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Structure–Activity Studies of Cysteine-Rich α-Conotoxins that Inhibit High-Voltage-Activated Calcium Channels via $GABA_B$ Receptor Activation Reveal a Minimal Functional Motif

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Abstract: *α-Conotoxins are disulfide-rich peptides that target nicotinic acetylcholine receptors. Recently we identified several α-conotoxins that also modulate voltage-gated calcium channels by acting as G protein-coupled $GABA_B$ receptor ($GABA_BR$) agonists. These α-conotoxins are promising drug leads for the treatment of chronic pain. To elucidate the diversity of α-conotoxins that act through this mechanism, we synthesized and characterized a set of peptides with homology to α-conotoxins known to inhibit high voltage-activated calcium channels via $GABA_BR$ activation. Remarkably, all disulfide isomers of the active α-conotoxins Pu1.2 and Pn1.2, and the previously studied Vc1.1 showed similar levels of biological activity. Structure determination by NMR spectroscopy helped us identify a simplified biologically active eight residue peptide motif containing a single disulfide bond that is an excellent lead molecule for developing a new generation of analgesic peptide drugs.*

Marine snails of the *Conus* genus produce venom, composed of a range of bioactive disulfide-rich peptides known as conotoxins, that is used for both defense and for prey capture.[1] Members of the α-conotoxin subfamily (Figure 1) are defined as neuronal or muscle nicotinic acetylcholine receptors (nAChRs) antagonists.[2]

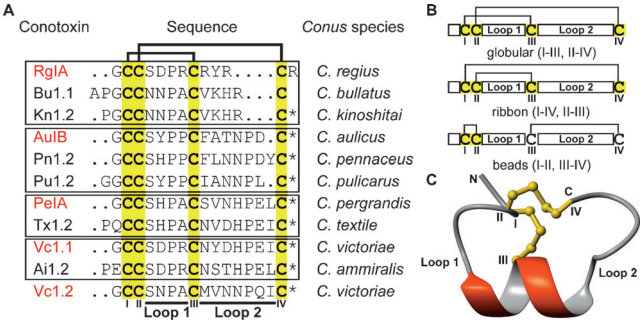

Figure 1. Selected α-conotoxins and their conserved structural framework. A) Sequences of α-conotoxins that inhibit high voltage-activated (HVA) calcium channel currents (I_{Ca}) via the $GABA_B$ receptor (in red) and homologous sequences that have been investigated in this study (in black). The boxes indicate groupings of homologous sequences; cysteines are highlighted in yellow and numbered with Roman numerals, and the native CysI–CysIII, CysII–CysIV connectivity is indicated by solid black lines (top). An asterix indicates an amidated C-terminus. The two loops of the α-conotoxins are indicated at the bottom. B) A schematic illustration of the three possible disulfide isomers for an α-conotoxin: the globular isomer (CysI–CysIII, CysII–CysIV, top), the ribbon isomer (CysI–CysIV, CysII–CysIII, middle), and bead isomer (CysI–CysII, CysIII–CysIV, bottom). C) The three-dimensional structure of Vc1.1 highlighting the helical nature of the α-conotoxins, the cysteine framework (Roman numerals), the two disulfide bonds (yellow ball and sticks) and the two sequence loops.

α-Conotoxins Vc1.1[3] and RgIA[4] have been of recent interest as drug leads, because they have analgesic properties in rat models of neuropathic pain.[5] Recently, a backbone cyclisation strategy was used to develop an orally active analogue of Vc1.1.[6] Vc1.1 and RgIA are α9α10 nAChR subtype antagonists, and potently inhibit N-type ($Ca_v2.2$) and R-type ($Ca_v2.3$) calcium channels via $GABA_B$ receptor ($GABA_BR$) activation.[7] $GABA_BR$ agonists have been shown to relieve neuropathic and chronic pain.[8] Subsequently, three other conotoxins, PeIA,[9] AuIB[5d] and Vc1.2,[10] have also been shown to inhibit HVA calcium channels via a $GABA_BR$-dependent mechanism (Figure 1 A).

α-Conotoxins contain four cysteine residues, so can potentially form three disulfide isomers during oxidative folding (Figure 1 B). These include the globular isomer (CysI–CysIII and CysII–CysIV), the ribbon isomer (CysI–CysIV and CysII–CysIII) and the beads isomer (CysI–CysII and CysIII–CysIV). The natively preferred disulfide connectivity for wild-

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type α-conotoxins is typically the globular isomer although the ribbon isomer of the α-conotoxin ImI was detected in the venom of *Conus imperialis*.[11] The three dimensional structure of the α-conotoxin globular isomer consists of a helical turn that is cross-braced by two disulfide bonds (Figure 1C). This structure orientates the side chains of the peptide to facilitate the binding of the conotoxin between two subunits of the pentameric nAChR.[12] Interestingly, the ribbon isomer of several α-conotoxins has been shown to inhibit nAChRs.[13] Therefore, exploring the effect of disulfide connectivity could provide valuable information on structure/activity relationships, and lead to the discovery of new bioactive molecules.

Here we focus on expanding our knowledge of the sequence and structural diversity of α-conotoxins that target the $GABA_BR$. Based on sequence homology to Vc1.1, RgIA, AuIB or PeIA, we chose six α-conotoxins from the ConoServer database (Figure 1A).[14] The globular isomers of each peptide were synthesized by Fmoc solid-phase peptide synthesis with regioselective disulfide bond formation using S-acetamidomethyl (Acm) protection on CysI and CysIII. Peptides were obtained in high purity and acceptable yield (6 to 18%) after HPLC purification (Figure S1 in the Supporting Information).

The purified globular isomers for all six peptides gave well dispersed signals in their ^{1}H NMR spectra, implying they adopt ordered structures in solution. They were further analyzed by two-dimensional NMR and secondary Hα shift analysis showed the peptides adopted a fold consistent with other α-conotoxins (Figure S3A–C), including a series of negative secondary shift values in the central portion of the peptide that is indicative of helical secondary structure.[15]

The conotoxins were tested for their ability to inhibit HVA calcium channel currents (I_{Ca}) in rat DRG neurons. α-Conotoxins Pn1.2, Pu1.2, Kn1.2 and Tx1.2 inhibited I_{Ca} by 22, 27, 13 and 8%, respectively. In comparison, Ai1.2 and Bu1.1 did not modulate I_{Ca} (Figures 2A and S9). These results expand the known number and sequence diversity of α-conotoxins that inhibit HVA calcium channel currents. It was previously shown that non-native disulfide isomers of α-conotoxins are biologically active.[13] Therefore, to explore the structural elements required for calcium channel inhibition via $GABA_BRs$, we synthesized the three possible disulfide isomers of Pn1.2, plus Pu1.2 and Vc1.1.

As described for the globular isomer syntheses, we used Fmoc SPPS and Acm protection on specific pairs of cysteines to produce the ribbon and beads isomers of Pn1.2, Pu1.2 and Vc1.1. NMR analyses revealed that all isomers adopted a single conformation in solution, except for the Pn1.2 globular isomer and all three beads' isomers. These peptides showed other minor conformations, presumably due to proline *cis*/*trans* isomerization. However this was only confirmed for the Pn1.2 globular isomer (Table S1). Chemical shift assignments and secondary shift analyses for all disulfide isomers are given in Tables S2–S4 and Figure S4.

Surprisingly, all isomers inhibited I_{Ca} in rat dorsal root ganglion (DRG) neurons (Figure 2B) and there was no significant difference in inhibitory activity between individual isomers of each peptide. All α-conotoxin isomers were stable during the assay and no disulfide shuffling between isomers

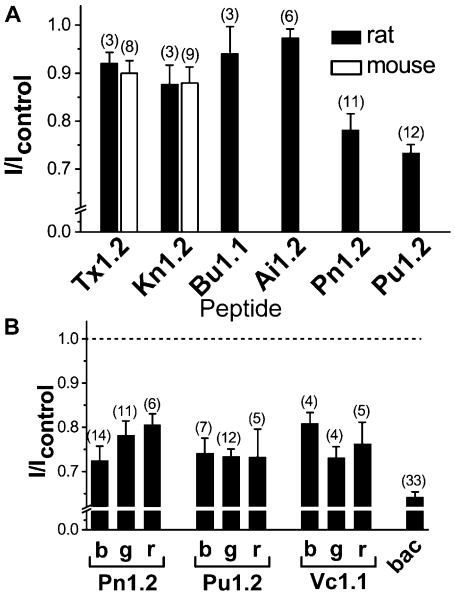

Figure 2. A) Averaged relative peak I_{Ca} amplitudes ($I/I_{control}$) ± SEM in the presence of various α-conotoxins (1 μM). The relative peak I_{Ca} amplitudes ($I/I_{control}$) in the presence of α-conotoxin Pn1.2, Pu1.2, Kn1.2 or Tx1.2 were 0.78 ± 0.034, 0.73 ± 0.019, 0.87 ± 0.040 and 0.92 ± 0.023 (mean ± SEM), respectively, whereas Bu1.1 and Ai1.2 did not modulate I_{Ca}. The number of experiments is in parentheses. B) The beads (b), globular (g) and ribbon (r) disulfide isomers of α-conotoxins Pn1.2, Pu1.2 and Vc1.1 inhibit I_{Ca} in rat DRG neurons. Mean relative peak I_{Ca} amplitudes ($I/I_{control}$) ± SEM in the presence of each α-conotoxin (1 μM) are shown. The number of experiments is in parentheses. The specific $GABA_BR$ agonist baclofen (bac, 50 μM) was used as a positive control. The dotted line indicates maximum current recorded in the absence of α-conotoxin (control).

was observed (Figure S8). We have shown that both reduced and alkylated Vc1.1 have no effect on HVA calcium channel currents.[7] Therefore, the observed activity can be attributed to specific isomers and suggests there might be a common bioactive motif between each isomer.

To further understand the common structural elements in the three isomers, we determined the three dimensional structures of the globular, ribbon and beads isomers of Pu1.2. Structures were calculated in CYANA 3.0[16] and refined in CNS[17] using NOE derived distance restraints, TALOS-N[18] derived dihedral angle restraints, and hydrogen bond restraints from D_2O exchange experiments. The structural statistics for the ensemble of the 20 lowest energy structures for each isomer are shown in Table S5. Figure 3 shows the lowest energy structure from each structural ensemble.

Both the globular and ribbon isomers had well-defined structures, with the globular isomer possessing the characteristic helical nature of an α-conotoxin. The beads isomer had two defined loop regions (Gly2–Pro8) and (Cys9–Cys16), separated by a flexible hinge (Figure S6). The surface features of all three isomers consist of a series of alternating polar and hydrophobic patches, with the major hydrophobic region formed by Pro7, Pro8, Ile10 and Ala11 (Figure S7). There were no obvious structural similarities between the three isomers, but there was some indication of common loop

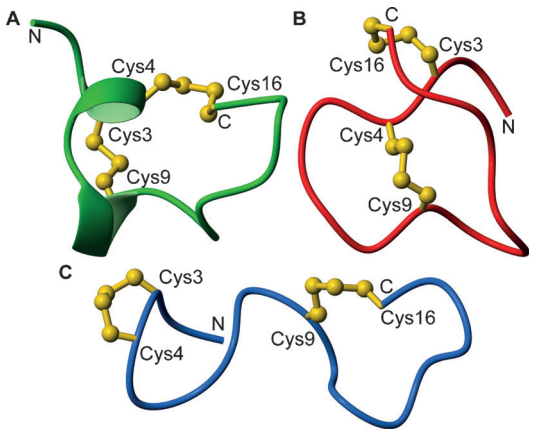

Figure 3. The three-dimensional structures of the A) globular (green); B) ribbon (red); and C) beads (blue) disulfide isomers of α-conotoxin Pu1.2. The structure shown for each isomer is the lowest energy conformer from an ensemble of the 20 lowest energy structures calculated using CYANA[16] and refined in CNS.[17] Structures are shown in ribbon format with disulfide bonds in ball and stick, and the cysteines and N and C termini labelled.

motifs. For example, the backbone RMSD from residues Cys3–Pro8 for all 60 structures was only 0.954 Å.

To see if any loop regions in the Pu1.2 isomers form the core requirement for HVA calcium channel inhibition, we made a set of truncated and disulfide deficient Pu1.2 mutants (Figure 4 A). These consisted of full-length ([Ser^3, Ser^9]Pu1.2-(1-16)), loop 1 alone ([Ser^4]Pu1.2(1-9)) and loop 2 (Pu1.2(9-16)) alone, each with a single disulfide bond restraint. The peptides were synthesized by Fmoc-based SPPS, and the disulfide bond formed by incubating the peptide in 0.1 M NH_4HCO_3 with 2.2′-bispyridyl disulfide, purified by HPLC and characterized by NMR spectroscopy (Figure S5).

The truncated loop 1 peptide, [Ser^4]Pu1.2(1-9), was sufficient to inhibit HVA calcium currents in rat DRG neurons and this effect could be blocked by a 4 min pre-incubation with the selective $GABA_BR$ antagonist CGP55 845 (Figure 4 B). The analogues [Ser^3, Ser^9]Pu1.2(1-16) and Pu1.2-(9-16) were inactive. As the loop 1 region of Pu1.2 was sufficient for biological activity, we decided to extrapolate this finding to Vc1.1. Interestingly, both Vc1.1 and RgIA, which were the first α-conotoxins shown to inhibit HVA I_{Ca} via the $GABA_BR$, have identical loop 1 sequences but vary significantly in length and composition of loop 2. Subsequent synthesis and testing of the loop 1 analogue of Vc1.1, [Ser^3]Vc1.1(1-8), revealed it inhibited HVA calcium currents in mouse DRG neurons with a potency comparable with full-length Vc1.1 (Figure 4 C). These truncated, single disulfide conotoxin analogues could be promising lead molecules for the development of new analgesics.

Vc1.1, RgIA and their backbone cyclic analogues have all been proposed as potential leads for drugs to treat neuropathic and chronic pain. Compared with these full-length peptides, the truncated versions have the advantage of structural simplicity, making their synthesis faster, less expensive and more efficient. Of note is that loop 1 of Vc1.1 and RgIA is also identical in sequence to that of ImI, which did not inhibit HVA calcium currents in rat DRG

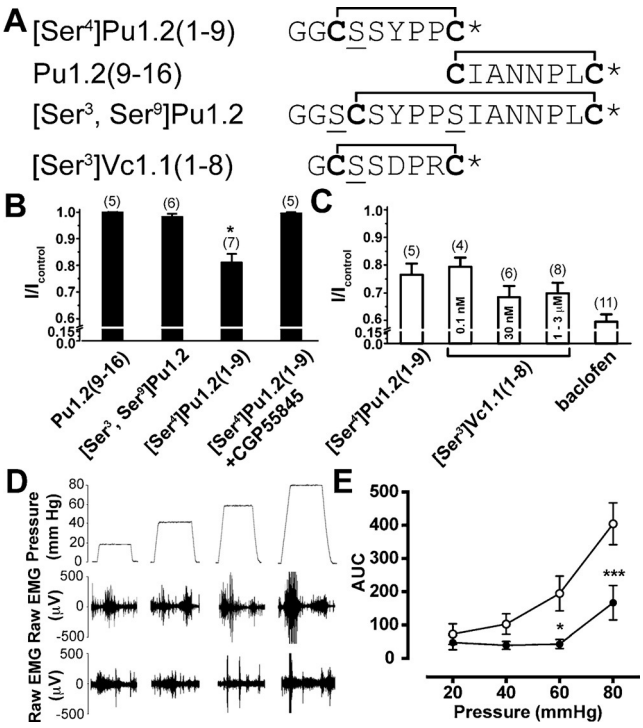

Figure 4. Truncated analogues of Pu1.2 and Vc1.1 inhibit HVA calcium currents in rat and mouse DRG neurons. A) The sequences of truncated and disulfide-deficient analogues of Pu1.2 and Vc1.1. Disulfide bonds are indicated by a solid line. The asterisk indicates an amidated C-terminus and the positions of the cysteines substituted with serine are underlined. B) I_{Ca} inhibition in the presence of full-length disulfide-deficient or truncated Pu1.2 analogues. Bar graphs represent averaged relative peak I_{Ca} amplitude ($I/I_{control}$) ± SEM values. The number of experiments is shown in parentheses. The asterisk indicates the difference between CGP55845 + [Ser^4]Pu1.2(1-9) and [Ser^4]Pu1.2(1-9) groups. Statistical significance was assessed using ANOVA (* $P<0.05$). C) Average $I/I_{control}$ data (± SEM) of peak I_{Ca} inhibition in mouse DRG neurons. I_{Ca} was inhibited by 23.5 ± 4% (1 μM [Ser^4]Pu1.2(1-9)), 30.2 ± 3.9%, 31.6 ± 4% and 20.6 ± 3.3% (1–3 μM, 30 nM and 100 pM [Ser^3]Vc1.1(1-8), respectively, or 41.5 ± 2.7% (50 μM baclofen). The number of experiments is in parentheses. The effect of Vc1.1(1-8)[Ser^3]Vc1.1(1-8) developed slowly, reached maximum inhibition within 10 min, and was irreversible. In comparison, the effect of [Ser^4]Pu1.2(1-9) was transient, with I_{Ca} showing partial recovery in the presence of this analogue (see also Figure S11). Similarly, ca. 20%, I_{Ca} inhibition values were obtained in rat DRG neurons in the presence of 1 μM Vc1.1(1-8) ($n=3$) (not shown). D) Representative tracing of the visceromotor response (VMR) to colorectal distension in mice. The upper panel shows the distension sequence (20 s duration, 4 min interval). The corresponding electromyography (EMG) recordings after intra-colonic administration with either vehicle or 1000 nM [Ser^3]Vc1.1(1-8) are illustrated in the middle and lower panel, respectively. VMR to colorectal distension were notably reduced by [Ser^3]Vc1.1(1-8). E) In healthy control mice intracolonic administration of [Ser^3]Vc1.1(1-8) (filled circles) significantly reduced VMR to 60 and 80 mmHg of distension, when compared to intracolonic vehicle treatment (open circles). Data expressed as area under the curve of the corresponding EMG signal for $n=4$ mice/group. (*$P<0.05$, ***$P<0.001$, $n=4$ mice, two-way ANOVA, Bonferroni posthoc).

neurons.[7b] This suggests residues in loop 2 can also influence interactions with $GABA_BR$, which is consistent with our previous study showing that post-translational modification of

Glu[14] in Vc1.1 to γ-carboxyglutamic acid resulted in a loss of HVA calcium channel inhibitory activity.[7b] Furthermore, post-translational modification of Pro[6] of Vc1.1 to hydroxyproline also caused a loss of HVA calcium current inhibition suggesting that this residue, which is present in both [Ser[3]]Vc1.1(1-8) and [Ser[4]]Pu1.2(1-9), might be a key interaction site with the $GABA_BR$.

We tested all Pu1.2 analogues and [Ser[3]]Vc1.1(1-8) for their ability to inhibit human α9α10 and α7 nAChRs expressed in *Xenopus* oocytes. None inhibited hα9α10 nAChRs, but 1 μM [Ser[3]]Vc1.1(1-8) fully inhibited hα7 nAChRs (Figure S12). This is consistent with a study showing a disulfide-deficient analogue of ImI, without CysII–CysIV, retained its affinity for rat α7 nAChRs comparable with the native peptide.[19] The key residues for inhibition of currents through α7 nAChRs by ImI is the Asp-Arg-Pro motif in loop 1, which is present in [Ser[3]]Vc1.1(1-8).[19] This key Asp-Arg-Pro motif is not present in [Ser[4]]Pu1.2(1-9), which likely explains why this peptide does not inhibit α7 nAChRs.

We determined if [Ser[4]]Pu1.2(1-9) or [Ser[3]]Vc1.1(1-8) could modify colonic nociceptor function.[20] To do this, we performed ex vivo afferent recordings from mouse splanchnic high-threshold nociceptors, which respond to focal compression and noxious stretch/distension.[21] We assessed nociceptor mechanosensitivity before and after increasing doses of each peptide. Both [Ser[4]]Pu1.2(1-9) and [Ser[3]]Vc1.1(1-8) dose-dependently inhibited colonic nociceptor mechanosensitivity, with greatest inhibition observed at a concentration of 1000 nM (Figure S14). We then assessed the effect of [Ser[3]]Vc1.1(1-8) in a mouse model of visceral pain. Noxious distension of the colorectum triggers the visceromotor response (VMR), a nociceptive brainstem reflex consisting of contraction of the abdominal muscles.[22] Abdominal electromyography (EMG), was used to assess visceral sensitivity in vivo in fully awake animals.[23] In this model, intracolonic treatment with [Ser[3]]Vc1.1(1-8) significantly reduced VMR in response to colorectal distension compared to vehicle treated mice (Figure 4C,D).

In conclusion, we have described four new α-conotoxins that inhibit HVA calcium channels via $GABA_B$ receptor activation in rodent DRG neurons. We have also identified a core peptide motif that inhibits HVA calcium channels and blocks nociceptor mechanosensitivity. Synthesis of peptides with multiple disulfide bonds can be technically challenging, and shuffling of disulfide bonds is problematic for drug development. Therefore, this simplified core peptide motif is a promising lead towards drugs to treat neuropathic and chronic pain. As such, further structure/activity studies are needed to fully understand the key determinants of its bioactivity and improve its drug-like properties.

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[1] a) B. M. Olivera, *J. Biol. Chem.* **2006**, *281*, 31173–31177; b) T. S. Han, R. W. Teichert, B. M. Olivera, G. Bulaj, *Curr. Pharm. Des.* **2008**, *14*, 2462–2479; c) S. Dutertre, A. H. Jin, I. Vetter, B. Hamilton, K. Sunagar, V. Lavergne, V. Dutertre, B. G. Fry, A. Antunes, D. J. Venter, P. F. Alewood, R. J. Lewis, *Nat. Commun.* **2014**, *5*, 3521.

[2] a) R. J. Lewis, S. Dutertre, I. Vetter, M. J. Christie, *Pharmacol. Rev.* **2012**, *64*, 259–298; b) J. L. Dutton, D. J. Craik, *Curr. Med. Chem.* **2001**, *8*, 327–344.

[3] D. W. Sandall, N. Satkunanathan, D. A. Keays, M. A. Polidano, X. Liping, V. Pham, J. G. Down, Z. Khalil, B. G. Livett, K. R. Gayler, *Biochemistry* **2003**, *42*, 6904–6911.

[4] M. Ellison, C. Haberlandt, M. E. Gomez-Casati, M. Watkins, A. B. Elgoyhen, J. M. McIntosh, B. M. Olivera, *Biochemistry* **2006**, *45*, 1511–1517.

[5] a) N. Satkunanathan, B. Livett, K. Gayler, D. Sandall, J. Down, Z. Khalil, *Brain Res.* **2005**, *1059*, 149–158; b) M. Vincler, S. Wittenauer, R. Parker, M. Ellison, B. M. Olivera, J. M. McIntosh, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 17880–17884; c) S. T. Nevin, R. J. Clark, H. Klimis, M. J. Christie, D. J. Craik, D. J. Adams, *Mol. Pharmacol.* **2007**, *72*, 1406–1410; d) H. Klimis, D. J. Adams, B. Callaghan, S. Nevin, P. F. Alewood, C. W. Vaughan, C. A. Mozar, M. J. Christie, *Pain* **2011**, *152*, 259–266; e) I. A. Napier, H. Klimis, B. K. Rycroft, A. H. Jin, P. F. Alewood, L. Motin, D. J. Adams, M. J. Christie, *Neuropharmacology* **2012**, *62*, 2202–2207.

[6] R. J. Clark, J. Jensen, S. T. Nevin, B. P. Callaghan, D. J. Adams, D. J. Craik, *Angew. Chem. Int. Ed.* **2010**, *49*, 6545–6548; *Angew. Chem.* **2010**, *122*, 6695–6698.

[7] a) B. Callaghan, D. J. Adams, *Channels* **2010**, *4*, 1–4; b) B. Callaghan, A. Haythornthwaite, G. Berecki, R. J. Clark, D. J. Craik, D. J. Adams, *J. Neurosci.* **2008**, *28*, 10943–10951; c) G. Berecki, J. R. McArthur, H. Cuny, R. J. Clark, D. J. Adams, *J. Gen. Physiol.* **2014**, *143*, 465–479.

[8] a) H. Pan, Z. Wu, H. Zhou, S. H. Chen, H. Zhang, D. Li, *Pharmacol. Ther.* **2008**, *117*, 141–161; b) D. Huang, S. Huang, C. Peers, X. Du, H. Zhang, N. Gamper, *Biochem. Biophys. Res. Commun.* **2015**, *465*, 188–193; c) T. G. Huynh, H. Cuny, P. A. Slesinger, D. J. Adams, *Mol. Pharmacol.* **2015**, *87*, 240–250.

[9] N. L. Daly, B. Callaghan, R. J. Clark, S. T. Nevin, D. J. Adams, D. J. Craik, *J. Biol. Chem.* **2011**, *286*, 10233–10237.

[10] H. Safavi-Hemami, W. A. Siero, Z. Kuang, N. A. Williamson, J. A. Karas, L. R. Page, D. MacMillan, B. Callaghan, S. N. Kompella, D. J. Adams, R. S. Norton, A. W. Purcell, *J. Biol. Chem.* **2011**, *286*, 22546–22557.

[11] H. Safavi-Hemami, D. G. Gorasia, A. M. Steiner, N. A. Williamson, J. A. Karas, J. Gajewiak, B. M. Olivera, G. Bulaj, A. W. Purcell, *J. Biol. Chem.* **2012**, *287*, 34288–34303.

[12] a) P. H. Celie, I. E. Kasheverov, D. Y. Mordvintsev, R. C. Hogg, P. van Nierop, R. van Elk, S. E. van Rossum-Fikkert, M. N. Zhmak, D. Bertrand, V. Tsetlin, T. K. Sixma, A. B. Smit, *Nat. Struct. Mol. Biol.* **2005**, *12*, 582–588; b) R. Yu, S. N. Kompella, D. J. Adams, D. J. Craik, Q. Kaas, *J. Med. Chem.* **2013**, *56*, 3557–3567.

[13] a) E. K. Lebbe, S. Peigneur, M. Maiti, B. G. Mille, P. Devi, S. Ravichandran, E. Lescrinier, E. Waelkens, L. D'Souza, P. Herdewijn, J. Tytgat, *Toxicon* **2014**, *91*, 145 – 154; b) Y. Wu, X. Wu, J. Yu, X. Zhu, D. Zhangsun, S. Luo, *Molecules* **2014**, *19*, 966 – 979; c) A. A. Grishin, C. I. Wang, M. Muttenthaler, P. F. Alewood, R. J. Lewis, D. J. Adams, *J. Biol. Chem.* **2010**, *285*, 22254 – 22263; d) J. L. Dutton, P. S. Bansal, R. C. Hogg, D. J. Adams, P. F. Alewood, D. J. Craik, *J. Biol. Chem.* **2002**, *277*, 48849 – 48857.

[14] Q. Kaas, R. Yu, A. H. Jin, S. Dutertre, D. J. Craik, *Nucleic Acids Res.* **2012**, *40*, D325 – 330.

[15] D. S. Wishart, C. G. Bigam, A. Holm, R. S. Hodges, B. D. Sykes, *J. Biomol. NMR* **1995**, *5*, 67 – 81.

[16] P. Güntert, C. Mumenthaler, K. Wüthrich, *J. Mol. Biol.* **1997**, *273*, 283 – 298.

[17] A. T. Brünger, P. D. Adams, G. M. Clore, W. L. DeLano, P. Gros, R. W. Grosse-Kunstleve, J. S. Jiang, J. Kuszewski, M. Nilges, N. S. Pannu, R. J. Read, L. M. Rice, T. Simonson, G. L. Warren, *Acta Crystallogr. Sect. D* **1998**, *54*, 905 – 921.

[18] Y. Shen, F. Delaglio, G. Cornilescu, A. Bax, *J. Biomol. NMR* **2009**, *44*, 213 – 223.

[19] D. Servent, H. L. Thanh, S. Antil, D. Bertrand, P. J. Corringer, J. P. Changeux, A. Menez, *J. Physiol.* **1998**, *92*, 107 – 111.

[20] J. Castro, A. M. Harrington, S. Garcia-Caraballo, J. Maddern, L. Grundy, J. Zhang, G. Page, P. E. Miller, D. J. Craik, D. J. Adams, S. M. Brierley, *Gut* **2016**, DOI: 10.1136/gutjnl-2015-310971.

[21] a) A. D. de Araujo, M. Mobli, J. Castro, A. M. Harrington, I. Vetter, Z. Dekan, M. Muttenthaler, J. Wan, R. J. Lewis, G. F. King, S. M. Brierley, P. F. Alewood, *Nat. Commun.* **2014**, *5*, 3165; b) J. Castro, A. M. Harrington, P. A. Hughes, C. M. Martin, P. Ge, C. M. Shea, H. Jin, S. Jacobson, G. Hannig, E. Mann, M. B. Cohen, J. E. MacDougall, B. J. Lavins, C. B. Kurtz, I. Silos-Santiago, J. M. Johnston, M. G. Currie, L. A. Blackshaw, S. M. Brierley, *Gastroenterology* **2013**, *145*, 1334 – 1346.

[22] T. J. Ness, G. F. Gebhart, *Brain Res.* **1988**, *450*, 153 – 169.

[23] a) J. A. Christianson, G. F. Gebhart, *Nat. Protoc.* **2007**, *2*, 2624 – 2631; b) A. Deiteren, J. G. De Man, N. E. Ruyssers, T. G. Moreels, P. A. Pelckmans, B. Y. De Winter, *Gut* **2014**, *63*, 1873 – 1882.

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