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SUPPLEMENTARY MATERIAL

Synthesis of Multivalent [lys^8]-Oxytocin Dendrimers that Inhibit Visceral Nociceptive Responses

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Abbreviations: Natural occurring amino acids are abbreviated to standard single or standard three letter codes; HBTU, N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uranium hexafluorophosphate); TFA, trifluoroacetic acid; DIEA, N,N'-diisopropylethylamine; DMF, N,N'-dimethylformamide; DCM, dichloromethane; ACN, acetonitrile; HATU, O-(7-Azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate; TBTA, Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine; Mtt, methyltrityl; DMSO, dimethyl sulfoxide; CHCA, α-cyano-4-hydroxycinnamic acid; TIPS, triisopropylsilane; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; RP-HPLC, reversed-phase high-performance liquid chromatography; LC-MS, liquid chromatography coupled mass spectrometry; NMR, nuclear magnetic resonance; NOESY, nuclear overhauser enhancement spectroscopy; HSQC, Heteronuclear single quantum coherence spectroscopy; TOCSY, total correlated spectroscopy; DOSY, diffusion-ordered spectroscopy; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; GPCR, G protein-coupled receptor; FLIPR, fluorescence imaging plate reader; TNBS, trinitrobenzene-sulphonic acid.

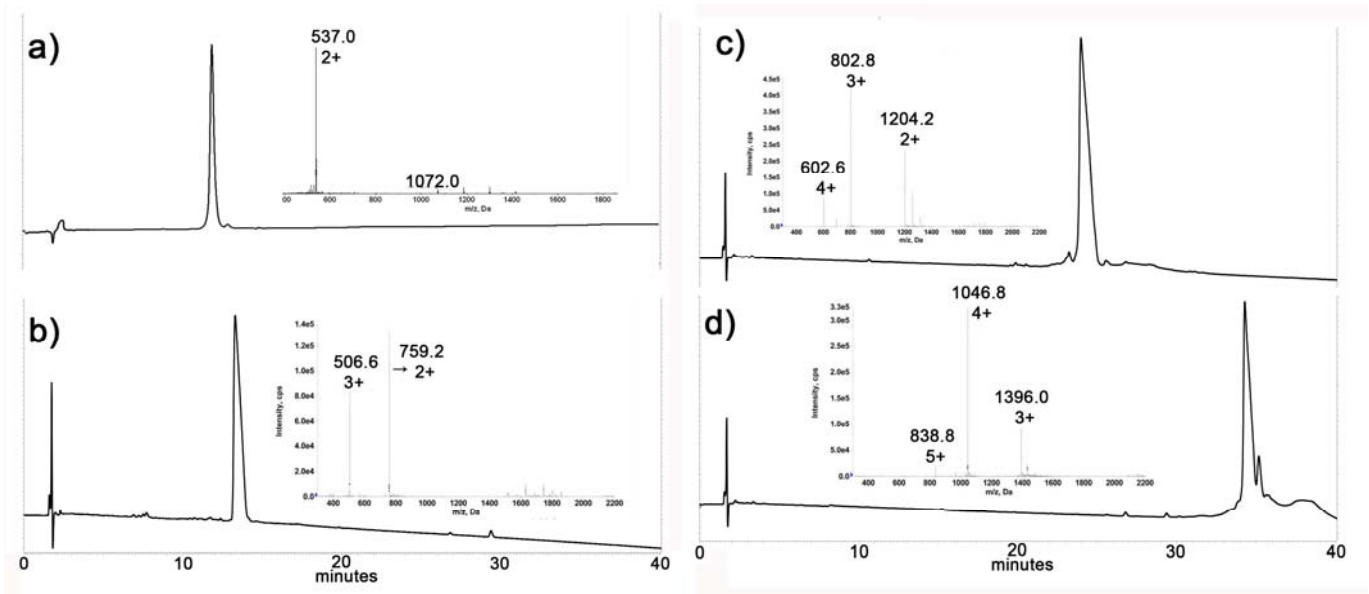
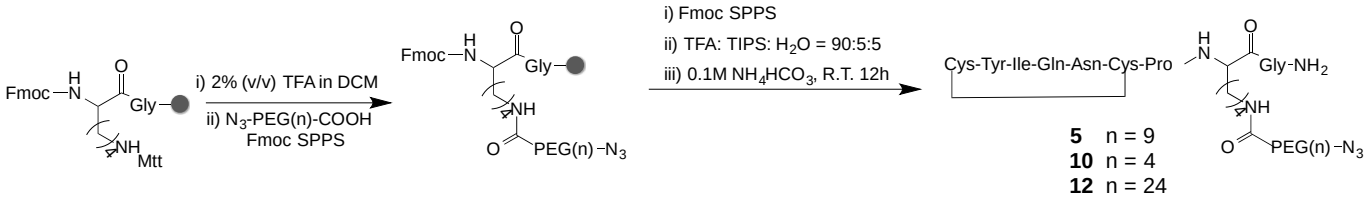


Figure S1. LC-ESI-MS results of a) G_0 alkyne dendron **1** b) G_1 alkyne dendron **2**, c) G_2 alkyne dendron **3** and d) G_3 alkyne dendron **4,** LC method: 1%/min linear gradient of 10-50% solvent B at a flow rate of 1 mL/min.



Scheme S1: Fmoc SPPS of [Lys[8]]-OT with an azido-PEG modification to yield **5**, **10** and **12**.

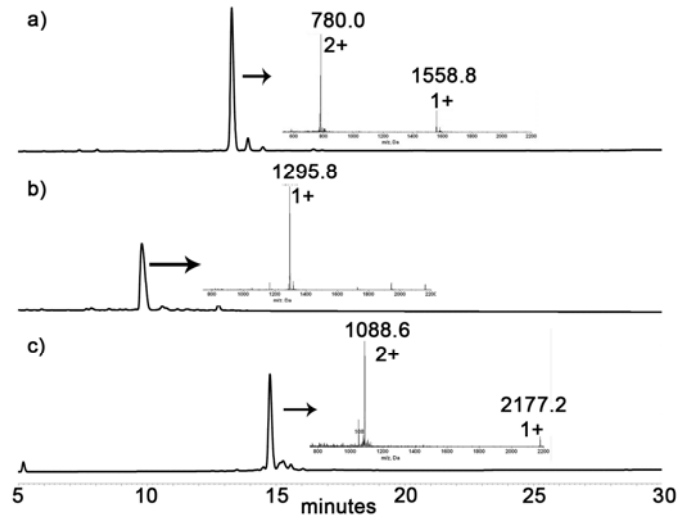
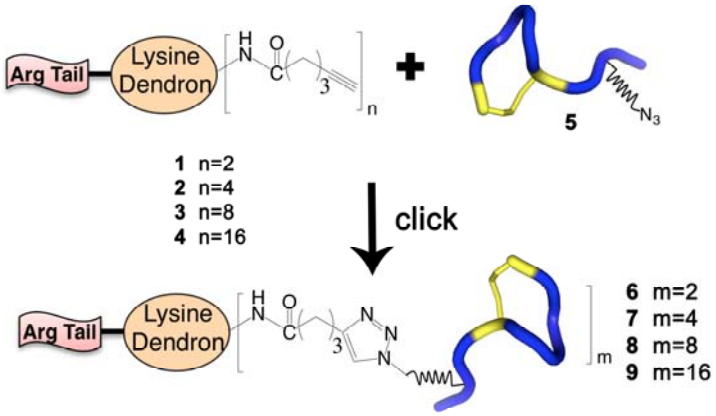


Figure S2. LC-ESI-MS results of a) azido-PEG(9)-LVT **5**, b) azido-PEG(4)-LVT **10** and c) azido-PEG(24)-LVT **12**, LC method: 1%/min linear gradient of 15-45 % solvent B at a flow rate of 1 mL/min.



Scheme S2: Cu-catalyzed click ligation of alkyne dendrimers **1-4** and azido-oxytocin analogue **5** resulting in final LVT dendrimers **6-9;**

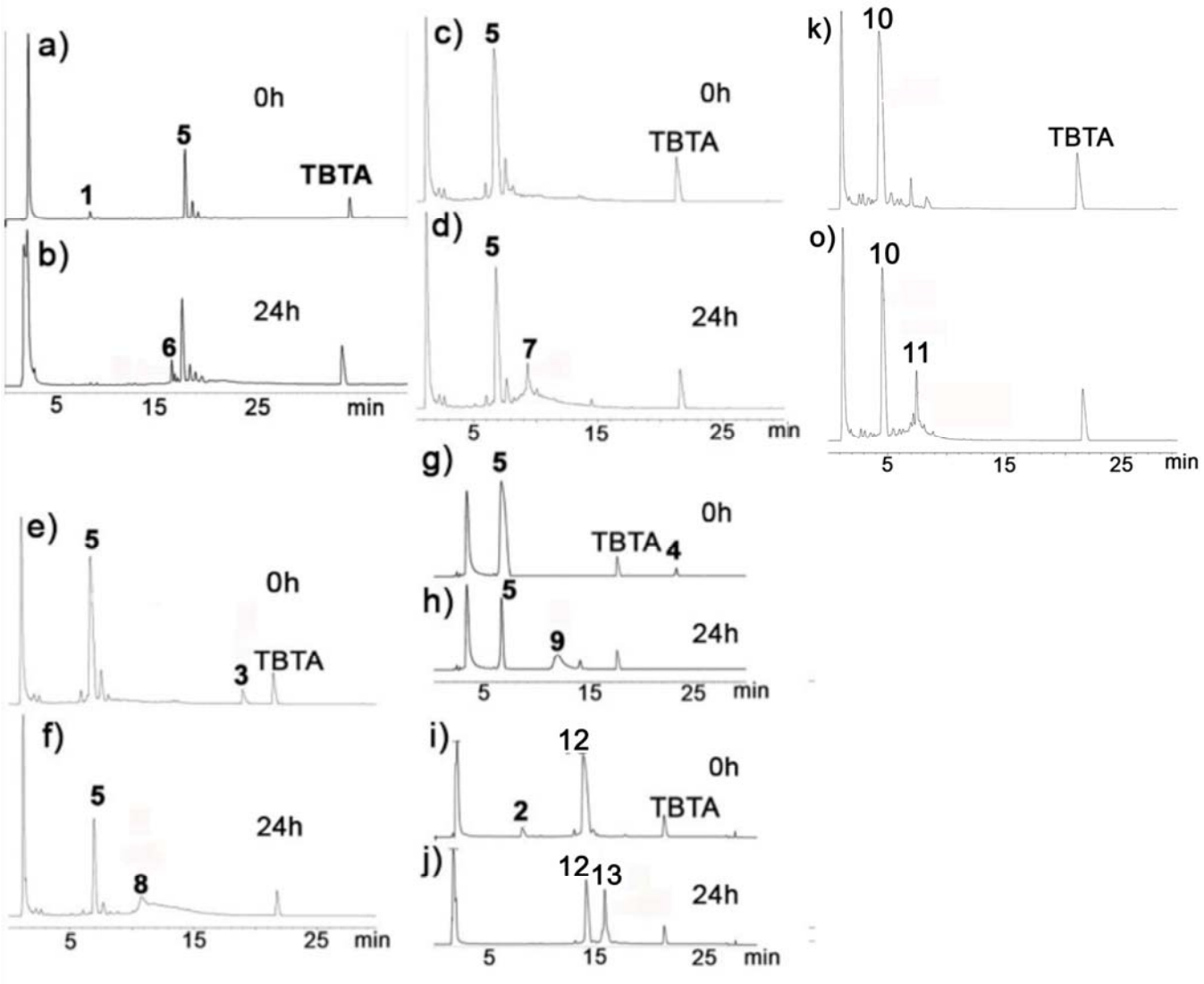


Figure S3. Analytical RP-HPLC analysis of click reaction between azido-PEG(9)-LVT **5** and 2-mer alkyne dendron **1** after a) 0 h and b) 24 h the LC method is: 1%/min linear gradient of 10-50% buffer B at

a flow rate of 1 mL/min; HPLC analysis of the click reaction between azido-PEG(9)-LVT **5** and the 4-mer alkyne dendron **2** after c) 0 h and d) 24 h; HPLC analysis of the click reaction between azido-PEG(9)-LVT **5** and the 16-mer alkyne dendron **3** after e) 0 h and f) 24 h; HPLC analysis of the click reaction between azido-PEG(9)-LVT **5** and the 16-mer alkyne dendron **4** after g) 0 h and h) 24 h; HPLC analysis of the click reaction between azido-PEG(24)-LVT **12** and the 4-mer alkyne dendron **2** after i) 0 h and j) 24 h, HPLC analysis of the click reaction between azido-PEG(4)-LVT **10** and the 4-mer alkyne dendron **2** after k) 0 h and o) 24 h LC method: 1%/min linear gradient of 20-50% solvent B at a flow rate of 1 mL/min. For the reaction between **10** and **2**, the LC method is: 1%/min linear gradient of 15-45% solvent B at a flow rate of 1 mL/min

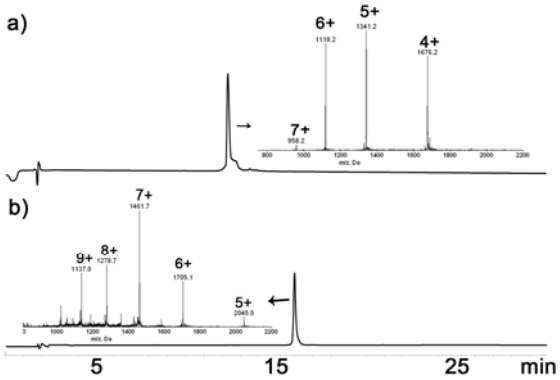


Figure S4. LC-ESI-MS results of purified a) 4xLVT-PEG(4)-D **11** and b) 4xLVT-PEG(24)-D **13**, LC method: 1%/min linear gradient of 15-45% solvent B at a flow rate of 1 mL/min.

Table S1 Theoretical mass and found molecular mass of compounds **1-11**

Entry	Compound	Theoretical Mass (Da)	Found Molecular Mass (Da)
1	$GlyArg_4Gly–[Lys][CO-(CH_2)_3-C{\equiv}CH]_2$	1071.9	1071.0
2	$GlyArg_4Gly-[Lys]_2[CO-(CH_2)_3-C{\equiv}CH]_4$	1516.6	1516.4
3	$GlyArg_4Gly-[Lys]_4[CO-(CH_2)_3-C{\equiv}CH]_8$	2405.9	2406.4
4	$GlyArg_4Gly-[Lys]_8[CO-(CH_2)_3-C{\equiv}CH]_{16}$	4184.5	4185.0
5	[Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys(ε-CO-PEG(9)-N_3)Gly-NH_2	1558.6	1557.8
6	$GlyArg_4Gly-[Lys][Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys(ε-CO-PEG(9)-1,2,3-triazole-(CH_2)_3-CO)Gly-NH_2]_2$	4189.1	4190.3
7	$GlyArg_4Gly-[Lys]_2[Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys(ε-CO-PEG(9)-1,2,3-triazole-(CH_2)_3-CO)Gly-NH_2]_4$	7751.3	7752.6
8	$GlyArg_4Gly-[Lys]_4$[Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys(ε-	14874.7	14876.8

	CO-PEG(9)-1,2,3-triazole-$(CH_2)_3$-CO)Gly-$NH_2]_8$		
9	GlyArg$_4$Gly-[Lys]$_8$[Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys(ε-CO-PEG(9)-1,2,3-triazole-$(CH_2)_3$-CO)Gly-$NH_2]_{16}$	29122.1	29210.0[a]
10	[Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys(ε-CO-PEG(4)-N_3)Gly-NH_2	1295.3	1294.8
11	GlyArg$_4$Gly-[Lys]$_2$[Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys(ε-CO-PEG(4)-1,2,3-triazole-$(CH_2)_3$-CO)Gly-$NH_2]_4$	6695.8	6700.7
12	[Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys(ε-CO-PEG(24)-N_3)Gly-NH_2	2176.8	2176.2
13	GlyArg$_4$Gly-[Lys]$_2$[Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Lys(ε-CO-PEG(24)-1,2,3-triazole-$(CH_2)_3$-CO)Gly-$NH_2]_4$	10223.8	10224.9

[a] Mass determined by 4700 MALDI-TOF MS in the positive ion linear mode using CHCA as matrix.

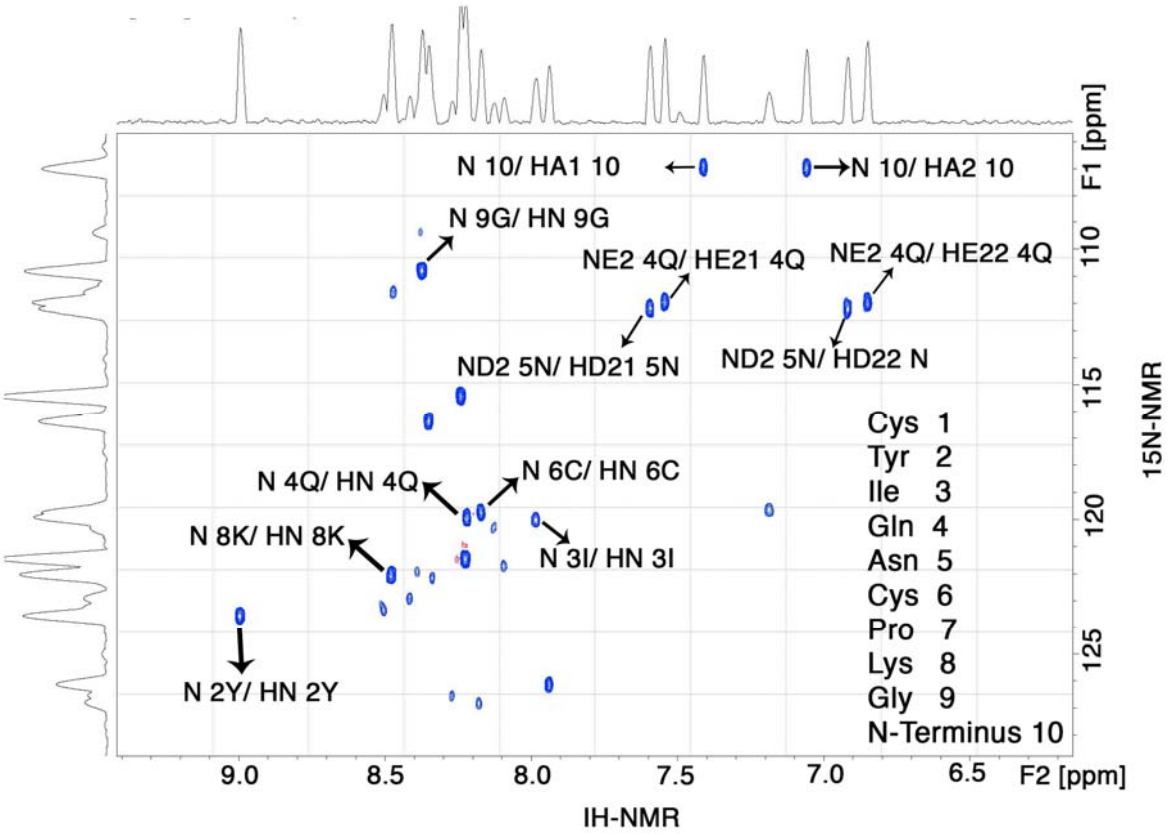


Figure S5. ^{1}H-^{15}N HSQC spectrum of the LVT dendrimer 4xLVT-PEG(9)-D **7.**

Table S2. Assigned αH chemical shift of analogues **6-9** and OT.

αH δ / ***(ppm)*** / **Residues**	**Analogue No.**				
	OT	**6**	**7**	**8**	**9**
1 Cys	4.260	4.274	4.286	4.275	4.276
2 Tyr	4.779	4.765	4.767	4.789	4.723
3 Ile	4.040	4.014	4.016	4.003	4.002
4 Gln	4.105	4.105	4.107	4.100	4.101
5 Asn	4.719	4.720	4.728	4.720	4.723
6 Cys	4.854	4.867	4.872	4.864	4.877
7 Pro	4.434	4.450	4.441	4.439	4.442
8 Lys or Leu [a]	4.295	4.240	4.223	4.232	4.232
9 Gly	3.895	3.900	3.903	3.901	3.900

[a] Leu residue is for OT, and Lys residue for analogues **6-9**

Table S3. *Diffusion coefficient of analogues* **5-9**

No.	**Analogue**	**Diffusion Coefficient (D, 10^{-10} m^2s^{-1})**	**Log (D)**
5	N_3-PEG(9)-LVT	2.6±0.1	-9.6
6	2xLVT-PEG(9)-D	1.5±0.1	-9.9
7	4xLVT-PEG(9)-D	1.2±0.1	-10.0
8	8xLVT-PEG(9)-D	0.9±0.1	-10.1
9	16xLVT-PEG(9)-D	0.7±0.1	-10.2

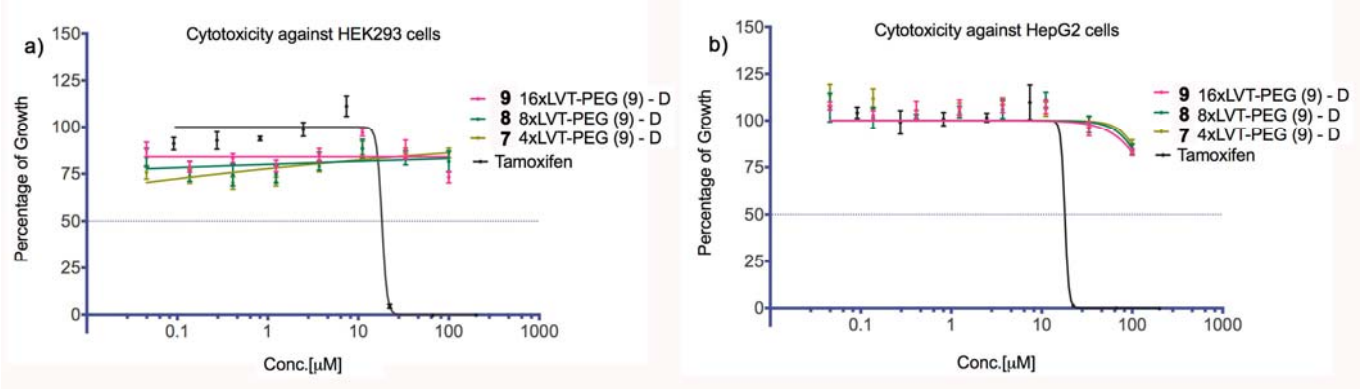


Figure S6. Cytotoxicity of LVT dendrimers **7-9** against HEK293 and HepG2 cells.

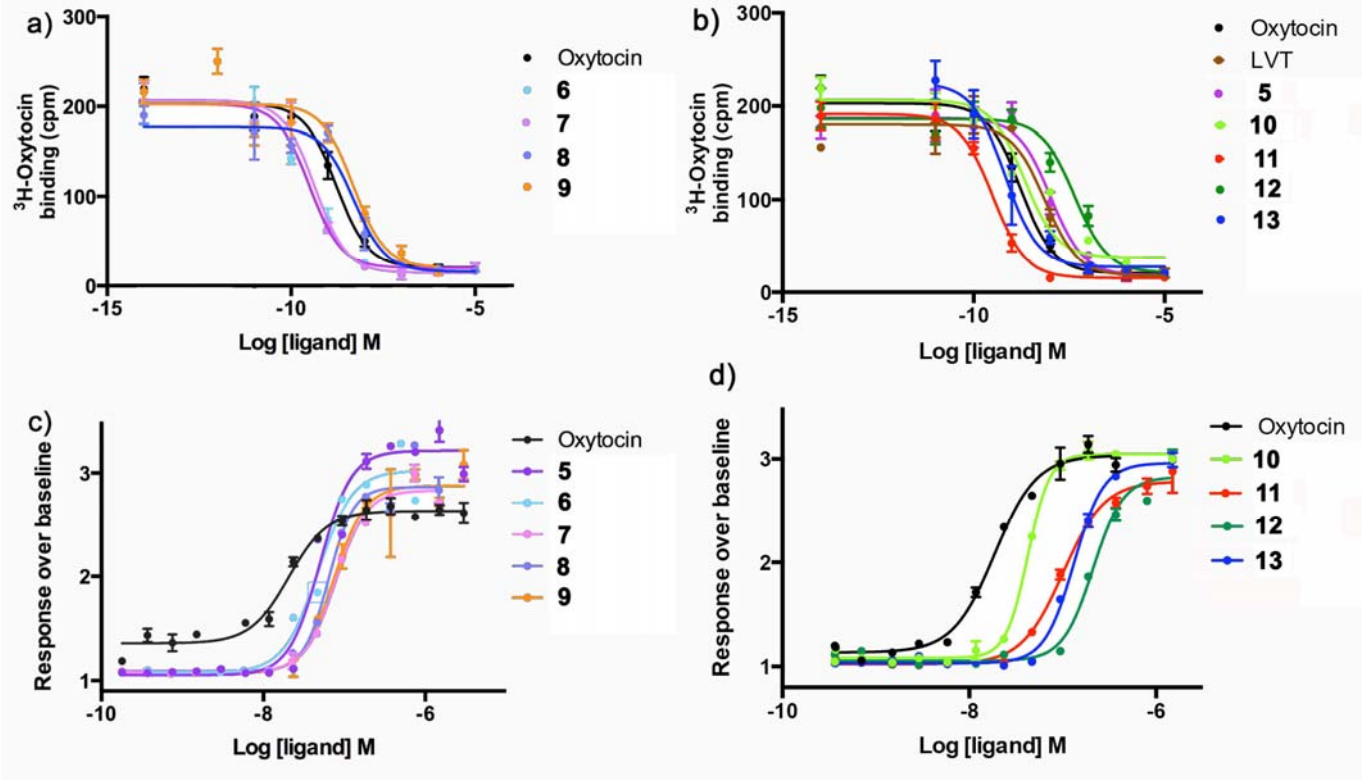


Figure S7. Binding and functional data of the LVT dendrimers (**6-9, 11** and **13**), monomer analogues (**5, 10** and **12**), LVT and OT on human OTR. Affinity data (a and b) were obtained via radioligand binding assays which measured displacement of ^{3}H-OT from the human OTR expressed in COS-1 cells. The functional EC_{50} values (c and d) were investigated by measuring the stimulation of Ca^{2+} responses from SH-SY5Y cells expressing the human OTR. Representative concentration-response curves performed in triplicate for each dendrimer at the human OTR.

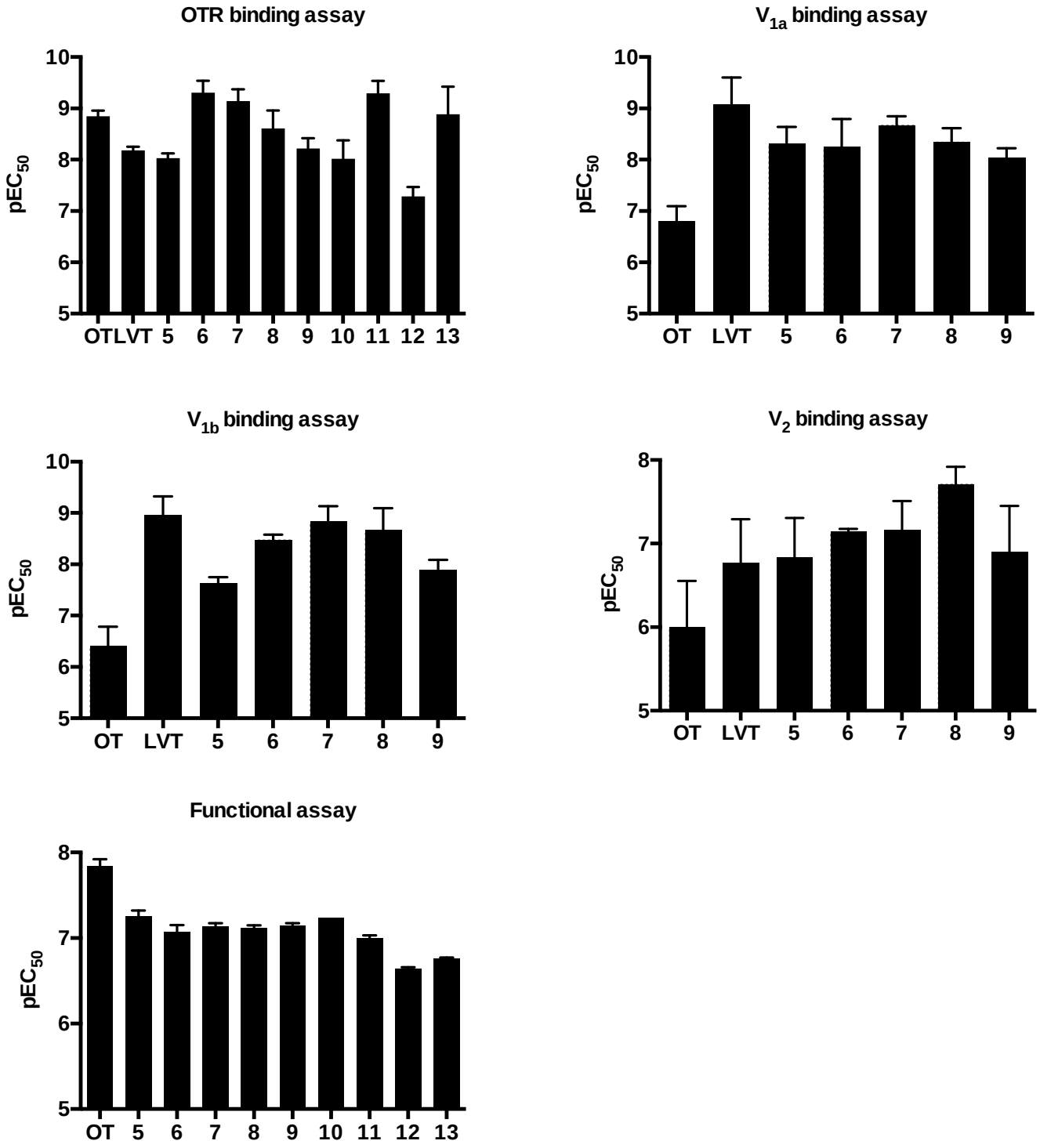


Figure S8. Binding data of LVT analogues at the human a) OTR, b) V_{1a} receptor, c) V_{1b} and d) V_2 receptor expressed in COS-1 cells; e) Functional data of LVT analogues at the human OTR in SHSY-5Y cells. Results are the means ± SEM of 3 experiments. Each experiment was performed in triplicate.

Table S4. Binding affinity of LVT dendrimers **6-9** and LVT analogues at the V_{1a}, V_{1b} and V_2 tested in radioligand binding assay. Ki values are means ± SEM of 3 separate experiments (performed in triplicate).

No.	Analogues	V_{1a} Binding Ki (nM)	V_{1b} Binding Ki (nM)	V_2 Binding Ki (nM)
	OT	78.15 ± 12.98	194.55 ± 42.30	568.76 ± 93.22
	LVT	0.43 ± 0.13	0.55 ± 0.12	84.64 ± 25.42
5	Azido-PEG(9)-LVT	2.39 ± 0.44	11.89 ± 0.86	72.40 ± 19.54
6	2xLVT-PEG(9)-D	2.81 ± 0.88	1.66 ± 0.09	35.70 ± 0.61
7	4xLVT-PEG(9)-D	1.05 ± 0.10	0.74 ± 0.13	34.20 ± 6.80
8	8xLVT-PEG(9)-D	2.22 ± 0.34	1.08 ± 0.27	9.68 ± 1.15
9	16xLVT-PEG(9)-D	4.51 ± 0.46	6.39 ± 0.71	62.46 ± 19.71

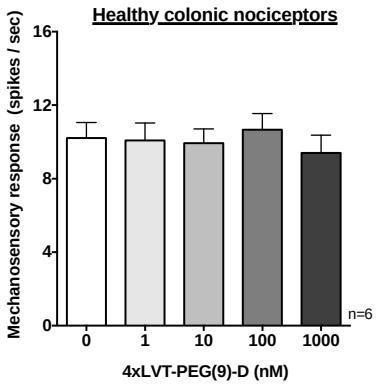


Figure S9. 4xLVT-PEG(9)-D **7** had no effect on healthy colonic nociceptors (***P*<0.001; ****P*<0.0001 compared with baseline response).