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Subtle modifications to oxytocin produce ligands that retain potency and improved selectivity across species

Markus Muttenthaler[1,*], **Åsa Andersson**[1], **Irina Vetter**[1], **Marta Busnelli**[2,3], **Lotten Ragnarsson**[1], **Rohit Menon**[4], **Christian Bergmayr**[5], **Sarah Arrowsmith**[6], **Jennifer R. Deuis**[1], **Han Sheng Chiu**[1], **Nathan J. Palpant**[1], **Margaret O'Brien**[7], **Terry J. Smith**[7], **Susan Wray**[6], **Inga Neumann**[4], **Christian W. Gruber**[5,8], **Richard J. Lewis**[1], and **Paul F. Alewood**[1,*]

[1]Institute for Molecular Bioscience, The University of Queensland, Brisbane, Australia [2]CNR - Institute of Neuroscience, Milan, Italy [3]Department of Biotechnology and Translational Medicine, University of Milan, Milan, Italy [4]Department of Behavioral and Molecular Neurobiology, Regensburg Center of Neuroscience, University of Regensburg, Germany [5]Center for Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria [6]Department of Cellular and Molecular Physiology, Harris-Wellbeing Preterm Birth Centre, Institute of Translational Medicine, University of Liverpool, Liverpool, United Kingdom [7]National Centre for Biomedical Engineering Science, National University of Ireland, Galway, Ireland [8]School of Biomedical Sciences, The University of Queensland, Brisbane, Australia

Abstract

Oxytocin (OT) and arginine-vasopressin (AVP) mediate fundamental functions in humans *via* four G protein-coupled receptors that are also involved in high-profile disorders. Therapeutic development has proven difficult due to the challenge of obtaining ligands that conserve selectivity across rodents and humans for translational studies. A medicinal chemistry strategy aimed at single atom modifications to the pharmacophore framework of OT and AVP yielded a more stable OT receptor (OTR) agonist that overcomes this hurdle: [Se-Se]-OT-OH has similar potency to OT, yet improved selectivity for OTR. This is conserved in mice, making it a powerful probe for distinguishing OTR against the V1a/V1b receptors. This was validated *in vivo*, where centrally infused [Se-Se]-OT-OH potently reversed social fear in mice, confirming that this action is mediated *via* OTR and not AVP receptors. [Se-Se]-OT-OH was furthermore tested in a preclinical labor induction/augmentation model, where it produced a more regular human uterine contraction pattern than OT with no activity at human cardiomyocytes, indicating an improved safety profile and therapeutic window compared to clinically used OT. Taken together, [Se-Se]-OT-OH is a novel and valuable probe to validate OTR as a therapeutic target in a wide range of biological systems and a promising new lead for new therapeutic candidate development. This medicinal chemistry

[*]Correspondence to: Markus Muttenthaler, m.muttenthaler@uq.edu.au, Tel: +61 7 3346 2985; Paul F. Alewood, p.alewood@uq.edu.au, Tel: +61 7 3346 2982.

Author Contributions

M.M. designed and performed the synthesis of the peptide analogues and developed the selenocysteine chemistry. A.A., I.V., M.B., L.R. C.B., R.J.L., C.W.G. performed the pharmacological analysis of the analogues. J.R.D., H.S.C. and N.J.P. performed the cardiomyocyte experiments. S.A., S.W., M.O. and T.J.S. performed the uterine contraction studies. R.M. and I.N. performed the behavioral *in vivo* experiments. M.M. and P.F.A. directed the project and wrote the paper. All authors contributed to the discussion, preparation of figures and interpretation of the results.

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approach which employs selenocysteine building blocks is highly applicable to similar complex peptidergic signaling systems that also struggle with selectivity issues.

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Introduction

Oxytocin (OT) and vasopressin (arginine-vasopressin, AVP) are closely related, multifunctional neurohypophysial neuropeptides. This family of neurohormones is mainly synthesized in the magnocellular and parvocellular neurons of the hypothalamus, stored in the neurohypophysis and released into systemic circulation after enzymatic cleavage in response to relevant physiological stimuli (1, 2). In the periphery, OT is involved in uterine smooth muscle contraction during parturition, milk ejection during lactation, ejaculation, and pain (3–5), while centrally released OT functions as a neurotransmitter or neuromodulator that promotes multiple behaviors (6–8), maternal care (9, 10), partnership bonding (8, 11, 12), social interactions (12), and stress and anxiety responses (13–15) mostly studied in rodents. A broad variety of behavioral effects were also described in humans through intranasal administration of OT, which led recently to a spike in clinical trials that study intranasal OT as a potential treatment option for psychopathologies characterized by social or emotional dysfunctions (14, 16–21). In contrast, AVP regulates fluid balance and blood pressure in the periphery (22–24); centrally released AVP is implicated in learning and memory (25–27), various social behaviors including pair bonding and aggression in rodents (6, 8, 28, 29), and in stress-related and anxiety disorders (28–30). The ubiquitous presence and involvement of the OT/AVP signaling system in so many diverse and fundamental physiological functions reflects its ancient character dating back at least 600 million years (8, 31). The OT/AVP receptors are members of the G protein-coupled receptor (GPCR) family (5, 32) and form attractive targets for a diverse range of high-profile disorders including cancer, pain, autism, schizophrenia, anxiety- and stress-related disorders, and reproductive and cardiovascular disorders (5, 8, 16, 33).

In humans and rodents, OT acts *via* one oxytocin receptor (OTR) and AVP *via* three vasopressin receptors (AVPRs – vasopressor V1aR, pituitary V1bR, renal/antidiuretic V2R). OT and AVP are structurally very similar nonapeptides differing only by two amino acids in position 3 and 8 (Figure 1A). Two cysteine residues in position 1 and 6 form the cyclic part of the molecules followed by a 3-residue amidated C-terminal tail. Their chemical similarity together with the high sequence homology of the extracellular binding domains of the OTR and AVPRs (~80%) leads to significant cross talk, with OT able to activate the AVPRs and AVP the OTR (34, 35). Specific receptor functionality is thus not controlled by ligand selectivity, but by a complex system of cell-specific up and down regulation of individual receptor expression, controlled release, receptor oligomerization, rapid clearance and enzymatic degradation (36). High OT/AVP receptor homology and overlapping distribution constitute a major hurdle in the development of selective receptor agonists, antagonists and therapeutic candidates (37, 38). The identification of drug leads is further complicated by significant species differences, where rodent and human selectivity typically do not overlap (Table 1) thus restricting translation into the clinic (37–39). For example, OT/AVP analogues used clinically that include desmopressin (40, 41), carbetocin (42) and atosiban (37, 43, 44) were shown to be receptor-subtype selective in rats though not in humans (Table 1); and it is

mainly due to rapid biodegradation, renal clearance and a limited administration window that they can be used clinically without severe side effects (40, 44). Despite these limitations, OT remains the ligand of 'choice' in the clinic to induce and progress labor (45). The lack of a complete set of selective receptor agonists/antagonists (Table 2) further limits our ability to characterize the physiological responses for each subtype receptor and their relevance in disease. Hence we commenced a program to overcome these limitations and yield better and more selective ligands for this fundamental signaling system (31, 46–50). In this study, we demonstrate that small modifications to the structural framework of the pharmacophore of the endogenous and pharmacologically unselective neuropeptides can be used to tune selectivity and generate analogues with an improved selectivity profile that is conserved across species (mouse and human), thereby facilitating future translational studies.

Results

Sixteen OT and AVP analogues with framework modifications including N-terminal deamination, C-terminal amide to acid change, deletion of residues 8 and 9, and disulfide bond replacement with the structurally isosteric yet slightly more hydrophobic and longer (0.3 Å, (51, 52)) diselenide bond were synthesized (Figure 1A, SI Table S1). The analogues were assessed for activity and selectivity at the human (h) OT, V1a, V1b and V2 receptors. Analysis of their structure-activity relationships (SAR) revealed that these modifications can lead to selectivity gains and revealed a valuable new ligand with significantly improved selectivity for OTR ([Se-Se]-OT-OH, **6**, >1400-fold to hV1aR, 500-fold to hV1bR, 15-fold to hV2R). This compound along with eight others (compounds **1, 2, 3, 4, 5, 7, 11, 12**) was chosen for a comprehensive pharmacological characterization that included radioligand displacement assays, Schild regression analysis, functional signaling assays, and human myometrial contraction assays to reveal the structural changes underlying the observed improvement in selectivity. [Se-Se]-OT-OH (**6**) plus d[Se-Se]-OT-OH **(7**) were then evaluated across all four murine (m) receptors to confirm whether activity and selectivity were conserved across species. Finally, the most selective compound, [Se-Se]-OT-OH (**6**), was assessed to modulate OTR action centrally in a mouse model of social fear, peripherally to induce and augment labor *via* human uterine contraction assays, and its stability was determined in human serum. [Se-Se]-OT-OH (**6**) was furthermore tested along with OT (**1**) and AVP (**11**) on human heart muscle cells to assess its cardiovascular safety profile.

Rationale for single atom modifications

Based on structural data of OT (beta-turn stabilized by disulfide) (4, 47, 53–55) and earlier studies (37, 49, 56, 57), it is known that OT/AVP binding and receptor activation are sensitive even to minor modifications (e.g. ring reduction by one sulfur atom or disulfide bond replacement by a dicarba bridge causes complete loss of activity (56); Gly9 to Val9 exchange causes agonist to antagonist switch (49); see further details in Supplementary Note 1 in SI). Additionally, traditional medicinal chemistry strategies that modified residues within the loop pharmacophore resulted in selectivity improvements that were not retained across species (Table 1) (37, 38). Based on these observations, we selected a range of single atom modifications of the pharmacophore framework (i.e. disulfide bridge and N/C-terminus

modifications summarized in Figure 1A and SI Table S1) to test the hypothesis that subtle framework modifications can yield selectivity gains that are retained across species.

Peptide synthesis

Two building blocks [Boc-L-Sec(Meb)-OH and dSec(Meb)-OH] were synthesized to access the desired sulfur/selenium modifications (Figure 1A & B, SI Table S1). To acquire Boc-L-Sec(Meb)-OH in desirable quantities, the protocols of Chocat *et al.* (58), Tanaka *et al.* (59) and Oikawa *et al.* (60) were optimized with the new synthetic chemistry able to provide 28.7 g of pure Boc-L-Sec(Meb)-OH in only three steps (Figure 1B, 62% overall yield, four days). A new synthetic strategy was also devised for dSec(Meb)-OH yielding the compound in a two-step synthesis with an overall yield of 12% (Figure 1B). With these building blocks in hand, all of the OT/AVP analogues **1-16** (Figure 1A, SI Table S1) were synthesized *via* Boc-SPPS using the HBTU-mediated *in situ* neutralization chemistry (51, 61), folded in 0.1M NH_4HCO_3 and purified by RP-HPLC.

Functional response of novel OT/AVP ligands

Agonist activity at the human OT, V1a and V1b receptors was determined using a Fluorescent Imaging Plate Reader (FLIPR) Ca^{2+} mobilization assay, and activity at hV2R was determined by measuring the production of second messenger cAMP upon receptor stimulation (Figure 1C & D, SI Table S2).

OT was able to activate all four receptors, yet with EC_{50} 10-300 fold higher at the hAVPRs (hOTR<hV2R<hV1bR<hV1aR) than at hOTR (0.52 nM). Deletion of the N-terminal amino group (dOT, **2**) improved activation at all of the AVPRs. Replacement of the disulfide bond with the isosteric, yet slightly more hydrophobic diselenide bond, ([Se-Se]-OT (**3**), d[Se-Se]-OT (**4**)) was well tolerated and did not lead to significant changes in EC_{50}. Exchange of the C-terminal amide to acid in OT (OT-OH, **5**) resulted in weaker potency at all receptors and truncations of the C-terminus of OT (**8**, **9**, **10**) yielded higher EC_{50} at all receptors with complete loss of activity at hV2R (up to 10 μM).

AVP also activated all four receptors including hOTR (hV2R>hV1bR>hV1aR>hOTR). Deamination of the N-terminus (dAVP, **12**) had little impact on receptor activation with EC_{50} values similar to AVP. The disulfide to diselenide exchange ([Se-Se]-AVP (**13)** and d[Se-Se]-AVP (**14**)) was again well tolerated and did not change the potency/selectivity profile significantly. Truncations of the C-terminus of AVP (**15**, **16**) led to significant loss in potency over all four receptors (Figure 1D, SI Table S2).

Interestingly, the combination of diselenide replacement and C-terminal amide to acid change yielded a partial (E_{max}=52%) agonist ([Se-Se]-OT-OH, **6**) with low nanomolar potency at hOTR (7.3 nM), no activation of hV1aR (>10 μM), a 600-fold higher EC_{50} at hV1bR (4319 nM) and a 15-fold higher EC_{50} at hV2R (109 nM). [Se-Se]-OT-OH (**6**) was found to be a weak antagonist at hV1aR (17% inhibition), with an IC_{50} of 132 nM (SI Figure S1), which was confirmed in a radioligand displacement assay (Figure 2D). Based on earlier SAR studies (37, 57, 62) it is known that N-terminal deamination of OT analogues often leads to improved OTR activation; hence we synthesized and assessed whether d[Se-

Se]-OT-OH (**7**) potency and selectivity for hOTR was improved. d[Se-Se]-OT-OH (**7**) (again a partial agonist at hOTR with E_{max}=59%), however, did not improve potency at OTR, yet improved potency at all three AVPRs thus yielding a less interesting selectivity profile than [Se-Se]-OT-OH (**6**) (Figure 1C, SI Table S2).

OT analogues **1-7**, AVP and dAVP (**11,12**) were further tested using the homogeneous time-resolved fluorescence (HTRF) inositol-1-phosphate (IP_1) functional assay to allow comparison with the downstream intracellular calcium changes measured using the FLIPR (Figure 1E, SI Table S3). The HTRF-IP_1 assay correlated well with the FLIPR assay (SI Table S5 and Supplementary Note 2 in SI) and the potency and selectivity trends, particularly with lead compound [Se-Se]-OT-OH (**6**) (Figure 2C), were confirmed. [Se-Se]-OT-OH (**6**) displayed again partial agonism (E_{max}=60%) at hOTR with an EC_{50} of 38 nM (2.6-fold less potent than OT) and no activity at hV1aR and hV1bR at concentrations up to 10 μM.

Radioligand displacement assay at human OT, V1a, V1b, and V2 receptors

Binding data for compounds **1-7** and **11**, **12** were obtained in radioligand displacement assays measuring the competition with ^{3}H-OT at hOTR,^{125}I-V1a antagonist at hV1aR, and ^{3}H-AVP at the human V1b and V2 receptors in cell membrane preparations of heterologously expressed receptor (SI Table S4). The experimental inhibition constants (K_i) correlated well with the functional data (SI Table S2 & S3). [Se-Se]-OT-OH (**6**) displaced ^{125}I-V1a antagonist at hV1aR with a K_i of 57 nM, confirming the weak antagonistic effects observed in the FLIPR assay (Figure 2D and SI Table S4).

Schild regression analysis for binding mode

To confirm competitive binding of [Se-Se]-OT-OH (**6**) at hOTR, we performed a series of Schild regression experiments with OT (**1**) and [Se-Se]-OT-OH (**6**) against the well-characterized hOTR antagonist atosiban (AT). Results of these experiments revealed that all three compounds were competitive agonists at hOTR (SI Figure S2).

[Se-Se]-OT-OH - a stable and selective OTR agonist for translational studies

Considering that [Se-Se]-OT-OH (**6**) is one of the most selective OTR agonists to date and represents an important molecular probe to study OTR, it was important to **(i)** prove the hypothesis that subtle pharmacophore framework modifications can yield selectivity gains that are conserved across murine and human receptors, **(ii)** characterize its activity in a physiological and clinical relevant setting, **(iii)** determine its metabolic stability compared to OT (which is used in the clinic) and **(iv)** assess its cardiovascular side effect profile. We therefore carried out the pharmacological characterization of **6** across the four murine receptors **(i)**, analyzed its role in central modulation of social anxiety/fear and tested it in one of the most important clinical applications of OT, namely labor induction/augmentation **(ii)**, determined its stability in human serum **(iii)**, and characterized its ability to activate cardiomyocytes that are linked to known cardiovascular side effects **(iv)**.

Binding and functional analysis at murine OT, V1a, V1b and V2 receptors (i)

To determine if the pharmacological profile observed in human receptors is retained in mice, we tested [Se-Se]-OT-OH (**6**) and d[Se-Se]-OT-OH (**7**) in functional (SI Table S3, SI Figure S3) and binding assays (SI Table S4, SI Figure S4) across the four murine receptor subtypes.

[Se-Se]-OT-OH (**6**) retained its functional selectivity for OTR in mice and was inactive at mV1aR and mV1bR up to concentrations of 10 μM (SI Table S3, SI Figure S3). It was able to activate mOTR with an EC_{50}=11.9 nM (E_{max}=43%), which is only slightly less potent in respect to OT (63). [Se-Se]-OT-OH (**6**) fully activated mV2R similarly to the human receptors – with an EC_{50} (183 nM) that is ~1000-fold larger compared to AVP (64) and 60-fold compared to OT (64). [Se-Se]-OT-OH (**6**) bound to the mOTR with a K_i=32.6 nM and we observed bi-phasic binding curves for mV2R (K_{i1}=0.71 nM and K_{i2}=1855 nM) and mV1aR (antagonist; K_{i1}=16.3 nM, K_{i2} >10 μM). [Se-Se]-OT-OH (**6**) was characterized by very low affinity binding for mV1bR (K_i >10 μM), which correlates well with the absence of activation (SI Table S4, SI Figure S3).

d[Se-Se]-OT-OH (**7**) also retained its selectivity profile in mice, again being less selective than [Se-Se]-OT-OH (**6**). Binding and functional activation was observed at all four murine receptors subtypes, including V1b, which displayed no binding/activity with [Se-Se]-OT-OH (**6**) (SI Table S3 & S4). d[Se-Se]-OT-OH (**7**) is a partial agonist at mOTR (EC_{50}=5.9 nM; E_{max}=38%) and at the mV1bR (EC_{50}=438 nM; E_{max}=68%), and a full agonist at mV1aR (EC_{50}=438 nM) and at mV2R (EC_{50}=41.6 nM). The binding affinity for mOTR was in the nanomolar range (K_i=5.9 nM), similar to OT (K_i=0.83 nM, (63)), and binding affinities for mV1aR and mV1bR were less potent, with K_i=293 nM and K_i=438 nM respectively. The binding of d[Se-Se]-OT-OH (**7**) to mV2R was characterized by a bi-phasic binding curve with K_{i1} of 0.71 nM and K_{i2} of 1855 nM.

Human serum stability assay (ii)

A human serum stability study was carried out on [Se-Se]-OT-OH (**6**), revealing that it was more stable than OT with a half-life of 25 h in human serum compared to 12 h of OT (Figure 3B).

Behavioral efficacy in a mouse model of social fear (iii)

[Se-Se]-OT-OH (**6**) was tested in the well-established social fear conditioning (SFC) paradigm (65), characterized by reduced social exploration of conspecifics 24 h after SFC. [Se-Se]-OT-OH (**6**) reduced social fear, as reflected by a facilitated extinction curve, 10 min after its intracerebroventricular (icv; 250 μM/2 μl/mouse) infusion compared with vehicle-treated social fear conditioned (SFC^+) mice. This effect was similar to that of synthetic OT, which also potently reversed social fear, when infused icv (250 μM/2 μl/mouse). All control mice, i.e. unconditioned (SFC^-) mice treated with either vehicle, [Se-Se]-OT-OH (**6**) or synthetic OT showed a similar level of social preference behavior as reflected by a high level of exploration of conspecifics. All SFC^+ and SFC^-groups, independent of subsequent treatment, also showed similar investigation of a non-social stimulus (small empty cage) indicating similar non-social anxiety (Figure 4).

Human myometrial contractility assays reflecting induction and augmentation of labor (iii)

To determine if the partial agonism observed with [Se-Se]-OT-OH (**6**) and d[Se-Se]-OT-OH OT (**7**) in the pharmacological studies would affect its physiological activity compared to the full agonist OT (**1**), we tested **1, 6** and **7**'s ability to induce contraction *via* activation of hOTR in myometrial human telomerase reverse transcriptase (hTERT-HM) cells (66) in a collagen gel contractility assay (67, 68). Both [Se-Se]-OT-OH (**6**) and d[Se-Se]-OT-OH (**7**) induced a contractile response comparable to OT (SI Figure S5). **6** and **7** increased contractility by 7.4% ($P < 0.01$) and 9% ($P < 0.001$) respectively, compared to 9% ($P < 0.001$) for OT. This was then confirmed in tissue bath studies using strips of pregnant human myometrium, where [Se-Se]-OT-OH (**6**) increased contraction amplitude in a dose-dependent manner that was comparable to OT (**1**). EC_{50} for [Se-Se]-OT-OH and OT were 5.89 ± 3.08 nM (N=5) and 1.14 ± 0.56 nM (N=8) respectively (P > 0.05, P=0.3718, F-test; Figure 3C & D). The contraction profile with [Se-Se]-OT-OH, however, was markedly different to OT, as it maintained a more phasic and frequent contractile activity pattern compared to a more tonic-like activity under OT at the respective concentrations (Figure 3C & D).

Human cardiomyocyte assay to assess cardiovascular safety profile (iv)

We tested the functional effects of OT (**1**), AVP (**11**) and [Se-Se]-OT-OH (**6**) on maximal intracellular Ca^{2+} levels in human cardiomyocytes (Figure 5) to determine if the improved selectivity of [Se-Se]-OT-OH (**6**) for hOTR over hV1aR would have reduced cardiovascular liability (which is regulated *via* hV1aR (69–71)) compared to OT. Both AVP and OT concentration-dependently caused an increase in maximal intracellular Ca^{2+} in human cardiomyocytes with EC_{50} values of 121 nM and 174 nM, respectively. OT only had a partial effect (53 ± 11 %) compared to AVP, which correlates well with our pharmacological results of hV1aR activation by OT (SI Table S2 & S3). Consistent with its improved selectivity, [Se-Se]-OT-OH had no effect on the maximal intracellular Ca^{2+} level in cardiomyocytes up to 10 μM.

Discussion

More than 60 years have passed since the discovery, synthesis and characterization of OT and AVP, yet there still remains a shortage of agonists and antagonists with good selectivity for the four known receptor subtypes (Table 2) (37, 38, 72). Physiological function and dysfunction of the individual receptor subtypes are therefore still studied by time- and cost-intensive knockout models, which have linked the subtypes to a range of high-profile disorders including autism, schizophrenia, epilepsy, stress, aggression, depression, anxiety and pain (5, 8, 16, 33). Therapeutic development targeting these disorders has so far failed due to the challenge of developing subtype-selective ligands that maintain their selectivity across species thereby enabling translational studies with clinically relevant animal models (Table 1) (37, 38, 73). While the chiral complexity and large surface area of peptides provide significant advantages in the development of selective ligands (particularly agonists), the traditional medicinal chemistry approach of modifying residues within the loop pharmacophore of OT/AVP struggled to overcome interspecies receptor differences, producing mainly ligands with non-correlating selectivity profiles between species (Table 1).

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We therefore explored a new medicinal chemistry strategy to address this issue in this current study.

Subtle pharmacophore framework modifications - a new strategy to retain selectivity across murine and human receptors

This SAR study (see Supplementary Note 1 in SI for a more in depth discussion of the SAR study) demonstrated that single atom modifications to the pharmacophore framework (C/N-terminus, disulfide bond) of the endogenous ligands OT/AVP can tune selectivity and yield ligands ([Se-Se]-OT-OH (**6**) and d[Se-Se]-OT-OH (**7**)) with an improved selectivity profile that is conserved across species (mouse and human, SI Table S3 and Figure 3A). In particular, the replacement of the disulfide bond with the slightly more hydrophobic and longer diselenide bond (51, 52) in combination with a C-terminal amide to acid change yielded the most pronounced selectivity gains for OTR ([Se-Se]-OT-OH (**6**), Figure 3A). This selectivity gain renders [Se-Se]-OT-OH (**6**) an excellent probe to delineate OTR function in the CNS in behavioral animal models (where V2R is not expressed) and a promising lead molecule for the clinic, which we further explored herein. Considering all pharmacological and physiological data from this study we recommend an effective dose of 50 nM or, for comparison studies with OT, a 10-fold higher concentration than OT to be used for future functional studies to ensure OTR-mediated action while retaining OTR preference.

General conclusions and guidelines for future OT/AVP design

(i) N-terminal deamination of OT ligands improves binding and potency at all four receptors, renders OT slightly more hydrophobic and improves its stability against proteases (62). N-terminal deamination of AVP shows little impact on potency and selectivity, yet we recommend deamination nevertheless since it offers enhanced stability against proteases. **(ii)** Recent development in the area of disulfide mimetics recommends the use of the superior seleno-ether bond instead of the disulfide or diselenide bond, mainly due to its enhanced metabolic properties against reduction (4). **(iii)** The underexplored C-terminus of OT and AVP has already yielded two interesting studies (conopressin agonist to antagonist switch (49) and this study) which form a good base for future antagonist designs: we envision more SAR studies around the C-terminus towards the development of a new class of OT/AVP antagonists that might be able to overcome species differences by harvesting antagonist design using the C-terminus in contrast to current antagonists that obtain antagonistic effects through sterically hindered modifications at the N-terminus. **(iv)** In terms of pharmacological characterization, the FLIPR is a valuable instrument for the primary screen at the OT/AVP receptors since it rapidly provides agonist/antagonist information with high-throughput capabilities. Follow-up characterization of selected leads by radioligand displacement, HTRF and/or Bioluminescence Resonance Energy Transfer (BRET) assays can then be used to better understand ligand kinetics and secondary messenger signaling pathways. The SAR study presented here is to our knowledge the first assessing affinity and functional data (FLIPR and IP_1) of the control compounds OT/dOT and AVP/dAVP over all four receptor subtypes in a single study, hence forming an important reference for future OT/AVP ligand development.

[Se-Se]-OT-OH modulating central actions (behavior) - reversal of social fear

OT has been linked to multiple high-profile disorders that are modulated centrally such as autism, bipolar disorders, schizophrenia, pain, anxiety and depression (5, 8, 16, 33). Recent studies showing that these central actions can be elicited *via* intranasal OT administration in humans spiked an enormous interest in this research area with numerous of clinical trials currently on-going that study the therapeutic effects of intranasal OT in these disorders. Considering that OT also activates V1aR and V1bR (SI Table S2-4), especially at high doses such as those used for intranasal application in humans, and that OTR can form homo and hetero dimers (50, 74–76), the exact target pharmacology and underlying mechanism of actions are still unclear and under debate. The receptor-specificity underlying these effects is of substantial interest given the partly opposite behavioral effects of OT and AVP especially in the context of anxiety and stress regulation (14). [Se-Se]-OT-OH (**6**) enables clear and simple distinction between the involvement of OTR versus V1aR and V1bR in the CNS without the use of time and cost intensive knock-out models or co-application of species selective V1a/V1bR antagonists. This is crucial for therapeutic target validation and important for future drug development efforts. We chose to behaviorally test our probe in the recently established mouse model of social fear conditioning, which has clinical implication to treat social anxiety disorders (15, 65, 77). SFC is a paradigm based on operant fear conditioning principles and generates robust social fear in mice without any confounding behavioral alterations, and icv infusion of OT has been shown to completely abolish social fear in mice (77). The use of [Se-Se]-OT-OH (**6**) in this study confirmed that this action is mediated *via* OTR with no influence of V1aR nor V1bR (Figure 4).

[Se-Se]-OT-OH modulating peripheral action - labor induction and augmentation

Selectivity against V1aR to reduce cardiovascular side effects is of particular importance in the periphery since it is the primary receptor subtype expressed in the vascular smooth muscle and heart (69–71, 78, 79). Clinically, OT is administered intravenously to induce labor, augment weak contractions in labor and treat postpartum hemorrhage, and intranasally to elicit lactation. Due to its activation of AVPRs, OT administration has only a limited therapeutic window of use and adverse effects reported in the mother include anaphylactic reaction, postpartum hemorrhage, cardiac arrhythmia, fatal afibrinogenemia, pelvic hematoma, subarachnoid hemorrhage, hypertensive episodes, rupture of the uterus, convulsions, coma and death due to water intoxication. In the neonate, adverse effects reported include bradycardia, cardiac arrhythmia, jaundice, retinal hemorrhage, permanent CNS or brain damage, seizures and death (source RxList and (45, 80)). Hence, there is a clear unmet need for better and more selective OTR agonists that are able to differentiate against the AVPRs to diminish side effects.

[Se-Se]-OT-OH (**6**) may be such a candidate as experiments showed no activity on human cardiomyocytes (in contrast to OT, which displayed nanomolar activity), where V1aR is highly expressed regulating many of the above-mentioned side effects (Figure 5). Furthermore, [Se-Se]-OT-OH (**6**) was able to induce augmentation of contractility in human myometrial preparations comparable to OT, though with a more regular contraction pattern (Figure 3C & D and SI Figure S4). By contrast, OT reduced contraction frequency in a dose dependent fashion but increased the contraction duration resulting in a much greater area

under the tension curve (a measure of the total contractile activity/work done) (Figure 3C & D); this can lead to uterine hyperstimulation and rupture, which are a life-threatening events for mother and fetus (81, 82). [Se-Se]-OT-OH (**6**) inactivity at V1aR, which is alongside with OTR also highly expressed in the myometrium during pregnancy and involved in contraction (83, 84), in combination with its partial agonism at OTR results in a more controlled and safer alternative to induce and progress labor in humans. Thus, [Se-Se]-OT-OH (**6**) not only reduces AVPR-related side effects but also offers additional advantages to aid labor induction and progression *via* a more regular increase of contraction strength without the risk of uterine hyperstimulation or rupture. This improved side effect profile is expected to lower associated risks for mother and fetus significantly and expand the therapeutic window of use, thereby lowering the incidence of cesarean sections and its associated costs.

Human serum stability of [Se-Se]-OT-OH

Enzymatic stability is a key characteristic for many peptide drug candidates, hence it was important to characterize the stability of [Se-Se]-OT-OH (**6**) compared to OT (**1**). Diselenide bonds are known to exhibit a lower redox potential than disulfide bonds, rendering such mimetics more difficult to reduce, a feature that can be exploited in improving the half-life of peptide drug candidates (4, 51, 52, 56, 85). C-terminal amides on the other hand are often used in nature to produce slightly more stable compounds compared to peptides that have C-terminal acids. The human serum stability study showed that [Se-Se]-OT-OH (**6**) displayed an improved half-life of 25 h compared to the half-life of 12 h of OT (Figure 3B), suggesting that the impact of the more redox stable diselenide bond outweighs the disadvantage of the C-terminal acid modification. While this increase in stability might only have limited effects for peripheral applications due to rapid renal clearance (minutes), it could be significant for potential therapeutic CNS applications through intranasal delivery (e.g. treatment of autism in children or migraines in adults) (21).

Conclusion

We were able to show that single atom modifications to the pharmacophoric framework of OT can lead to enhanced selectivity profiles that are conserved across species (mouse and human), thereby overcoming a significant hurdle in ligand development. This is an important proof-of-concept study towards a better molecular understanding of ligand pharmacology and development, not only for the four OT/AVP receptor subtypes, but also for similar complex peptidergic signaling systems. The lead ligand derived from this study, [Se-Se]-OT-OH (**6**), is a new valuable probe that can effectively delineate the physiological responses of OTR and is particularly suited for use in animal behavior studies looking at the central roles of OTR. Its functional selectivity and close structural similarity to OT are important features that will help to clarify the contributions of the OT/OTR signaling system in health and disease. The improved selectivity, stability and safety profile of [Se-Se]-OT-OH could lead to superior alternatives to OT in the clinic for peripheral indications such as labor induction/progression and postpartum hemorrhage, as well as for central indications such as autism, migraine, schizophrenia, anxiety and stress, where recent clinical trials have shown that intranasal administered OT is safe and therapeutically effective (20, 21, 86–89). Taken

together, [Se-Se]-OT-OH (**6**) has great potential to advance our understanding of OTR as a therapeutic target in different animal models and represents a promising lead molecule for a variety of high-profile OTR-related indications.

Materials and Methods

Detailed materials and methods are provided in the Supplementary Information (SI).

Selenocysteine building block synthesis

An optimized protocol with yield-improving modifications was developed based on Chocat *et al.* (58), Tanaka *et al.* (59) and Oikawa *et al.* (60) enabling the synthesis of the Boc-L-Sec(Meb)-OH at a scale of 20 g with an overall yield of 62%. A new two-step strategy was devised for the synthesis of dSec(Meb)-OH with an overall yield of 12% (Figure 1).

Peptide synthesis

Peptides **1-16** were assembled manually *via* Boc-SPPS using the HBTU-mediated *in situ* neutralization protocol (51, 61). The analogues were cleaved by HF (90), purified by RP-HPLC, folded over 24 h in 0.1M NH_4HCO_3 buffer at pH 8.2 (1 mg/10 mL, ~100 μM) and purified by RP-HPLC to >95% purity.

FLIPR assay for the human OT, V1a and V1b receptors

Ca^{2+} responses were assessed in COS-1 cells transfected with OTR, V1a or V1b using a $FLIPR^{TetraPlus}$ fluorescent plate reader (Molecular Devices, Sunnyvale, CA) as previously described (4). In brief, cells were loaded with Calcium-4 No Wash dye (Molecular Devices) for 30 min and responses to addition of test compounds were measured using a cooled CCD camera with excitation at 470–495 nM and emission at 515–575 nM. Data was analyzed using ScreenWorks 3.2 (Molecular Devices) and plotted using GraphPad Prism 6.

cAMP assay for hV2R

Cloning of hV_2R, transfection and propagation of HEK293 cells, and assay to determine formation of ^{3}H-cAMP was performed according to published protocols (47, 91, 92).

HTRF-IP_1 assay for the human receptors

COS-1 cells were cultured and transiently transfected with plasmid DNA encoding the human OTR, V1aR or V1bR following the manufacturer's protocol (Lipofectamine 2000, Invitrogen or FuGENE HD, and Roche Applied Science) (56). Assays measuring IP_1 accumulation were performed 48 h post-transfection following the manufacturer's instructions (IP_1-HTRF assay kit, Cisbio International).

Radioligand displacement assay for the human receptors

Receptor affinity assays were performed using FlashBLUE GPCR scintillation beads (PerkinElmer) (56). Reactions containing increasing concentrations of competing OT/AVP analogues (10 pM to 10 μM), FlashBLUE GPCR beads (100 μg for hOTR and hV1aR/200 μg for hV1bR and hV2R), human OT/V1a/V1b/V2 receptor membrane preparation (5 μg

protein) and radioligand (^{3}H-OT (2 nM)/^{3}H-AVP (0.5 nM)/^{3}H-AVP (3 nM)) in assay buffer (50 mM Tris-HCl, 10 mM $MgCl_2$ (5 mM $MgCl_2$ for OT), 0.1% BSA, pH 7.4) were established in 96-well white polystyrene plates with clear flat bottoms in a total reaction volume of 80 μL.

Radioligand displacement assay for the murine receptors

Competitive binding assays were performed using ^{3}H-OT and ^{3}H-AVP (Perkin Elmer) and increasing concentration of the indicated peptides at 30°C on membranes prepared from HEK293 cells transfected with OTR, V1aR, V1bR and V2R murine receptors as in Busnelli *et al.* (63).

HTRF-IP_1 assay and cAMP determination for the murine receptors

IP_1 and cAMP accumulation were determined in HEK293 cells transiently transfected with DNA plasmids encoding the murine OT/V1a/V1b or V2 murine receptors using HTRF assays (IP_1 and cAMP assays, CisBio International, Bagnols-sur-Cèze, France) as previously described (63).

Stability assays

Peptides were added to human serum and aliquots were taken and analyzed by RP-HPLC and LCMS at 1, 2, 3, 4, 12, 24 and 48 h time points (56).

Social fear conditioning (SFC) mouse model

SFC was performed as previously described on adult mice (65). Briefly, on day 1 (social fear acquisition), the SFC^- mice were allowed to investigate a social stimulus (male conspecific in a small cage) in the conditioning chamber for 3 min without receiving any foot shocks, whereas SFC^+ mice were given a 1-s electric foot shock (0.7 mA) each time they investigated (sniffed) the social stimulus. On day 2 (social fear extinction), SFC^+ and SFC^- mice were first exposed to 3 non-social stimuli (empty small cages) to assess non-social investigation as a parameter of non-social fear and anxiety-related behavior. Mice were then exposed to 6 different conspecific males (social stimulus) in 3 min intervals, each in a different small cage to assess social investigation as a parameter of social fear. Mice received icv infusions of either vehicle (2 μl; sterile Ringer solution), OT (250 μM/2 μl; 500 pmol) or [Se-Se]-OT-OH (250 μM/2 μl; 500 pmol) 10 min prior to social fear extinction on day 2 as previously described (65, 77). All infusions were performed 10 min prior to social fear extinction on day 2 of the SFC paradigm. The dose and timing of OT was selected based on previous studies (77).

Human myometrial cell contractility assay

Human uterine myometrial smooth muscle cells (hTERT-HM) were cultured, collagen-gels were prepared, and the assays were performed as described previously (47, 68).

Tissue bath myometrial contractility assays

Strips of myometrium were dissected from biopsies obtained from the lower uterine incision site during Cesarean section (93). All women gave written informed consent to participate.

The study was approved by the North West (Liverpool East) Research Ethics committee (Ref: 10/H1002/49) and by the Research and Development director at Liverpool Women's Hospital NHS Foundation Trust, Liverpool, UK. [Se-Se]-OT-OH (**6**) or OT (**1**) was added to the organ baths and amplitude of contraction was measured and compared to control activity occurring immediately prior to application of the first concentration (100%). Data were analyzed using Origin Pro 9.0 and Graphpad Prism 5 ($N \geq 5$) as described previously (47, 94, 95).

Human cardiomyocyte assay

Changes in intracellular Ca^{2+} levels were assessed in cardiomyocytes differentiated from WT WTC11 human induced pluripotent stem cells (hiPSCs) using a FLIPR^{Tetra} fluorescent plate reader (Molecular Devices, Sunnyvale, CA, USA). In brief, cells were loaded with Calcium-4 No Wash dye (Molecular Devices) according to the manufacturer's instructions and incubated at 37 °C for 30 min. Responses to addition of test compounds were measured using a cooled CCD camera with excitation at 470–495 nM and emission at 515–575 nM. Data was analyzed using ScreenWorks 3.2 (Molecular Devices) and plotted using GraphPad Prism 6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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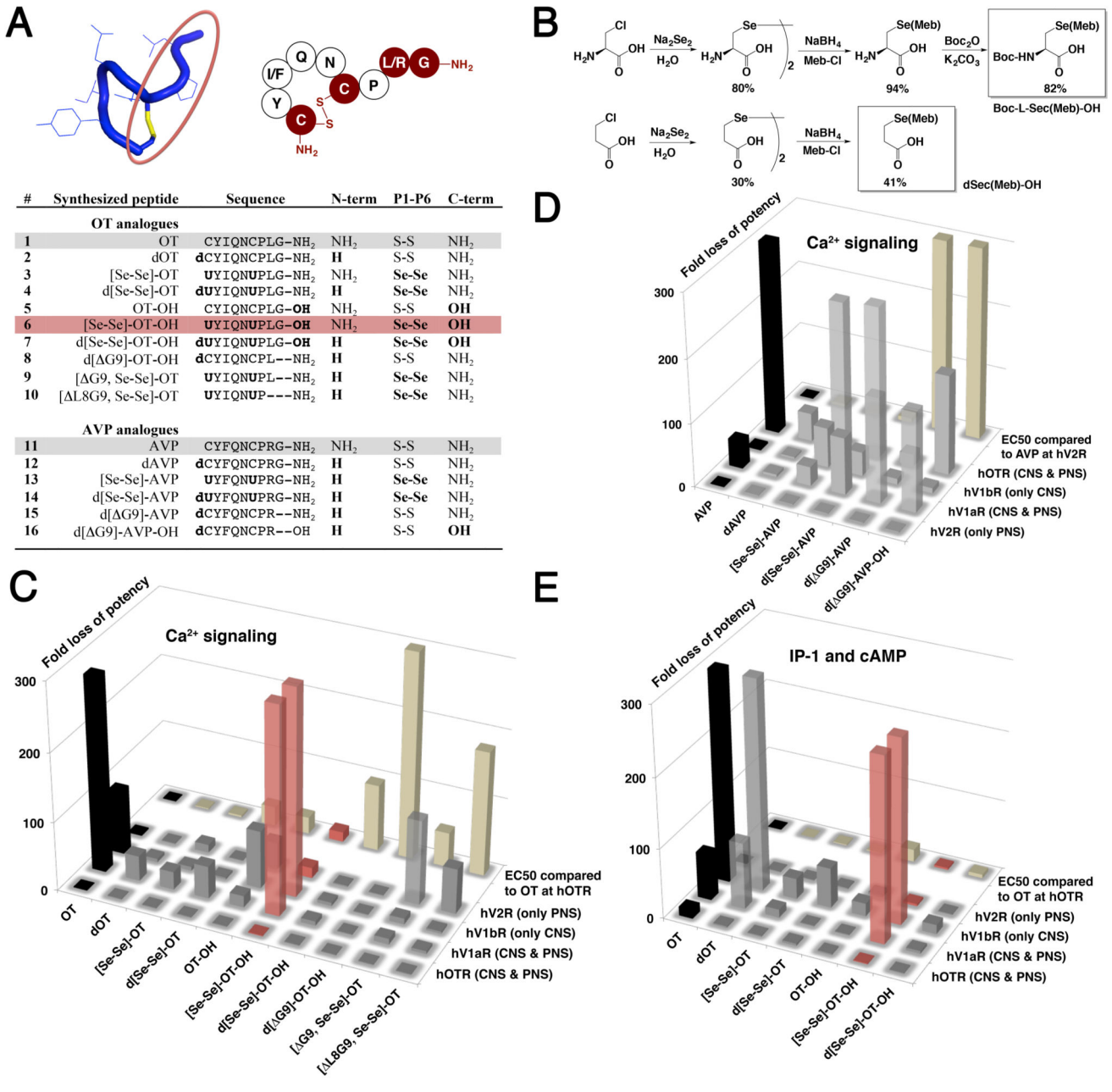
One Sentence Summary

Novel medicinal chemistry strategy yields potent oxytocin receptor agonist that can clearly differentiate between the oxytocin receptor and the closely-related vasopressin receptors in mice and humans.



#	Synthesized peptide	Sequence	N-term	P1-P6	C-term
	OT analogues				
1	OT	CYIQNCPLG-NH_2	NH_2	S-S	NH_2
2	dOT	**d**CYIQNCPLG-NH_2	**H**	S-S	NH_2
3	[Se-Se]-OT	**U**YIQN**U**PLG-NH_2	NH_2	**Se-Se**	NH_2
4	d[Se-Se]-OT	**dU**YIQN**U**PLG-NH_2	**H**	**Se-Se**	NH_2
5	OT-OH	CYIQNCPLG-**OH**	NH_2	S-S	**OH**
6	[Se-Se]-OT-OH	**U**YIQN**U**PLG-**OH**	NH_2	**Se-Se**	**OH**
7	d[Se-Se]-OT-OH	**dU**YIQN**U**PLG-**OH**	**H**	**Se-Se**	**OH**
8	d[ΔG9]-OT-OH	**d**CYIQNCPL--NH_2	**H**	S-S	NH_2
9	[ΔG9, Se-Se]-OT	**U**YIQN**U**PL--NH_2	**H**	**Se-Se**	NH_2
10	[ΔL8G9, Se-Se]-OT	**U**YIQN**U**P---NH_2	**H**	**Se-Se**	NH_2
	AVP analogues				
11	AVP	CYFQNCPRG-NH_2	NH_2	S-S	NH_2
12	dAVP	**d**CYFQNCPRG-NH_2	**H**	S-S	NH_2
13	[Se-Se]-AVP	**U**YFQN**U**PRG-NH_2	**H**	**Se-Se**	NH_2
14	d[Se-Se]-AVP	**dU**YFQN**U**PRG-NH_2	**H**	**Se-Se**	NH_2
15	d[ΔG9]-AVP	**d**CYFQNCPR--NH_2	**H**	S-S	NH_2
16	d[ΔG9]-AVP-OH	**d**CYFQNCPR--OH	**H**	S-S	**OH**

Figure 1. SAR study of OT/AVP with a focus on pharmacophore framework modifications.

A NMR structure of OT and framework residues marked in red that were the focus of this study. The table provides an overview of all synthesized peptides with details on their SAR modifications. *U…selenocysteine; d…deamino (N-terminus); Δ…deletion of residue; bold…modification; P…position; term…terminus.* **B** Synthesis of selenocysteine building blocks for Boc-SPPS to enable sulfur to selenium replacements. **C & D** Functional screen of OT analogues **1-10** (**C**) and AVP analogues **11-16 (D)** at human OTR, V1aR, V1bR (FLIPR Ca^{2+} signaling assay) and V2R (cAMP assay). Bar graphs illustrate selectivity gains across all four receptors with increased bars indicating loss of function at that particular receptor.

[Se-Se]-OT-OH (red) displayed remarkable selectivity gains for hOTR with >300-fold loss of potency at hV1aR and hV1bR. The last line (beige) illustrates how modifications affected potency compared to OT at hOTR and AVP at hV2R (black). **E** Functional follow-up study for OT analogues **1-7** using second messenger IP_1 signaling for human OTR, V1aR and V1bR (V2R same cAMP values as in **C**) confirming the selectivity gains of [Se-Se]-OT-OH. For exact EC_{50} values please refer to SI Table S2 & S3.

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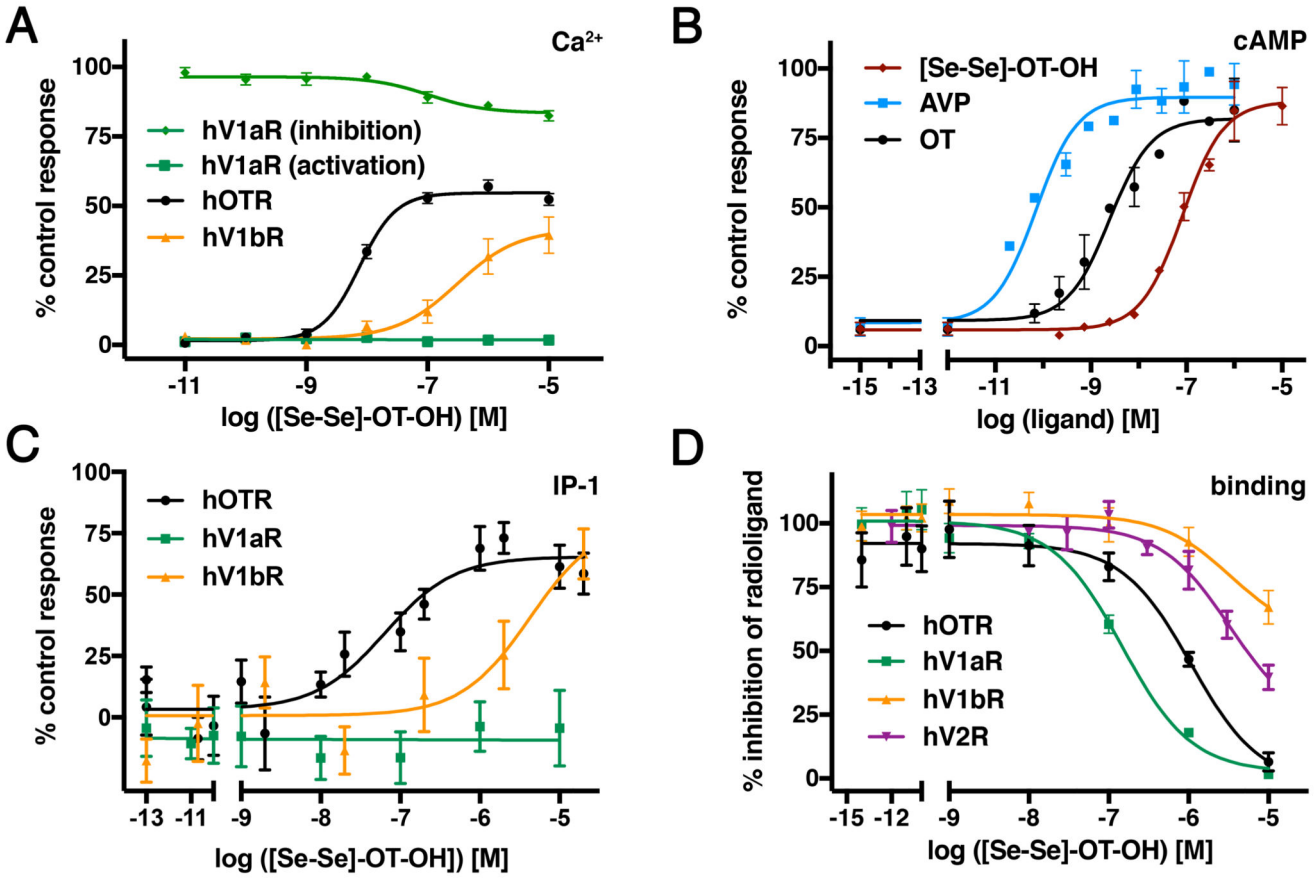

Figure 2. [Se-Se]-OT-OH (6) characterization over all four human OT/AVP receptors.

A Representative concentration-response curves of [Se-Se]-OT-OH (**6**) at the human OT, V1a and V1b receptors measuring calcium signaling using a FLIPR. **B** Representative concentration-response curves of OT, AVP and [Se-Se]-OT-OH at hV2R measuring the production of cAMP. **C** Representative concentration-response curves of [Se-Se]-OT-OH at the hOTR, hV1aR and hV1bR measuring the IP_1 production. **D** Representative concentration-response curve of the radioligand displacement assay of [Se-Se]-OT-OH at human OT, V1a, V1b and V2 receptors. All curves were normalized to % of response of control ligand (OT for OTR, AVP for AVPRs). Data is presented as means ± SEM of results obtained from at least three separate experiments, each performed in triplicate.

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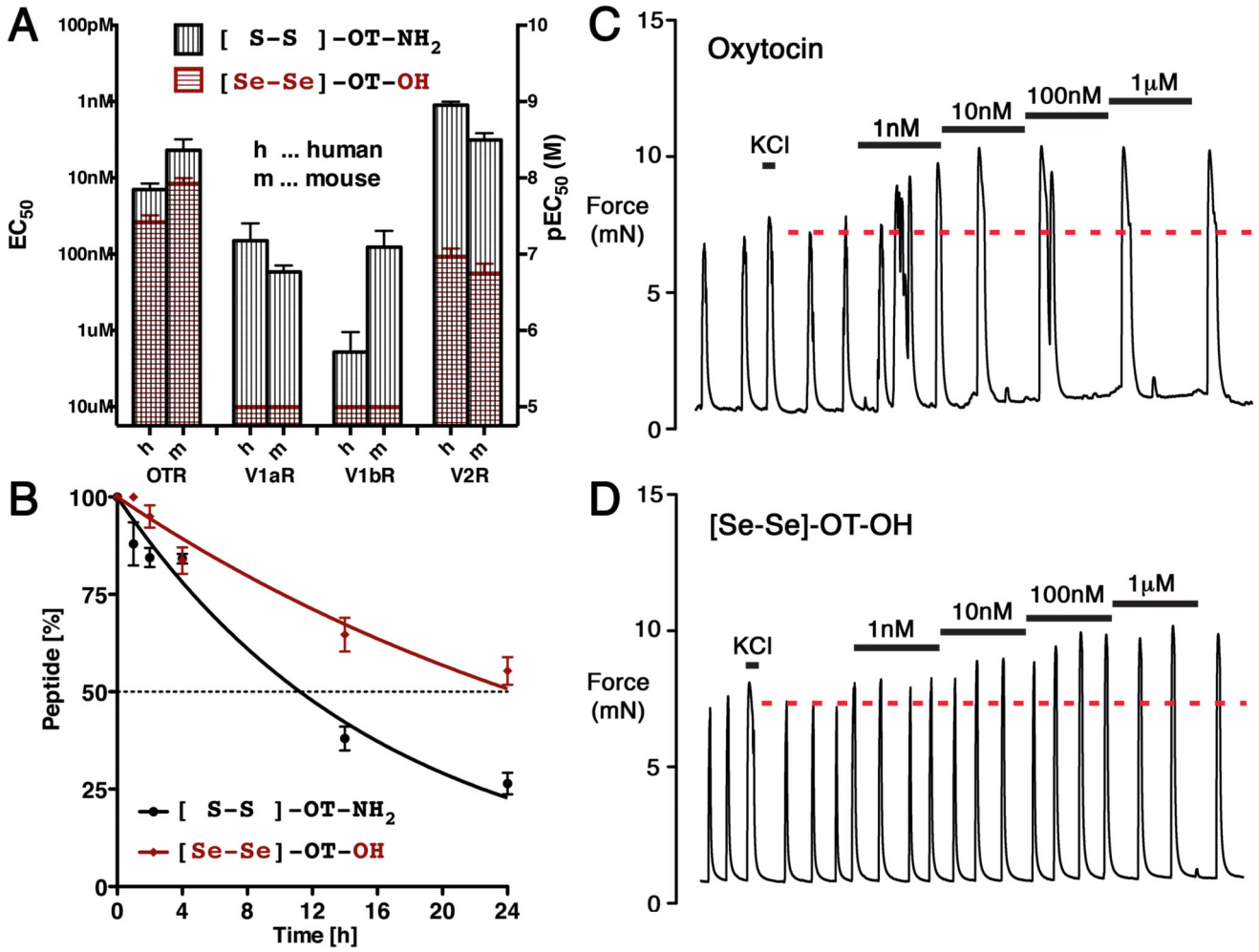

Figure 3. Comparison of [Se-Se]-OT-OH (6) with endogenous OT (1).

A Functional selectivity profile of [Se-Se]-OT-OH and OT over all four human (h) and murine (m) OT/AVP receptors (IP_1 for OTR, V1aR, V1bR; cAMP for V2R) illustrating that [Se-Se]-OT-OH is more selective for the OTR than OT and also retains its selectivity profile across species. **B** Metabolic stability of [Se-Se]-OT-OH ($t_{1/2}$=25 h) compared to OT ($t_{1/2}$=12 h) in human serum. Data (**A, B**) is presented as means ± SEM of results obtained from at least three separate experiments, each performed in triplicate. **C & D** Representative tissue bath study showing the dose-dependent effect of OT (**C**) compared with [Se-Se]-OT-OH (**D**) on contractions of human myometrium. [Se-Se]-OT-OH increases contraction amplitude in a similar manner to OT, yet maintains a more phasic and frequent contractile activity pattern compared to a more tonic-like activity under OT. N=5 for [Se-Se]-OT-OH, N=8 for OT.

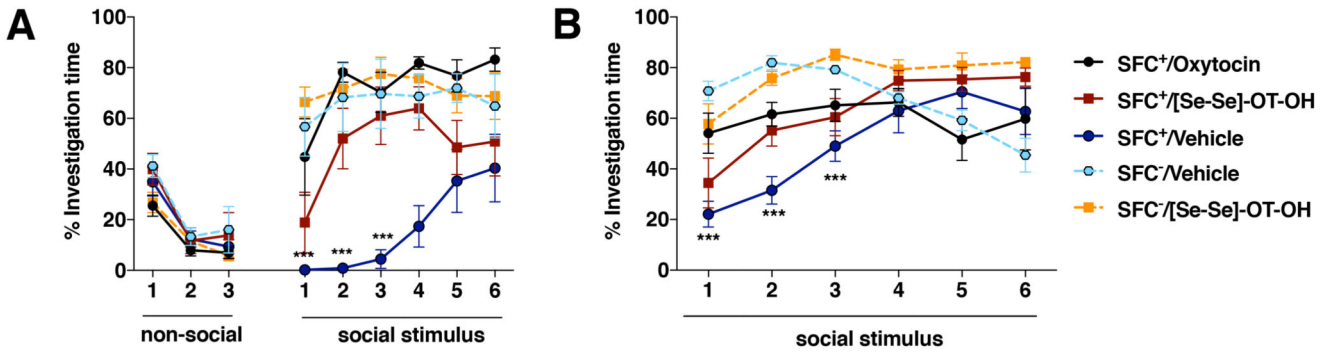

Figure 4.

Social fear conditioning (SFC) is a paradigm based on operant fear conditioning principles and generates robust social fear in mice without any confounding behavioral alterations (77). SFC-induced social fear could be reversed in male mice by icv administration of [Se-Se]-OT-OH (**6**) (in contrast to vehicle) thus confirming that this action is mediated *via* OTR and not *via* AVPRs. Percentage investigation of 3 non-social (empty cage) and 6 social (cage with a conspecific) stimuli during social fear extinction (day 2; **A**) and social fear extinction recall (day 3; **B**) was measured. Unconditioned (SFC^-) and conditioned (SFC^+) mice were icv infused with vehicle, OT (**1**) (250 μM/2 μl; 500 pmol) or [Se-Se]-OT-OH (**6**) (250 μM/2 μl, 500 pmol) 10 min prior to extinction training. Data represents mean ± SEM. *** $p<0.001$ *vs.* SFC^+/[Se-Se]-OT-OH (**A** and **B**).

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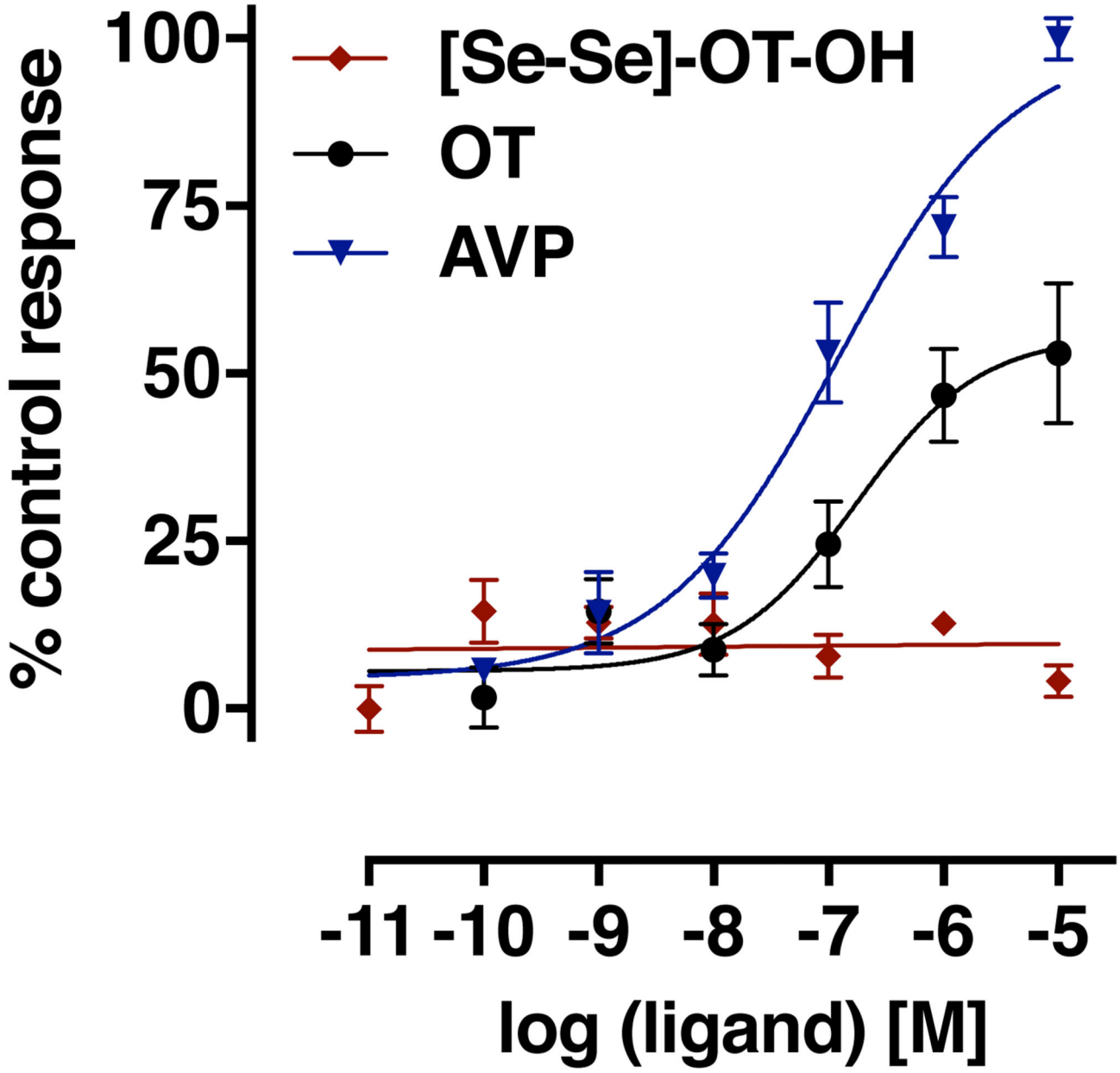

Figure 5.

Effects of AVP, OT and [Se-Se]-OT-OH on maximal intracellular Ca^{2+} in human cardiomyocytes assessed by the FLIPR. AVP and OT concentration-dependently increased the maximal intracellular Ca^{2+} concentrations while [Se-Se]-OT-OH had no effect. Data is presented as mean ± SEM, N=4 wells per data point.

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Table 1

Overview of commonly used molecular probes and the long-standing problem of producing ligands that retain selectivity across species for translational studies.

Ligand	Species	Selectivity	Subtype preference
Oxytocin	human	unselective	OT≈V1a>>V1b≈V2
	mouse	OT preference	OT>V1a≈V1b≈V2
	rat	OT preference	OT>V1a≈V1b≈V2
Vasopressin	human	unselective	OT≈V1a≈V1b≈V2
	mouse	unselective	OT≈V1a≈V1b≈V2
	rat	unselective	OT≈V1a≈V1b≈V2
Atosiban	human	V1a preference	V1a>OT≈V1b>V2
	mouse	OT selective	OT>>V1a≈V1b≈V2
	rat	unselective	OT≈V1a≈V1b≈V2
T4G7-OT	human	OT preference	OT>V1a>V1b≈V2
	mouse	OT selective	OT>>V1a≈V1b
	rat	OT selective	OT>>V1a≈V1b≈V2
d[Cha4]-AVP	human	V1b selective	V1b>>V1a≈OT≈V2
	rat	unselective	V1b≈V2>>OT≈V1a
F-180	human	V1a preference	V1a>OT≈V1b>V2
	rat	unselective	V1a≈V1b≈V2

≈…within 10-fold, >10-100-fold; >> more than 100-fold of EC_{50}; Refs: (37, 38, 41, 48, 49, 63, 96–99)

Table 2

Molecular probes to study the OT/AVP receptor system. For ligands to be called selective they need to be tested across all four receptor subtypes and display at least 100-fold selectivity compared to the other three receptor subtypes. As seen in this table, there still exists an urgent need for selective agonists and antagonists, preferably ligands that retain their selectivity profile across species. Reported K_i values are listed in parentheses in nM.

Selective Agonists	Subtype	Selective Antagonists
carba-1-[4-FBzlGly7]-dOT (FE 202767) (0.08)[a]	*human OTR*	SSR126768 (0.9)
[Thr4,Gly7]-OT (1.0)	*rat OTR*	
	mouse OTR	
[Phe2,Ile3,Hgn4,Orn(iPr)8]-VP (FE 202158) (4.4)[b], [Hmp1,Phe2,Ile3,Hgn4,Dab8]-VP (F-180) (11.7)	*human V1aR*	$d(CH_2)_5$[D-Ile2,Ile4,Tyr9]-AVP (3.7), SR49059 (0.9)
	rat V1aR	$d(CH_2)_5$[Tyr(Me)2]-AVP (0.7), SR49049 (2.2)
	mouse V1aR	
d[Cha4]-AVP (1.2)	*human V1bR*	Org52186 (4.0)
d[Leu4, Lys8]-VP (1.2)	*rat V1bR*	SSR149415 (1.3)[c]
	mouse V1bR	
	human V2R	SR121463 (4.1)
dDAVP (0.3)	*rat V2R*	SR121463 (1.5)
	mouse V2R	SR121463 (11.2)

Table was adapted from Chini et al.(38).

d … N-terminal deamination; carba-1 … S to CH_2 replacement for Cys1; $d(CH_2)_5$ … β-mercapto-β,β-cyclopenthamethylenepropionic acid was used instead of Cys1; desGly … desglycineamide; Orn … ornithine; Tyr(Me) … O-methyltyrosine; 4-FBzlGly … N-(4-fluorobenzyl)glycine; iPr … isopropyl; Hmp … 2-hydroxy-3-mercaptopropionic acid; Hgn … homoglutamine; Dab … 2,4-diaminobutyric acid

References for new ligands: a … (96) [only EC_{50} determined, no K_i]; b … (100, 101)

c … correction for SSR149415: SSR149415 is a selective V1bR antagonist for rat but not for mice, as it was reported in Chini et al.(38) (<100-fold selectivity in mice mV1bR vs. mV1a) (102, 103).