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Supporting Information

Self-assembly of Alkylated Lysine-dendron Oxytocin Amphiphiles for Enhanced Stability and Sustained Pharmacological Activity to Oxytocin Receptors

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Experimental section

Materials

Rink amide 4-methyl benzhydryl amine (MBHA) resin, Fmoc-protected amino acids, 2-(7-Azabenzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HATU), and O-benzotriazole-N, N, N', N' tetramethyl-uronium-hexafluorophosphate (HBTU) were purchased from GL Biochem (Shanghai, China) Ltd. Piperidine and diethyl ether were purchased from Sinopharm Chemical Reagent Co Ltd. TFA, DCM, DMF and acetonitrile (ACN) were purchased from Adamas. N, N-diisopropylethylamine (DIPEA), azide acetic acid, 5-hexynoic acid, copper sulfate, sodium ascorbate, dimethyl sulfoxide (DMSO), a-cyano-4-hydroxycinnamic acid (CHCA), and triisopropylsilane (TIPS) as well as all other reagents and solvents were obtained from Sigma-Aldrich (USA). Water measuring 18.2 $M\Omega \cdot cm^{-1}$ (Millipore Co) was used for all buffers for liquid chromatography.

General Fmoc-based solid phase peptide synthesis

Prior to peptide synthesis, a 1 mmol scale of rink amide MBHA resin was swollen in DMF overnight. The first 4 equiv of Fmoc-protected amino acid was conjugated to the resin with *in situ* neutralization. It was noted that the first amino acid should be reacted twice to ensure the conjugation successfully. Piperidine (30%) in DMF was used twice for Fmoc deprotection. 4 equiv of HBTU and 4 equiv of DIEA in DMF solution acted as coupling reagent and base. Then, the next amino acids were subsequently attached to the free amino group of the previous amino acid followed by repeated above steps. The mixture was kept to react for 1 h. Cleavage of the peptide from the resin was performed by adding 95% v/v TFA containing 2.5% v/v triisopropylsilane and 2.5% v/v H_2O for 2 hours. After the cleavage was complete, ice-cold diethyl ether was used to precipitate the peptide, and the dried sediments were obtained with a vacuum pump. Subsequently, 50% ACN in water was used to dissolve the peptide. Filtration and lyophilization of the filtrate yielded crude peptides. The crude peptide was purified by high-performance liquid chromatography (HPLC) using 50% water-ACN containing 0.05% TFA as eluent, and then lyophilized. Finally, the purity and molecular weights were confirmed by analytical HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), respectively. The HPLC solvents are as follows: solvent A was 0.05% TFA in Millipore water and solvent B was 0.05% TFA in ACN/H_2O (90/10, v/v). For peptide purification, two types of columns were used: a Shimadzu C18 column (10 μm, 250×20 mm) and an Agilent C18 column (5 μm, 250×9.4 mm). Peptide purification was carried out by preparative RP-HPLC using a Shimadzu LC-16P solvent delivery system. Peak absorbance was monitored at 214 nm with a Shimadzu SPD-16AV UV-vis detector. For MALDI-TOF MS, the products were analyzed using CHCA as the matrix, made as follows: 4 $mg \cdot mL^{-1}$ CHCA in ACN/H_2O (50/50, v/v) with 0.5% TFA.

Synthesis of native OT and its derivatives

The sequences of unoxidized OT Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly and unoxidized oxytocin analogue (OTK) Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys-Gly were synthesized following Fmoc-based manual solid phase peptide synthesis (SPPS) protocols. Detailed procedures for peptide assembly, cleavage and purification are described in methods. Of note, Fmoc-Phe-OH was required to be double coupled to improve the yield. For the unoxidized azido oxytocin (azido-OTK) Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys (azido-acetic acid)-Gly, after connecting the second amino acid Fmoc-Lys (Mtt)-OH, the protective group Mtt was removed by 1% TFA/DCM several times prior to the Fmoc group. Then, azido-acetic acid, activated by 0.5 M HATU, was connected for 24 h to achieve a high yield. The next steps are followed by SPPS protocols described in methods.

The crude peptides (OT, OTK and azido-OTK) were oxidized at 1 $mg \cdot mL^{-1}$ concentration in ammonium bicarbonate (0.1 M, pH=8) buffer. The oxidized products were purified using preparative RP-HPLC after adjusting pH of the mixture to 2 with TFA aqueous solution.

Synthesis of alkyne-modified lipidated lysine dendrons

The alkyne-modified lipidated lysine dendrons were synthesized by Fmoc-SPPS (Figure 1), the procedures

described in methods. Specifically, a linker with a sequence of RRRR (arginine-arginine-arginine-arginine) was first coupled to the resin. Then, Fmoc-Lys (Mtt)-OH was added to the coupling system. For 1G-dendron, Fmoc group of lysine was deprotected and 2 equiv of excess 5-hexynoic acid activated by HATU was coupled for 24 h. The remaining Mtt was removed by 1% TFA/DCM several times and 2 equiv of excess dodecanoic acid activated by HATU was coupled for 24 h. Whereas for the 2G-dendron, Fmoc group of lysine was deprotected and Fmoc-Lys (Fmoc)-OH was coupled to the system. 5-hexynoic acid, activated by HATU, was coupled to the dendron for 24 h with 2 equiv excess per dendron-branch. The remaining Mtt was removed by 1% TFA/DCM several times. Fmoc-Lys (Fmoc)-OH and dodecanoic acid were coupled sequentially to the system. For the resin cleavage and final purification, the resin was treated as described in methods.

Synthesis of ALOAs (1G-OTK and 1G-OTK)

The first-generation lipidated lysine dendron (52 mg, 0.005 mmol) and azido-OTK (110 mg, 0.01 mmol) were dissolved in H_2O/DMSO (30/70, v/v, 100 µL) in the presence of $CuSO_4$ (0.1 M, 20 µL). A solution of sodium ascorbate (0.1 M, 75 µL) was injected into the mixture solution under nitrogen atmosphere. The reaction was allowed to proceed with stirring for 24 h at 25 °C. After that, the reaction was monitored by analytical HPLC, and the resulted product was purified by RP-HPLC with 1 %/min linear gradient of 25-65% buffer B at a flow rate of 5 mL/min. The concentration of copper in the products was detected by inductively coupled plasma-mass spectrometry (ICP-MS). The step to synthesize 2G-OTK was the same as above.

Preparation of ALOA (1G-OTK and 1G-OTK) nanostructures

In brief, 5 mg of 1G-OTK was dissolved in 500 µL DMSO and slowly added to 10 mL of water. After 30 min stirring, the solution was dialyzed against pure water for three days to remove organic solvents. The self-assembled nanoparticles were obtained by lyophilization. The steps to prepare 2G-OTK self-assembled nanostructures were the same as above. In order to characterize the morphology of 1G-OTK and 1G-OTK, the hydrodynamic sizes and zeta potential were determined with dynamic light scattering (DLS). Transmission electron microscope (TEM) was performed to observe the self-assembled nanostructures.

Circular dichroism (CD) Spectrum

All CD spectra were recorded on a Jasco J-810 spectropolarimeter by continuously purging dry N_2 gas at 25 °C. Each spectrum was collected from 300 nm to 190 nm and averaged from three scans in a quartz cuvette with a 0.1 mm path length for 10 mM peptide solution.

Temporal stability assay

50 µM of OT, 1G-OTK and 2G-OTK were dissolved in water. Then, 1 mL of each sealed solution was placed inside a glass at 25 °C for different days. 50 µL of samples were taken at t = 0, 3, 7, 14 and 21 d by RP-HPLC. HPLC method of OT: 1 %/min linear gradient of 5-45 % solvent B at a flow rate of 1 mL/min. HPLC method of 1G-OTK and 2G-OTK: 1 %/min linear gradient of 20-65 % solvent B at a flow rate of 1 mL/min. The number of remaining products (%) was determined by integrating the area underneath the corresponding peak (monitored at 214 nm) and compared with that of the control.

Thermal stability assay

OT, 1G-OTK and 2G-OTK were dissolved in water to a concentration of 50 µM. Then, 1 mL of each solution was placed inside a glass vial, sealed and incubated at 55 °C for different time. Samples (50 µL) were taken at t = 0, 1, 2, 4, 8, 12, 24, 48 and 72 h, quenched with 50 µL of 2% TFA in water. The remaining samples were analyzed using HPLC. HPLC method: 1 %/min linear gradient of 15-70% solvent B at a flow rate of 1 mL/min. The number of remaining products (%) was determined by integrating the area underneath the corresponding peak (monitored at 214 nm) and compared with that of the control.

Human serum stability assay

Human serum (Sigma-Aldrich) was centrifuged at 13000 rpm for 15 min and then the supernatant was collected to

pre-warm to 37 °C. 500 μM of OT, 1G-OTK and 2G-OTK were respectively diluted in PBS. The sample solutions (300 μL) were mixed with 300 μL serum and incubated at 37 °C. At various time points (t = 0, 1, 2, 4, 8, 12 and 24 h), 50 μL aliquots were withdrawn, quenched with 100 μL of 2% TFA/50% acetonitrile in water and cooled at 4 °C for 5 min.[1, 2] After that, the mixture was centrifuged at 2000 rpm for 5 min and the final supernatant was immediately analyzed by HPLC. HPLC method: 1 %/min linear gradient of 15-70% solvent B at a flow rate of 1 mL/min. The number of remaining products (%) was determined by integrating the area underneath the corresponding peak (monitored at 214 nm) and compared with that of the control.

Cytotoxicity assay

The cytotoxicity of OT, 1G-OTK and 2G-OTK on the treated cells was determined by an MTT assay. The cells were seeded in 96-well plates with 10^4 cells per well for 24 hours before MTT assay. Then, the cells were treated with different concentrations of OT, 1G-OTK and 2G-OTK for 24 hours. The viability of treated cells was measured by a standard MTT assay. All measurements were performed in quintuplicate.

Functional activity assay

HEK293 cells were cultured in RPMI media supplemented with 15% fetal bovine serum at 37 °C and transiently transfected with plasmid DNA encoding the OT/V1a/V1b receptors using Lipofectamine 2000 following the manufacturer's protocol. At 24 h post-transfection, cells were seeded at a density of 15,000 cells per well in black-walled imaging plates and maintained for another 24 h at 37 °C in a 5% humidified CO_2 incubator. Assays measuring ligand-induced Ca^{2+} responses were performed at 48 h post-transfection using a Fluorometric Imaging Plate Reader (FLIPR).

Author Contributions

F. Xie conducted the experiment and wrote the manuscript. Y. Lin assisted the experiment; A. Andersson and I. Veter performed the data analysis; L. Zhao reviewed and edited the initial manuscript; J. Wan designed and orchestrated the study.

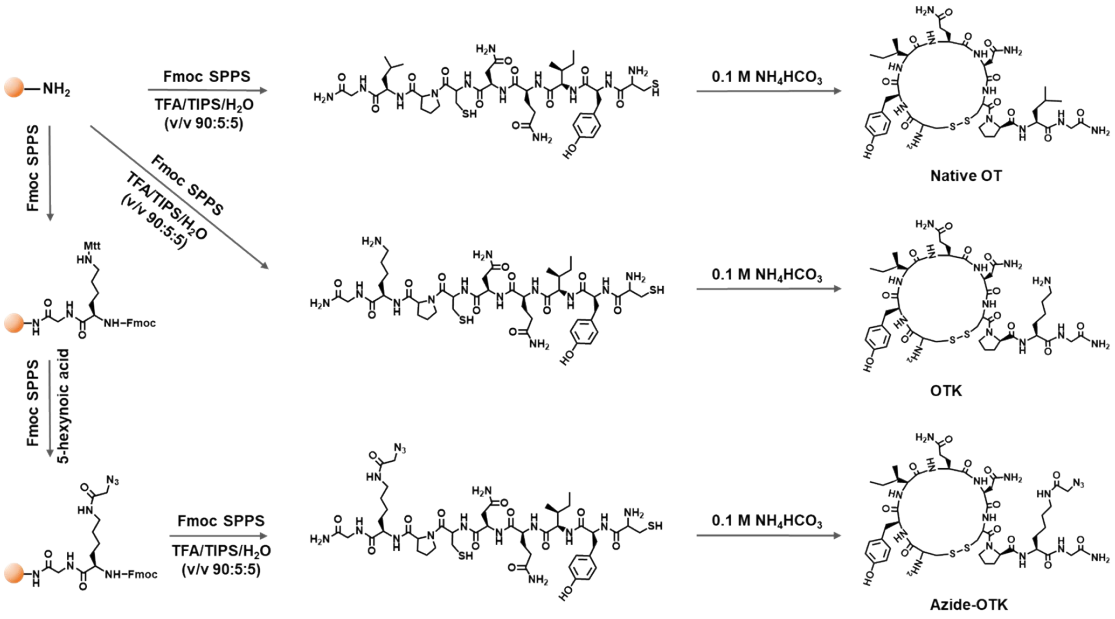

Figure S1. Synthetic route of OT, OTK and Azide-OTK.

Table S1. Theoretical molecular and measured molecular mass of the synthetic compounds 1-7.

Entry	Compound	Theoretical Molecular Mass (Da)	Measured Molecular Mass in MALDI-MS (Da)
1	OT	1006.44	1007.76
2	OTK	1021.45	1022.71
3	Azide-OTK	1104.46	1105.62
4	Dendron-1G	1045.73	1046.85
5	Dendron-2G	1578.13	1579.58
6	1G-OTK	2150.19	2151.99
7	2G-OTK	3787.05	3790.48

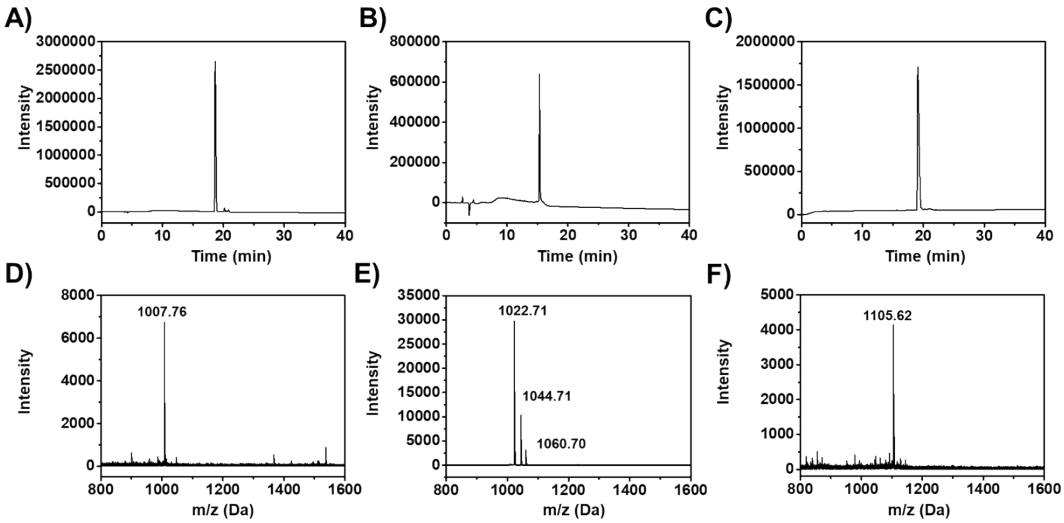

Figure S2. HPLC of A) OT, B) OTK, and C) Azide-OTK. MALDI-TOF-MS of D) OT, E) OTK and F) Azide-OTK.

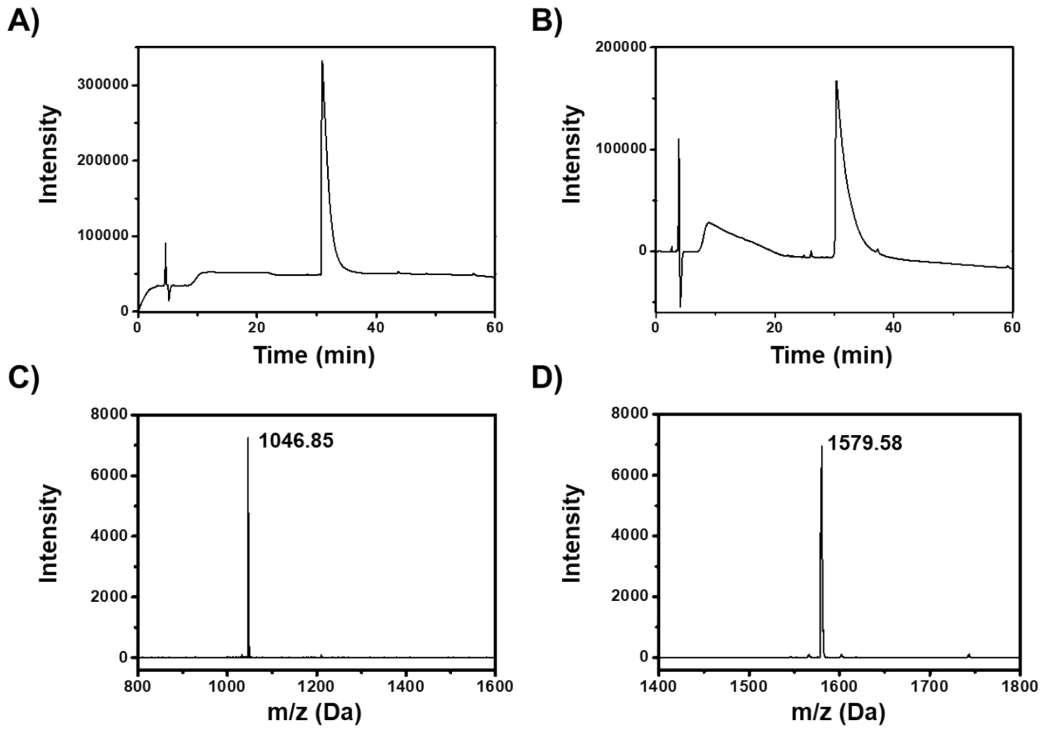

Figure S3. HPLC of A) Dendron-1G and B) Dendron-2G. MALDI-TOF-MS of C) Dendron-1G and D) Azide-OTK.

Table S2. Copper content, Zeta Potential and LogP value of Azido-OTK, Dendron-1G, Dendron-2G, 1G-OTK and 2G-OTK.

Compound	Azido-OTK	Dendron-1G	Dendron-2G	1G-OTK	2G-OTK
Copper content	——	——	——	49.5 ppb	13.0 ppb
Zeta Potential	——	——	——	8.24±0.46mV	11.75±0.56mV
LogP value	-4.80	2.19	6.68	-3.46	-4.61

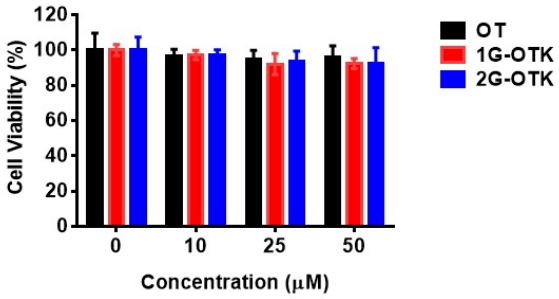

Figure S4. Cell viability of NIH/3T3 cells incubation with OT, 1G-OTK and 2G-OTK of different concentrations for 24 h.

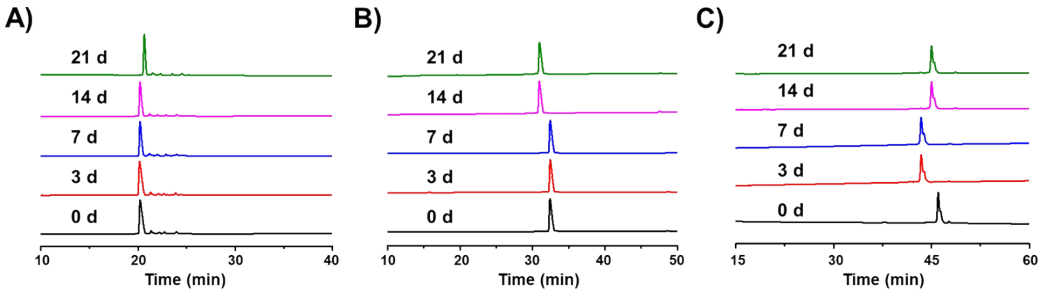

Figure S5. Temporal stability of A) OT, B) 1G-OTK and C) 2G-OTK in water at 25 °C.

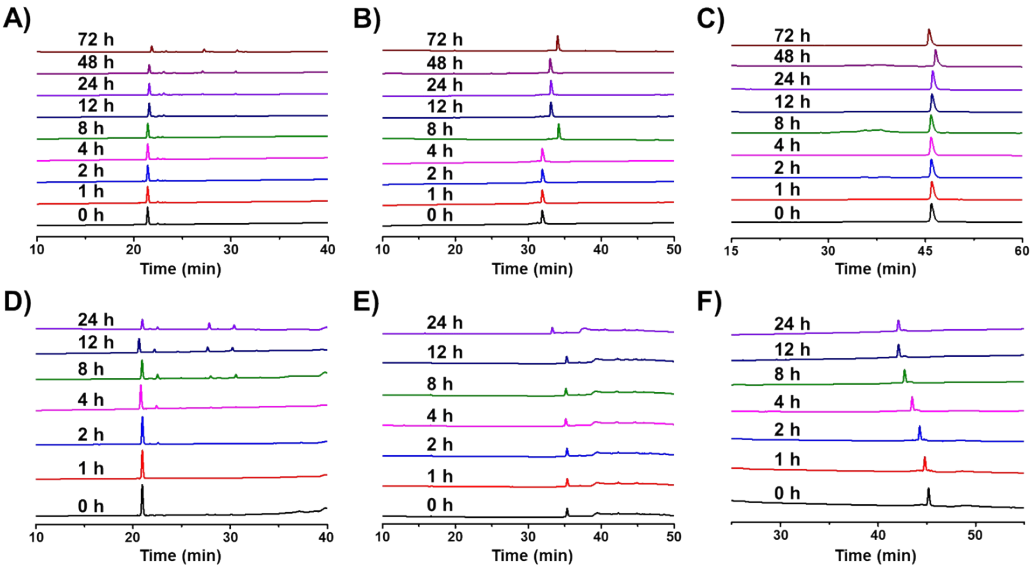

Figure S6. Thermal stability of A) OT, B) 1G-OTK and C) 2G-OTK in water at 55 °C; Serum stability of D) OT, E) 1G-OTK and F) 2G-OTK in human serum at 37 °C.

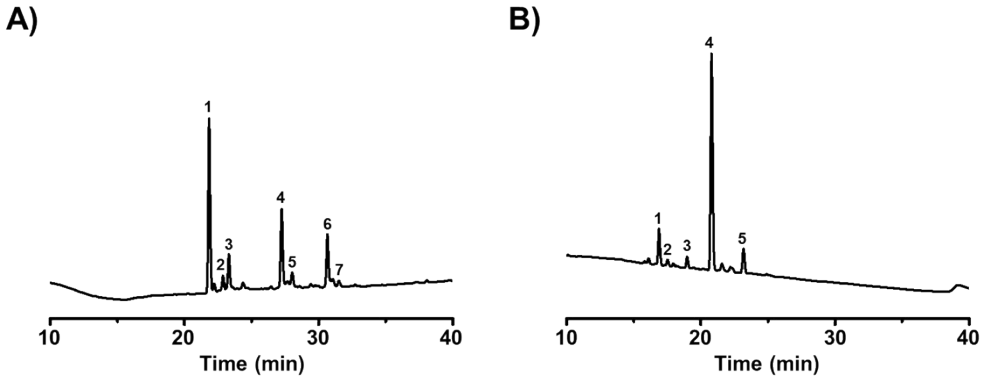

Figure S7. A) HPLC traces of OT in water at 55°C after 72 h; B) HPLC traces of OT degraded by proteinase K at 37 °C after 12 h.

Table S3. Identification of OT degradation products in water at 55°C for pH 7.0 formulations from LC and MS data.

Peak Number	*m/z*	Charge	Degradation Products
1	1007.44	1+	Oxytocin
2, 3	1008.42	1+	Mono-deamidation at Asn, Gly or Gln
4	1039.41	1+	Trisulfide
5	1071.38	1+	Tetrasulfide
6	1007.44	2+	Dimer
7	975.44	2+	bis-deamidated

Table S4. Identification of OT degradation products degraded by proteinase K at 37°C formulations from LC and MS data.

Peak Number	*m/z*	Charge	Degradation Products
1, 2	513.23	2+	Mono peptide-bond broken oxytocin species
3	485.21	2+	Mono peptide-bond broken oxytocin species
4	1007.44	1+	Oxytocin
5	476.21	2+	Bis peptide-bond broken oxytocin species

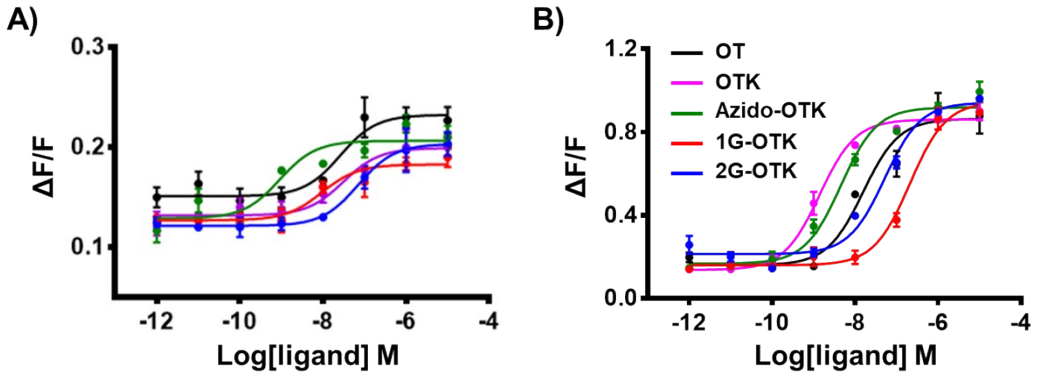

Figure S8. Function activity of OT, OTK, Azido-OTK, 1G-OTK and 2G-OTK expressing A) human V1aR and B) human V1bR. All data are Mean ± SEM (*n*=3).

Reference

1 A. D. de Araujo, M. Mobli, J. Castro, A. M. Harrington, I. Vetter, Z. Dekan, M. Muttenthaler, J. J. Wan, R. J. Lewis, G. F. King, S. M. Brierley and P. F. Alewood, *Nat. Commun.*, 2014, **5**, 3165.

2 M. Muttenthaler, A. Andersson, A. D. de Araujo, Z. Dekan, R. J. Lewis and P. F. Alewood, *J. Med. Chem.*, 2010, **53**, 8585-8596.