I8-arachnotocin—an arthropod-derived G protein-biased ligand of the human vasopressin V2 receptor

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The neuropeptides oxytocin (OT) and vasopressin (VP) and their G protein-coupled receptors OTR, V1aR, V1bR, and V2R form an important and widely-distributed neuroendocrine signaling system. In mammals, this signaling system regulates water homeostasis, blood pressure, reproduction, as well as social behaviors such as pair bonding, trust and aggression. There exists high demand for ligands with differing pharmacological profiles to study the physiological and pathological functions of the individual receptor subtypes. Here, we present the pharmacological characterization of an arthropod (Metaseiulus occidentalis) OT/VP-like nonapeptide across the human OT/VP receptors. I8-arachnotocin is a full agonist with respect to second messenger signaling at human V2R (EC50 34 nM) and V1bR (EC50 1.2 µM), a partial agonist at OTR (EC50 790 nM), and a competitive antagonist at V1aR [pA2 6.25 (558 nM)]. Intriguingly, I8-arachnotocin activated the Gαs pathway of V2R without recruiting either β-arrestin-1 or β-arrestin-2. I8-arachnotocin might thus be a novel pharmacological tool to study the (patho)physiological relevance of β-arrestin-1 or -2 recruitment to the V2R. These findings furthermore highlight arthropods as a novel, vast and untapped source for the discovery of novel pharmacological probes and potential drug leads targeting neurohormone receptors.

Oxytocin (OT) and vasopressin (VP) are prototypical neuropeptides that together with their G protein-coupled receptors (GPCRs), OTR, V1aR, V1bR, and V2R, form a versatile neuroendocrine signaling system in humans. Peripherally, OT is important in the regulation of labor1 and milk let-down2, while VP is crucial for water homeostasis and vasoconstriction3,4. Centrally, OT and VP influence different behaviors, such as empathy5, social recognition6–8, attachment9, parental care10, and anxiety-related behavior11. Consequently, its dysregulation is associated with a wide range of disorders, including post-partum complications, cardiovascular diseases, diabetes insipidus and dysmenorrhea as well as social anxiety disorders, autism, schizophrenia, Prader-Willi syndrome and depression12–18.

OT and VP differ only in two amino acids and their receptors share ~80% binding site sequence homology19, which results in OT and VP being unselective and able to activate all four receptors. Consequently, neuroscientists are looking for a repertoire of ligands with different pharmacological profiles to study the (patho)physiology of the individual receptor subtypes of this fundamental signaling system. In an attempt to provide such a pharmacological toolbox, we started to explore different animal species in the search for OT/VP-like ligands that are active on the human receptors, yet better at discriminating signaling between the four OT/VP receptors. Our strategy14 relies on the fact that OT/VP-like signaling system is highly conserved and widely distributed across vertebrates, including fish and amphibians, as well as in several invertebrate species such as mollusks, annelids, nematodes, insects, starfish, and hydra20. Its origin can be traced back ~600 million years to the closely-related ancestral vertebrate nonapeptide vasotocin and similar invertebrate nonapeptides (Fig. 1)20,21. Our strategy has already yielded several important pharmacological probes, including a selective human V1aR antagonist based on an OT/VP-like peptide isolated from the black garden ant Lasius niger19, a selective antagonist at the human V1aR
Figure 1. Phylogenetic relationship and molecular sequence of OT/VP-like neuropeptides. Taxonomic groups and neuropeptide names (in brackets). Conserved cysteines are highlighted in yellow, and the disulfide bond between Cys5 and Cys8 is indicated. *C-terminus amidated; #Renamed to arachnotocin to simplify nomenclature. Analogous to the existing phylum nomenclature, we refer to the mite-derived peptide by I8-ARACHNOTOCIN.

In this work, we report the chemical synthesis and pharmacological characterization of I8-arachnotocin across the four human OT/VP receptors using second messenger quantification as well as β-arrestin-1 and -2 recruitment assays in cells heterologously expressing the individual receptor subtypes OTR, V1aR, V1bR, and V2R.

Materials and Methods

Peptide synthesis. I8-arachnotocin was produced by solid phase peptide synthesis (SPPS) using fluorenylmethyloxycarbonyl (Fmoc) chemistry, purified and analyzed using methods previously described. Briefly, the peptide was synthesized on a Rink amide 4-methylbenzyloxycarbonyl (Fmoc) resin (Auszep, Australia) using 4-fold excess of protected amino acids, 4 equivalents of hexafluorophosphozone benzotriazol-1-ylmethyl (HTU) (Iris Biotech, Germany) and N,N-diisopropylmethylethylamine (DIPEA) (Auszep, Australia) in dimethylformamide (DMF) (Auszep, Australia) and a coupling time of 15 min. The peptide was cleaved from the resin and the side chain protecting groups were removed by treatment with trifluoroacetic acid (TFA) (Auspep, Australia), triisopropylsilane (TIPS) (Sigma Aldrich, Australia), ethandithiol (EDT) (Sigma Aldrich, Australia): H2O (90:2.5:2.5:5) for 2 h. The crude peptide was folded for 24 h at 100 µM at 25°C in 0.1 M NH4HCO3 (Auspep, Australia), pH 8.2, and purified using a Vydac Protein and Peptide C18 preparative column. Analytical HPLC was performed with column heating at 40°C and detection at 214 nm. The final product was analyzed by LC-MS on an API QSTAR PULSAR from PE Sciex, used in series with Agilent 1100 series HPLC system with a Kromasil C18 column at a gradient of 0–40% eluent B (90% acetonitrile, 0.045% trifluoroacetic acid) in 20 min.

Cell culture and transient receptor expression. All cell culture work was performed with human embryonic kidney cells 293 (HEK293). Unless otherwise stated, cells were incubated at 37°C and 5% CO2 and grown in Dulbecco’s Modified Eagle’s Medium (DMEM; Thermo Fisher Scientific, Australia and Fisher Scientific, Austria) containing 10% fetal bovine serum (GE Life Sciences, Australia and Sigma-Aldrich, Germany), 50 U/mL penicillin and 50 µg/mL streptomycin (Thermo Fisher Scientific, Australia and Sigma-Aldrich, Germany). Transient transfections were performed via Lipofectamine 2000 (Thermo Fisher Scientific, Australia) or jet-PRIME (Polyplus transfection, France) using 2 µg of pEGFP-N1 plasmid DNA coding for EGFP-tagged human OT/VP receptors or a combination of 2 µg each of receptor-encoding plasmids and a plasmid coding for β-arrestin-1- or -2-Nluc (subcloned into pcDNA3, with NanoLuc provided under a Limited Use Label License from Promega, Madison, USA).

Second messenger quantification. Inositol-1-phosphate (IP1) accumulation in response to Gα1 coupled activation of human OTR, V1aR, and V1bR were measured using the IP-One Gq assay kit (Cisbio, France). Cells were seeded 4 h after transfection onto 384-well plates at a density of 10,000 cells per well and incubated for 2 days. At the time of the assay, all media was removed and the cells equilibrated to the provided stimulation buffer for 15 min at 37°C. The cells were stimulated with peptide ligands at varying concentration (10 µM–30 µM) for 1 h at 37°C. Cyclic adenosine monophosphate (cAMP) accumulation induced by Goi, coupling of V2R activation was determined using the LANCE Ultra cAMP detection kit (Perkin Elmer, Waltham, USA). Cells were re-passaged 4 h after transfection at a 1:2 ratio and incubated overnight. The next day, all media was removed, and cells were suspended with CAMP stimulation buffer (5 mM HEPES, 0.5 mM 3-isobutyl-1-methylxanthine, 0.1% bovine serum albumin in Hank’s balanced salt solution, HBSS, pH 7.4) and the cells transferred onto a 384-well plate at a density of 300 cells per well. Stimulation was carried out for 30 min at 25°C. Second messenger levels were measured by homogenous time-resolved fluorescence resonance energy transfer (FRET) measurement on a Flexstation 3 (Molecular Devices, San Jose, USA) using the ratios 665/620 nm (IP1) and 665/615 nm (cAMP) at an excitation wavelength of 340 nm. For antagonist screening, cells were stimulated with the endogenous ligand OT at OTR (50 nM), or VP for V1aR (1 nM), V1bR (3 nM) and V2R (0.5 pM), in presence (1 or 10 µM) and absence of I8-arachnotocin, as well as with 10 µM of the respective endogenous ligand.

Antagonism of I8-arachnotocin at V1aR was characterized by Schild regression analysis (as published earlier). Briefly, several concentration-response curves of the endogenous agonist VP (as described above) were measured...
in the presence (1 μM, 3 μM and 10 μM) and absence of I8-arachnotocin. The logarithm of the dose-ratio (A'/A-1) was plotted vs. the logarithm of the respective concentration of 18-arachnotocin (B) to obtain the pA2 value.

β-arrestin-1 and -2 recruitment. Recruitment of β-arrestin-1 and -2 upon receptor stimulation was measured via real-time measurement of bioluminescence resonance energy transfer (BRET) between β-arrestin-1/2-luciferase and EGFP-tagged receptors. Cells were co-transfected with β-arrestin-1/2-Nluc and OT/VP receptor encoding plasmids at a ratio of 1:10. At 6 h post-transfection, the cells were transferred onto a white, clear bottom 96-well plate at 50,000 cells/well in phenol-red free DMEM containing 10% fetal bovine serum. The following day, the cells were serum starved for 1 h in phenol-free DMEM. Furimazine (Promega, Madison, USA), diluted 1:50 in HBSS, was added to the cells 5 min prior to monitoring at a 1:1 ratio. Light emissions were measured at 460 nm (Nluc) and 510 nm (EGFP) on a Flexstation 3 (Molecular Devices, San Jose, USA). After establishment of a baseline for 5 min, peptides diluted in HBSS were added and the response measured for 35 min. The ligand-induced BRET signal was calculated as: (emission EGFPligand/emission Nlucligand) – (emission EGFPHBSS/emission NlucHBSS). Concentration-response curves were generated from the BRET signal at 5 min after addition of various peptide concentrations (10 pM – 30 μM).

Immunoblotting for ERK 1/2. Immunoblotting was performed as described previously. Briefly, following an overnight incubation and 16 h starvation HEK293 cells transiently expressing human V2R were treated with 1 μM 18-arachnotocin or 1 μM VP prepared in DMEM. Cells were incubated with peptide ligands at 37 °C for indicated periods and 1 mL of chilled 1x phosphate-buffered saline were used to terminate the incubation. After freeze-thaw cycle in liquid nitrogen cells were solubilized in 100 μL of lysis buffer (50 mM HEPES, 0.5% NP-40, 50 mM glycerol–2-phosphate, 250 mM NaCl, 5 mM EDTA, 2 mM imidazole, 1 mM Na3VO4, 1 mM hepta-molybdate, pH adjusted to 7.0 with NaOH), freshly added 1 mM PMSF, one Complete Mini EDTA-free tablet (Roche) and one PhosSTOP tablet (Roche) and then centrifuged at 10,000 × g for 10 min. The bicinchoninic acid assay (Micro-BCA kit, Pierce) was used to measure total protein content. Phosphorylated and total ERK 1/2 were detected by immunoblotting on the same membrane using the same exposure method with an anti-phospho-p44/42 MAPK antibody (ERK 1/2) (1:1,000; Cell Signaling Technology) and an anti-p44/42 MAPK (ERK 1/2) (1:1,000; Cell Signaling Technology), respectively. Detection and quantification of resulting bands were executed by secondary antibodies (Donkey anti rabbit 680RD and 880RD) and Odyssey Clx (LiCor Biosciences) infrared fluorescent imaging system, respectively.

Data analysis. All data were analyzed using GraphPad Prism (GraphPad Software, San Diego) and all graphs were normalized to the activity of OT/VP above baseline. Concentration response curves were fitted to three-parameter non-linear regression curves with a bottom constrained to zero, a slope of one and sigmoidal shape at logarithmic scale to derive estimates of potency (EC50) and maximum efficacy (Emax). Concentration response curves for Schild regression analysis were additionally constrained to a top value of one hundred. All data were presented as mean ± SEM of at least three independent experiments (unless otherwise stated) conducted in triplicate.

Ethics Statement. The study presented in this manuscript did not involve human or animal subjects.

Results  I8-arachnotocin is an agonist at human OTR, V1aR and V2R. Fmoc-SPPS, cleavage, oxidative folding, followed by preparative C18-HPLC purification yielded I8-arachnotocin in >95% purity (15% overall yield) (Fig. 2). The concentration-response curves of 18-arachnotocin at the human OT/VP receptors (Fig. 3) indicated discriminatory effects in terms of Emax and EC50. At both V1aR and V2R, it was a full agonist, yet with highly disparate potencies, namely potencies of 1.2 μM (logEC50 = −5.93 ± 0.15) and 34 nM (logEC50 = −7.47 ± 0.09) respectively. At OTR, 18-arachnotocin was a partial agonist (Emax = 62%) with an EC50 of 790 nM (logEC50 = −6.11 ± 0.20) (Fig. 3). At all three receptors, I8-arachnotocin was less potent compared to the respective endogenous peptide (OTR 65-fold, V1aR 750-fold, V2R 5,000-fold). No activation of V1aR by I8-arachnotocin was observed for concentrations up to 10 μM (Table 1).

I8-arachnotocin is a competitive antagonist at human V1aR. Based on the lack of V1aR activation, we performed an antagonist screen of 18-arachnotocin across all four receptors (Fig. 4a). Receptor stimulation with VP (0.55 nM) in the presence of 1 and 10 μM I8-arachnotocin, yielded lower IP1 levels than VP alone (Student’s t-test, p = 0.0226). The concentration-response curves of VP on V1aR in the absence and presence of 18-arachnotocin (1 μM, 3 μM, 10 μM) indicated an 18-arachnotocin-mediated dextral shift of the potency proportionally to its concentration, without affecting Emax, typical for a competitive antagonist (Fig. 4b). The dextral shift was evaluated via Schild regression analysis yielding a linear regression slope of 1.28 ± 0.02 and a pA2 of 6.253 (−3.58 nM), thus demonstrating that 18-arachnotocin is a competitive antagonist of the V1aR.

I8-arachnotocin does not induce β-arrestin-1 or -2 recruitment at V2R. With ligand bias becoming more relevant in understanding (patho)physiological responses, we also measured I8-arachnotocin-induced β-arrestin-2 recruitment. A concentration of 10 μM of the endogenous ligand induced rapid recruitment of β-arrestin-2 across all human OT/VP receptors, as judged by the increasing BRET signal from basal to maximum in 50–100 s. However, this effect was absent upon stimulation with 10 μM of 18-arachnotocin at OTR-, V1aR- and V2R-expressing cells (Fig. 5).

To further explore this effect, we measured concentration-response curves of β-arrestin-2 recruitment across all four receptors (Fig. 6, Table 2). At the V2R, 18-arachnotocin did not recruit β-arrestin-2...
up to a concentration of 100 µM, in contrast to VP which recruited β-arrestin-2 with an EC$_{50}$ of 60 nM (logEC$_{50}$ = −7.22 ± 0.07). At the V$_{1b}$R, I8-arachnotocin recruited β-arrestin-2 (E$_{max}$ = 65%) with an EC$_{50}$ of 1.2 µM (logEC$_{50}$ = −5.92 ± 0.14), compared to VP, which recruited β-arrestin-2 with an EC$_{50}$ of 6.9 nM (logEC$_{50}$ = −8.16 ± 0.09). At the OTR, I8-arachnotocin recruited β-arrestin-2 with low efficacy (E$_{max}$ = 32%) with an EC$_{50}$ of 5.7 µM (logEC$_{50}$ = −5.24 ± 0.26), compared to OT which recruited β-arrestin-2 with an EC$_{50}$ of 170 nM (logEC$_{50}$ = −6.77 ± 0.15). At the V$_{1a}$R, no I8-arachnotocin-induced β-arrestin-2 recruitment was detected at a concentration up to 100 µM, in contrast to VP-induced β-arrestin-2 recruitment with an EC$_{50}$ of 28 nM (logEC$_{50}$ = −7.55 ± 0.12); this aligned with our findings of I8-arachnotocin being a competitive antagonist to VP.

Figure 2. Quality of synthetic I8-arachnotocin. Analytical RP-HPLC of the purified I8-arachnotocin (purity > 95%). Inset: high resolution MS of the product with the observed molecular weight of 964.44 Da (theoretical: 964.44 Da). ACN = acetonitrile; TFA = trifluoroacetic acid; *C-terminus amidated.

Figure 3. I8-arachnotocin is an agonist at human V$_2$R, V$_{1b}$R, and OTR. Concentration-dependent accumulation of second messengers (cAMP and IP$_1$) after receptor stimulation of (a) OTR, (b) V$_{1b}$R, (c) V$_{1a}$R and (d) V$_2$R with I8-arachnotocin (30 pM – 30 µM). Results were normalized to accumulation of IP$_1$ and cAMP above baseline and maximal activation of the receptors by their endogenous ligands (OT for OTR and VP for VPRs). Data points were fitted by nonlinear regression curves (sigmoidal, slope = 1); error bars depict SEM; n = 3. For EC$_{50}$ and E$_{max}$ values refer to Table 1.
Table 1. Potency and efficacy (G protein-mediated) of I8-arachnotocin at human receptors. EC50 is given in nM or µM (as indicated) and as pEC50 as logEC50 ± SEM. **controls were VP at V2R, V1aR, V1bR and OT at OTR.

| Receptor | EC50  | pEC50  | Emax  | V1aR  | 1.2 µM | −5.93 ± 0.15  | 104 ± 8% | 1.6 nM | −8.80 ± 0.09  | 100% |
|----------|-------|--------|-------|-------|--------|-------------|----------|-------|-------------|------|
| V2R      | 34 nM | −7.47 ± 0.09 | 99 ± 3% | 6.7 µM| −11.17 ± 0.10 | 100% |
| V1bR     | antagonist pA2 of 6.253 (−558 nM) | 0.56 nM | −9.25 ± 0.07 | 100% |
| OTR      | 790 nM | −6.11 ± 0.20 | 62 ± 6% | 12 nM | −7.91 ± 0.09 | 100% |

Figure 4. I8-arachnotocin is a competitive antagonist at V1aR. (a) Accumulation of second messengers (IP1 and cAMP) with partial activating concentrations of endogenous ligands (50 nM OT at OTR; 1 nM VP at V1aR; 3 nM VP at V1bR; 0.5 pM at V2R) in the absence and presence of 1 or 10 µM I8-arachnotocin in comparison to a saturating concentration of endogenous ligand (10 µM). All data were normalized to second messenger accumulation above baseline (0%) and maximum (100%) activity of the endogenous ligand. The dashed line depicts IP1/cAMP accumulation in the absence of I8-arachnotocin. Error bars depict SEM. n = 3, except n = 2 for V1aR (error bars depict SD). The asterisk (*) indicates significance in Student's t-test (p = 0.0226). (b) Accumulation of IP1 by stimulation of human V1aR with VP (10 pM – 10 µM) alone or in the presence of 1, 3 or 10 µM I8-arachnotocin. Receptor activation was normalized to the accumulation of IP1 above baseline. Data points were normalized to the maximum (100%) and minimum (0%) response generated by VP. Error bars depict SEM; n = 3. Insert: Schild regression analysis: A = EC50 of VP in presence of I8-arachnotocin; A’ = EC50 of VP; B = logarithm of I8-arachnotocin concentration; Schild slope 1.28 ± 0.02 (SEM), R2 = 0.9998. The dotted line represents a reference line with a slope of 1; n = 3.
at this receptor subtype. We furthermore quantified bias on OTR and V1bR following the method outlined by Kenakin27, but did not detect differences compared to OT or VP.

Since recent studies uncovered an overlapping role of β-arrestin-1 and β-arrestin-2 with regard to V2R-dependent agonist-induced endocytosis and ERK activation28, we probed whether I8-arachnotocin is capable of recruiting β-arrestin-1 in a BRET-based assay. In comparison to VP (10 µM), which robustly induced β-arrestin-1 recruitment, I8-arachnotocin (10 µM) also failed to recruit β-arrestin-1 in a time-dependent manner (Fig. 7a). Moreover, VP treatment resulted in a concentration-dependent β-arrestin-1 recruitment with an EC50 of 56 nM (logEC50 = -7.25 ± 0.07), but receptor stimulation with I8-arachnotocin exhibited no β-arrestin-1 recruitment up to 100 µM (Fig. 7b). Overall, data obtained by BRET studies clearly demonstrate that modulation of the V2R with I8-arachnotocin results in neither recruitment of β-arrestin-1 nor β-arrestin-2, thereby confirming bias of this ligand towards G protein-coupling.

I8-arachnotocin modulates V2R-mediated ERK signaling. Having established that I8-arachnotocin is a G protein-biased peptide ligand, we examined its ability to modulate V2R-mediated ERK 1/2 phosphorylation. Previous studies reported that V2R activates ERK by two different pathways hypothesized to be dependent on either Gαs or β-arrestin signaling with distinct temporal patterns; the early phase (≤5 min) being mediated by the G protein-dependent pathway, while the later phase (>10 min) being β-arrestin-dependent29. More recently, a study utilizing a combination of CRISPR/Cas9 genome-editing and pharmaceutical inhibition to generate HEK293 cells with ‘zero functional G’ indicated that ERK signaling mediated by V2R may still be dependent upon G protein even at later time points at which it may also be β-arrestin-dependent30. HEK293 cells transiently transfected to express the human V2R were stimulated with 1 µM I8-arachnotocin and 1 µM vasopressin for time periods between 1 min and 2 h. The immunoblotting data demonstrate that the late phase of V2R-dependent ERK 1/2 activation induced by I8-arachnotocin is significantly different from the late phase of VP-stimulated ERK 1/2 activation (30–120 min; Fig. 7c,d). While both peptides provoked an early and rapid ERK 1/2 phosphorylation peaking at 5 min, I8-arachnotocin-stimulated ERK 1/2 phosphorylation decreased over time in contrast to VP-elicited ERK 1/2 activation that resulted in a more sustained and prolonged ERK 1/2 activation (Fig. 7c,d). These data further support that I8-arachnotocin is a biased peptide ligand that modulates ERK 1/2 activity by preferentially activating the early phase G protein-dependent pathway at the plasma membrane over the late phase pathway that is β-arrestin-dependent.
Peptides are gaining momentum in the drug development field, due to their (i) ability to interact with proteins on a large surface and (ii) structural and chiral complexity, which allows for improved discrimination between highly homologous targets as compared to small molecules. This is particularly the case for the OT/VP signaling system, where multiple small molecule drugs failed due to selectivity issues and the majority of approved therapeutics are peptide drugs. The study of the complex signaling pathways of the widely-distributed and fundamental OT/VP signaling system remains however challenging due to the limited availability of pharmacological probes.

Differentiating between signaling events that occur pre or post β-arrestin recruitment has become an important focus for studies looking at understanding the (patho)physiological roles of β-arrestin-mediated receptor internalization, desensitization and trafficking. In addition, β-arrestin-dependent signaling has been linked to chronic stress-evoked melanoma metastasis via OTR, increased neonatal rat cardiac fibroblast proliferation via V1aR, morphine tolerance via V1bR and sustained non-canonical signaling after receptor internalization via V2R, resulting in strong antidiuretic and anti-natriuretic effects. Biased ligands such as I8-arachnotocin discovered in this work are thus important tools to advance our understanding in these areas of interest.

Table 2. Potency and efficacy (β-arrestin recruitment) of I8-arachnotocin at human receptors. EC50 is given in nM or µM (as indicated) and as pEC50 as logEC50 ± SEM. Controls were VP at V2R, V1aR, V1bR and OT at OTR; n.d., not detectable; β-arrestin.

Discussion
Peptides are gaining momentum in the drug development field, due to their (i) ability to interact with proteins on a large surface and (ii) structural and chiral complexity, which allows for improved discrimination between highly homologous targets as compared to small molecules. This is particularly the case for the OT/VP signaling system, where multiple small molecule drugs failed due to selectivity issues and the majority of approved therapeutics are peptide drugs. The study of the complex signaling pathways of the widely-distributed and fundamental OT/VP signaling system remains however challenging due to the limited availability of pharmacological probes. Differentiating between signaling events that occur pre or post β-arrestin recruitment has become an important focus for studies looking at understanding the (patho)physiological roles of β-arrestin-mediated receptor internalization, desensitization and trafficking. In addition, β-arrestin-dependent signaling has been linked to chronic stress-evoked melanoma metastasis via OTR, increased neonatal rat cardiac fibroblast proliferation via V1aR, morphine tolerance via V1bR and sustained non-canonical signaling after receptor internalization via V2R, resulting in strong antidiuretic and anti-natriuretic effects. Biased ligands such as I8-arachnotocin discovered in this work are thus important tools to advance our understanding in these areas of interest.

By utilizing a drug discovery strategy on the synthesis of evolutionarily-conserved, yet distinct, peptides, we were able to bypass the time- and resource-consuming fractionation, isolation and identification steps
associated with the discovery of plant- or venom-derived compounds such as kalata B723, inotocin19 and cono-
pressin T22. This strategy led us to explore the vast and untapped arthropod kingdom and resulted in the discov-
ery and pharmacological characterization of I8-arachnotocin.

I8-arachnotocin activated the $\alpha_s$ (cAMP) pathway, inducing ERK 1/2 phosphorylation without detecta-
ble recruitment of $\beta$-arrestin-1 or -2 at V2R, despite the capability of this receptor to form stable and strong
interactions with $\beta$-arrestins42. These findings are consistent with the observation that I8-arachnotocin induced
substantially lower levels of ERK 1/2 phosphorylation at later time points compared to VP, leading us to con-
clude that I8-arachnotocin displays a clear bias away from $\beta$-arrestin-dependent signaling at V2R. We are not
aware of another V2R ligand capable of selectively activating the non-$\beta$-arrestin-dependent ($EC_{50}$ = 50 nM)
vs $\beta$-arrestin-dependent ($EC_{50}$ > 100 $\mu$M) signaling pathway (>2,000-fold difference). Such biased ligands are
highly sought-after for research tools that allow for the discrimination between multiple active conformations of
GPCRs32,33. Since the pharmacology of the $\beta$-arrestin-1 and -2 pathways in respect to the OT and VP receptors
is not fully elucidated yet43,44, I8-arachnotocin represents a valuable first probe to advance our understanding of
this pathway at the human V2R.

Our data suggest that I8-arachnotocin can only effectively recruit $\beta$-arrestin-2 at the V1bR ($Emax$ = 65%). To
try to understand the structural differences resulting in bias between the four receptors, we compared the bind-
ing site residues of the V1bR vs. the OTR, V1aR and V2R; there are two positions that differ in these receptors, i.e.
position 7.30 (Thr vs. Glu) and 7.42 (Asn vs. Ser)19. Although there are only a limited number of reports dealing
with structural changes in GPCRs responsible for arrestin recruitment, it has been suggested for instance that
residues in TM6 and TM7 are important for pathway selectivity45,46. Hence, the identified residues in positions

Figure 7. Characterization of $\beta$-arrestin-1 recruitment and ERK 1/2 phosphorylation induced by I8-
arachnotocin vs. VP at the human V2R. (a) Kinetic profile of VP- and I8-arachnotocin-mediated $\beta$-arrestin-1
recruitment in HEK293 cells co-expressing EGFP-V2R and $\beta$-arrestin-1-Nluc. Cells were stimulated by 10 $\mu$M of
VP or I8-arachnotocin, respectively, 5 min after addition of the luciferase substrate (furimazine). The results are
shown as differences in the BRET signals in the presence of ligands and are expressed as the mean value ± SD;
n = 2. (b) Concentration-response curves of VP and I8-arachnotocin at V2R using HEK 293 cells co-expressing
EGFP-tagged V2R and $\beta$-arrestin-1-Nluc. Cells were pretreated with furimazine and measurements were
taken 5 min after addition of ligands. Ligand-induced BRET was calculated as: (emission $\text{EGFP}_{\text{ligand}}$/emission $\text{NLuc}_{\text{ligand}}$) − (emission $\text{EGFP}_{\text{HBSS}}$/emission $\text{NLuc}_{\text{HBSS}}$). Results were normalized to $\beta$-arrestin-1 recruitment in
response to VP. Data points were fitted by nonlinear regression curves (sigmoidal, slope = 1); error bars indicate
SEM; n = 3. (c) Representative Western blot images of ERK 1/2 phosphorylation by stimulation of V2R with
I8-arachnotocin vs. VP and (d) quantification of I8-arachnotocin- and VP-induced phosphorylated ERK 1/2
(pERK) relative to total ERK 1/2 (tERK) from four independent experiments (±SEM). Cells were transiently
transfected with EGFP-V2R encoding plasmid and treated with 1 $\mu$M I8-arachnotocin or 1 $\mu$M VP at 37 °C
for indicated time periods. Immunoblots were prepared from the same membranes using the same exposure
method. Regions of interest were cropped from the full image (see Supplementary Information). Statistical
significance was determined by Student’s t test (*P < 0.05; **P < 0.01; ns non-significant).
7.30 and 7.42 of the ligand binding pocket of OT/VP receptors, could contribute to the observed bias of the bound Bα-arachnotocin ligand, by altering the interaction of the receptor C-tail with the N-terminal domain of arrestin37. However, this remains speculative until future studies reveal further details.

Overall, the GPCRs are prime drug targets45 and the vast chemical diversity of nature will continue to deliver novel pharmacological and therapeutic leads, particularly with technological advances that accelerate the drug discovery pipeline36. This work follows this innovative trend by exploiting the evolutionary conservation and ubiquity of neuropeptides across the animal kingdom95,31. In particular, it highlights the abundance of neuropeptides in arthropods: e.g., there are >50–150 neuropeptides reported in the model species Tribolium castaneum52, Nasonia vitripennis53, Apis mellifera54 and Drosophila melanogaster55. We thus argue that arthropods represent a novel, vast and untapped source for the discovery of pharmacological probes and potential therapeutic leads for a broad range of signaling systems.

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C.W.G. designed research. L.D., J.G., E.M., P.K., H.C.M. and M.M. performed research. L.D., J.G., E.M., P.K., K.D.G.P., M.M. and C.W.G. analyzed data. All authors wrote the paper and approved the final version.

Competing interests
The authors declare no competing interests.

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