci*. 1997; 17:5711–5725. [PubMed: 9221770]
- Rachline J, Perin-Dureau F, Le Goff A, Neyton J, Paoletti P. The micromolar zinc-binding domain on the NMDA receptor subunit NR2B. *J Neurosci*. 2005; 25:308–317. [PubMed: 15647474]
- Rigby AC, Baleja JD, Li L, Pedersen LG, Furie BC, Furie B. Role of gamma-carboxyglutamic acid in the calcium-induced structural transition of conantokin G, a conotoxin from the marine snail *Conus geographus*. *Biochemistry*. 1997; 36:15677–15684. [PubMed: 9398296]
- Schüler T, Mesic I, Madry C, Bartholomäus I, Laube B. Formation of NR1/NR2 and NR1/NR3 heterodimers constitutes the initial step in N-methyl-D-aspartate receptor assembly. *The Journal of biological chemistry*. 2008; 283:37–46. [PubMed: 17959602]
- Schwieters CD, Clore GM. Internal coordinates for molecular dynamics and minimization in structure determination and refinement. *J Magn Reson*. 2001; 152:288–302. [PubMed: 11567582]
- Schwieters CD, Kuszewski JJ, Tjandra N, Clore GM. The Xplor-NIH NMR molecular structure determination package. *J Magn Reson*. 2003; 160:65–73. [PubMed: 12565051]
- Sheng Z, Dai Q, Prorok M, Castellino FJ. Subtype-selective antagonism of N-methyl-D-aspartate receptor ion channels by synthetic conantokin peptides. *Neuropharmacology*. 2007; 53:145–156. [PubMed: 17588620]
- Sheng Z, Liang Z, Geiger JH, Prorok M, Castellino FJ. The selectivity of conantokin-G for ion channel inhibition of NR2B subunit-containing NMDA receptors is regulated by amino acid residues in the S2 region of NR2B. *Neuropharmacology*. 2009; 57:127–136. [PubMed: 19427876]
- Sheng Z, Prorok M, Castellino FJ. Specific determinants of conantokins that dictate their selectivity for the NR2B subunit of N-methyl-D-aspartate receptors. *Neuroscience*. 2010; 170:703–710. [PubMed: 20688135]
- Simeone TA, Sanchez RM, Rho JM. Molecular biology and ontogeny of glutamate receptors in the mammalian central nervous system. *Journal of child neurology*. 2004; 19:343–360. discussion 361. [PubMed: 15224708]
- Skjaerbaek N, Nielsen KJ, Lewis RJ, Alewood P, Craik DJ. Determination of the solution structures of conantokin-G and conantokin-T by CD and NMR spectroscopy. *The Journal of biological chemistry*. 1997; 272:2291–2299. [PubMed: 8999936]
- Teichert RW, Jimenez EC, Twede V, Watkins M, Hollmann M, Bulaj G, Olivera BM. Novel conantokins from *Conus parvus* venom are specific antagonists of N-methyl-D-aspartate receptors. *The Journal of biological chemistry*. 2007; 282:36905–36913. [PubMed: 17962189]
- Tsai VWW, Dodd PR, Lewis RJ. The effects of alanine-substituted conantokin-G and ifenprodil on the human spermine-activated N-methyl-D-aspartate receptor. *Neuroscience*. 2005; 130:457–464. [PubMed: 15664702]
- Twede VD, Miljanich G, Olivera BM, Bulaj G. Neuroprotective and cardioprotective conopeptides: an emerging class of drug leads. *Current opinion in drug discovery & development*. 2009a; 12:231–239. [PubMed: 19333868]
- Twede VD, Teichert RW, Walker CS, Gruszczynski P, Kałmierkiewicz R, Bulaj G, Olivera BM. Conantokin-Br from *Conus bretinghami* and selectivity determinants for the NR2D subunit of the NMDA receptor. *Biochemistry*. 2009b; 48:4063–4073. [PubMed: 19309162]

- Warder SE, Blandl T, Klein RC, Castellino FJ, Prorok M. Amino acid determinants for NMDA receptor inhibition by conantokin-T. *Journal of Neurochemistry*. 2001; 77:812–822. [PubMed: 11331410]
- Warder SE, Chen Z, Zhu Y, Prorok M, Castellino FJ, Ni F. The NMR solution structure of the NMDA receptor antagonist, conantokin-T, in the absence of divalent metal ions. *FEBS Letters*. 1997; 411:19–26. [PubMed: 9247135]
- Warder SE, Prorok M, Chen Z, Li L, Zhu Y, Pedersen LG, Ni F, Castellino FJ. The roles of individual gamma-carboxyglutamate residues in the solution structure and cation-dependent properties of conantokin-T. *The Journal of biological chemistry*. 1998; 273:7512–7522. [PubMed: 9516452]
- Wilson MJ, Yoshikami D, Azam L, Gajewiak J, Olivera BM, Bulaj G, Zhang M. μ -Conotoxins that differentially block sodium channels NaV1.1 through 1.8 identify those responsible for action potentials in sciatic nerve. *Proceedings of the National Academy of Sciences*. 2011; 108:10302–10307.
- Wittekindt B, Malany S, Schemm R, Otvos L, Maccacchini ML, Laube B, Betz H. Point mutations identify the glutamate binding pocket of the N-methyl-D-aspartate receptor as major site of conantokin-G inhibition. *Neuropharmacology*. 2001; 41:753–761. [PubMed: 11640930]

Highlights

- A phylogeny-directed search found five peptide toxins of the conantokin family.
- Three new toxins were active, inhibiting NR2D-containing receptors most potently.
- NMR spectroscopy of an ultra-short conantokin shows an alpha-helical conformation.
- Mutational analysis highlights conantokin residues imparting NR2D potency.
- Conantokins hold promise for developing a differentiating NMDAR pharmacology.

Fig. 1.

The shells of four specimens of *Conus bocki* from various localities in the Central Philippines. Specimens are generally collected using tangle nets at depths of ~100 m.

Fig. 2.

Relative potencies of conantokins from *Conus bocki* tested on NMDA receptors. One of the four NR2 subunits was co-expressed with NR1-3b in *Xenopus oocytes*. Concentration-response relationships obtained utilizing two-electrode voltage clamp electrophysiology are shown for conBk-A (A), conBk-B (B) and conBk-C (C). Each data point represents the average peak current response from a minimum of three independent oocytes. Error bars represent $\pm$ SEM. These three conantokins from *C. bocki* showed the highest potency on receptors with the NR1-3b/NR2D subunit combination (diamonds).

Fig. 3.

CD spectra of *conBk-A*, *conBk-B*, and *conBk-C*. A. CD spectra for *conBk-A* and *conBk-C* in the presence and absence of 2 mM CaCl_2 . B. CD spectra for *conBk-B* without CaCl_2 , with 2 mM CaCl_2 , 60% TFE, or both 2 mM CaCl_2 and 60% TFE. The dual minima at 208 and 222 nm are characteristic of an α -helical conformation. Ellipticity is plotted on a per-peptide bond basis to simplify comparison of these conantokins that vary in length. Data points represent the average of 5 independent scans.

Fig. 4.

NMR data for conBk-B. (A) Amide-alpha proton (HN-HA) region of the 2D NOESY spectra recorded for conBk-B shows complete residue connectivity (red dotted lines). NOEs from HA atoms of Gly-1 are indicated by astrisk (*). Multiple i to $i+3$ HA/HN NOEs can be seen, consistent with an α helix. (B) Amide-aliphatic-aromatic proton region of the 2D NOESY spectra shows NOEs between Tyr-5 and Ile-9, indicating proximity for these side chains within the structure of conBk-B. (C) Effects of calcium ions and TFE on conBk-B structure. Changes in ^{13}C chemical shifts (δ) were obtained by subtracting the chemical shift values observed in buffer with no added calcium salt and no added TFE. Positive values of δ indicate increased helical content due to addition of 10 mM CaCl_2 (blue), 40% TFE (green), and both calcium and TFE (yellow).

Fig. 5.

Calcium binding to conBk-B monitored by NMR. Chemical shifts were obtained from 1D NMR spectra measured for conBk-B peptide solutions at 20 °C containing 40% TFE and varying concentrations of calcium chloride. Response of each amide proton is plotted with the following symbols: + Glu 2, ○ Gla-3, □ Gla-4, × Tyr-5, △ Ser-6, ◇ Gla-7, ▽ Ala-8, + Ile-9. Gamma proton chemical shifts for the three Gla residues in conBk-B are plotted with symbols: ● Gla-3, ■ Gla-4, and ◆ Gla-7. Model curves depict the result of non-linear least-squares fitting of these data (and other data not shown) with global parameters that define a system of two calcium-binding sites with differing intrinsic affinities and modest positive cooperativity between sites.

Fig. 6.

ConBk-B NMR structure. (A) Twenty energy-minimized structures, shown with colored tubes, highlight the helical conformation of conBk-B. (B) An ensemble of energy-minimized conBk-B structures is shown with all-atom stick models in stereo. Atoms are color coded as follows: H white, C charcoal, O red, N blue. For clarity, only ten (of twenty available) randomly selected models are shown. A representative model, selected because it most closely resembles the ensemble average, is highlighted with thicker sticks. (C) ConBk-B is approximately amphipathic with respect to two perpendicular axes (dotted lines). Charged Glu and other polar moieties are clustered at the N-terminal half of conBk-B and along one face of the helix, while more hydrophobic residues are grouped together at the C-terminal half of the opposite face.

Fig. 7.

Comparison of conantokin structures determined by NMR. Representative models of conBk-B (A) [9 residues, pdb id *2m7r*], conG (B) [17 residues, pdb id *1onu*], and conT (C) [21 residues, pdb id *1ont*] were aligned by superposition of residues 3–9. All

three structures are α -helices, depicted here as stick models inside semi-transparent accessible surfaces. Glu residues (highlighted with red dots) are positioned along a nearly contiguous ridge for conBk-B and conG and appear more distributed for conT. Differences in side chain volume at position 8 (highlighted with black dots) may contribute to NMDA receptor subtype selectivity.

Table 1Nucleotide and amino acid sequences of cloned *Conus bocki* conantokins

Conantokin	Nucleotide and predicted amino acid sequences ^a
conBk-A	CACAGAGGCGATGAAGAATATTGAGAGTTTATAGAGAGAGACGCGAACTCGTCAGTTCTAAATCCCCAGATAG H R G D E E Y S E F I E R E R E L V S S K I P R
conBk-B	GCCAGAGGCGAAGAAGAATATTGAGAGGCTATAGGAAGAAATAA A R G E E E Y S E A I G R K
conBk-C	GCCAGAGACGATGAAGAATATGAGAGTTTATAGAGCAACAACGCGAAGCAGGATTAGTGTA A R D D E E Y A E F I E Q Q R E A G L V
conBk-D	GCCAGAGGCAAGAAGAACTTGCAGAGAACGCGCGGAGTTTGCCAGAGAACTAGCTACAAATGGAAAAAGATAA A R G K E E L A E N A P E F A R E L A T N G K R
conBk-E	GCCAGAGGCGAAGAAGAATGTTGAGAGGCTATAGGAAGAAATAA A R G E E E C S E A I G R K
Conantokin	Predicted mature amino acid sequences ^b
conBk-A	GDYYSYFIYRERYLVSSKIPR ^
conBk-B	GEYYSYAI *
conBk-C	DDYYAYFIYQQRAGLV ^
conBk-D	GKYLAYNAPYFARYLATN *
conBk-E	GEYYSYCSYAI *
conBr ^c	GDYYSKFIYRERYAGRLDLSKFP ^
conG ^d	GEYYLQYNQYLIRYKSN *
conT ^e	GEYYQKMLYNLRYAEVKKNA *

^a COOH;^{*} CONH₂^a predicted cleavage site for mature toxins are shown in bold and stop codons are underlined^b conserved and predicted gla residues are highlighted^c Twede et al., 2009^d Mena et al., 1990^e Haack et al., 1990

Table 2

Comparison of IC₅₀ values measured for *Conus bocki* conantokins and others

	NMDA receptor subtype IC ₅₀ value ± SEM (μM) [hill coefficient]				IC ₅₀ ratio		
	NR2A	NR2B	NR2C	NR2D	D/B	A/B	C/B
conBk-A	0.9 ± 0.3 [-1.9]	0.92 ± 0.07 [-1.9]	1.4 ± 0.2 [-1.1]	0.46 ± 0.04 [-1.5]	0.50	0.92	1.5
conBk-B	3.9 ± 0.2 [-1.3]	5.1 ± 0.2 [-1.2]	3.9 ± 0.1 [-0.8]	1.91 ± 0.06 [-0.9]	0.37	0.76	0.76
conBk-C	0.9 ± 0.1 [-1.4]	0.37 ± 0.07 [-1.6]	1.5 ± 0.07 [-1.2]	0.17 ± 0.02 [-0.93]	0.46	2.5	4.1
conR1-A	0.52 ^b	0.11 ^b	6.1 ^b	0.48 ^b	4.4	4.7	55
conG	>10 ^c	0.1 ^c	1 ^c	1 ^c	10	~100	10
conR	0.53 ^a	0.35 ^a	~10 ^a	~10 ^a	~30	1.5	~29

Ratios in bold are the extremes; in combination, two conantokins with extreme ratios may help discriminate between NR2 subtypes specified in each column. IC₅₀ values were determined using a curve fit of data from n=3–6 oocytes. Standard error of mean (SEM) was determined by analysis of IC₅₀ values calculated from individual experimental trials. ConBk-D and conBk-E were inactive at concentrations tested.

^aTwede et al., 2009

^bGowd et al., 2010

^cTeichert et al., 2007

Table 3Amino acid sequences of *conBk*-B and analogs

Conantokin	Amino acid sequence
<i>conBk</i> -B	GE $\gamma\gamma$ YS γ AI *
<i>conBk</i> -E	GE $\gamma\gamma$ CS γ AI *
<i>conBk</i> -B[COOH]	GE $\gamma\gamma$ YS γ AI ^
<i>conBk</i> -C [1–9]	DD $\gamma\gamma$ YA γ FI *
<i>conBk</i> -B[A8F]	GE $\gamma\gamma$ YS γ FI *
chimera <i>Bk</i> -BC [1–9]	GE $\gamma\gamma$ YA γ FI ^
chimera <i>Bk</i> -CB [1–9]	DD $\gamma\gamma$ YS γ AI *

 γ Gla residues

* C-terminal amidation

^ C-terminal carboxyl

Table 4

IC₅₀ values measured for conBk-B analogs

	NMDA receptor subtype IC ₅₀ Values ± SEM (μM) [hill coefficient]					NR2D/NR2B IC ₅₀ Ratios
	NR2A	NR2B	NR2C	NR2D		
conBk-B	3.9 ± 0.2 [-1.3]	5.1 ± 0.2 [-1.2]	3.9 ± 0.1 [-0.8]	1.91 ± 0.06 [-0.9]		0.37
conBk-E	>10	>10	>10	>10		ND
conBk-B[COOH]	>10	>10	-	>10		ND
conBk-C [1-9]	>10	>10	>10	>10		ND
conBk-B[A8F]	>10	2.1 ± 0.6 [-1.0]	-	2.9 ± 0.3 [-1.3]		1.4
chimeraBk-BC [1-9]	>10	3.3 ± 0.6 [-0.7]	-	6.2 ± 0.2 [-1.0]		1.9
chimeraBk-CB [1-9]	>10	>10	-	>10		ND

Each IC₅₀ value represents the average from n=3-6 oocytes.