Supplemental Information

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"Photoaffinity labeling of the Sigma-1 Receptor with N-(3-(4-nitrophenyl)propyl)-N-dodecylamine (4-NPPC12) – Evidence for Receptors Dimers"

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Experimental Procedures

"In Gel" digestion with trypsin and mass spectrometry determination. Coomassie Blue R-250 stained gel pieces were de-stained twice for 5 min in MeOH/CH_3COOH/H_2O (45%:45%:10%), dehydrated for 5 min in ACN/ H_2O/NH_4HCO_3 (50%:50%:25 mM) then once more for 1 min in 100% ACN, and then dried in a Speed-Vac for 2 min, samples were reduced in 25 mM DTT (Dithiothreitol in 25 mM NH_4HCO_3) for 30 min at 56°C, alkylated with 55 mM IAA (Iodoacetamide in 25 mM NH_4HCO_3) in the dark at room temperature for 30 min, washed twice in H_2O for 30 s, equilibrated in 25 mM NH_4HCO_3 for 1 min, dehydrated for 5 min in ACN/$H_2$0/NH_4HCO_3 (50%:50%:25 mM) then once more for 30 s in 100% ACN. The samples were dried again and rehydrated with 20 µl of trypsin solution (10 ng/µl trypsin Gold (PROMEGA, Madison, WI) in 25 mM NH_4HCO_3 / 0.01% ProteasMAX w/v (PROMEGA, Madison, WI)). Additional 30 µl of digestion solution (25 mM NH_4HCO_3 / 0.01% ProteasMAX w/v (PROMEGA, Madison, WI)) was added to facilitate complete rehydration and excess overlay needed for peptide extraction. The digestion was conducted for 2 h at 42°C. Peptides generated from digestion were transferred to a new tube and acidified with 2.5% TFA (Trifluoroacetic Acid) to 0.5% final then solid phase extracted (*ZipTip® C18* pipette tips Millipore, Billerica, MA).

Mass spectrometry MALDI-TOF-TOF analysis and MS/MS. Peptides were eluted from the C18 column with 1 µl of acetonitrile/H_2O/TFA (60%:40%:0.2%) into a 0.5 ml Protein LoBind tube (Eppendorf); 0.5 µl was deposited onto the Opti-TOF™ 384 Well plate (Applied Biosystems, Foster City, CA) and re-crystallized with 0.50 µl of matrix (10 mg/ml Cyano-4hydroxycinnamic acid in acetonitrile/H_2O/TFA (60%:40%:0.2%)). Peptide Map Fingerprint result-dependent MS/MS analysis was performed on a 4800 Matrix-Assisted Laser

Desorption/Ionization-Time of Fhv-Time of Flight (MALDI TOF-TOF) mass spectrometer (Applied Biosystems, Foster City, CA). A peptide fingerprint was generated by scanning a mass range of 700-4,000 Da using 1000 shots acquired from 20 randomized regions of the sample spot at 4200 intensity of OptiBeam™ on-axis Nd:YAG laser with 200Hz firing rate and 3 to 7 ns pulse width in positive reflectron mode. The fifteen most abundant precursors, excluding trypsin autolysis peptides and sodium/potassium adducts, were selected for subsequent tandem MS analysis where 1200 total shots were taken with 4700 laser intensity and 2 kV collision induced activation (CID) using air. Post-source decay (PSD) fragments from the precursors of interest were isolated by timed-ion selection and reaccelerated into the reflectron to generate the MS/MS spectrum. Raw data was deconvoluted using GPS Explorer™ software and submitted for peptide mapping and MS/MS ion search analysis against user defined database with an in-house licensed Mascot search engine (Matrix Science, London, UK).

Figure S1

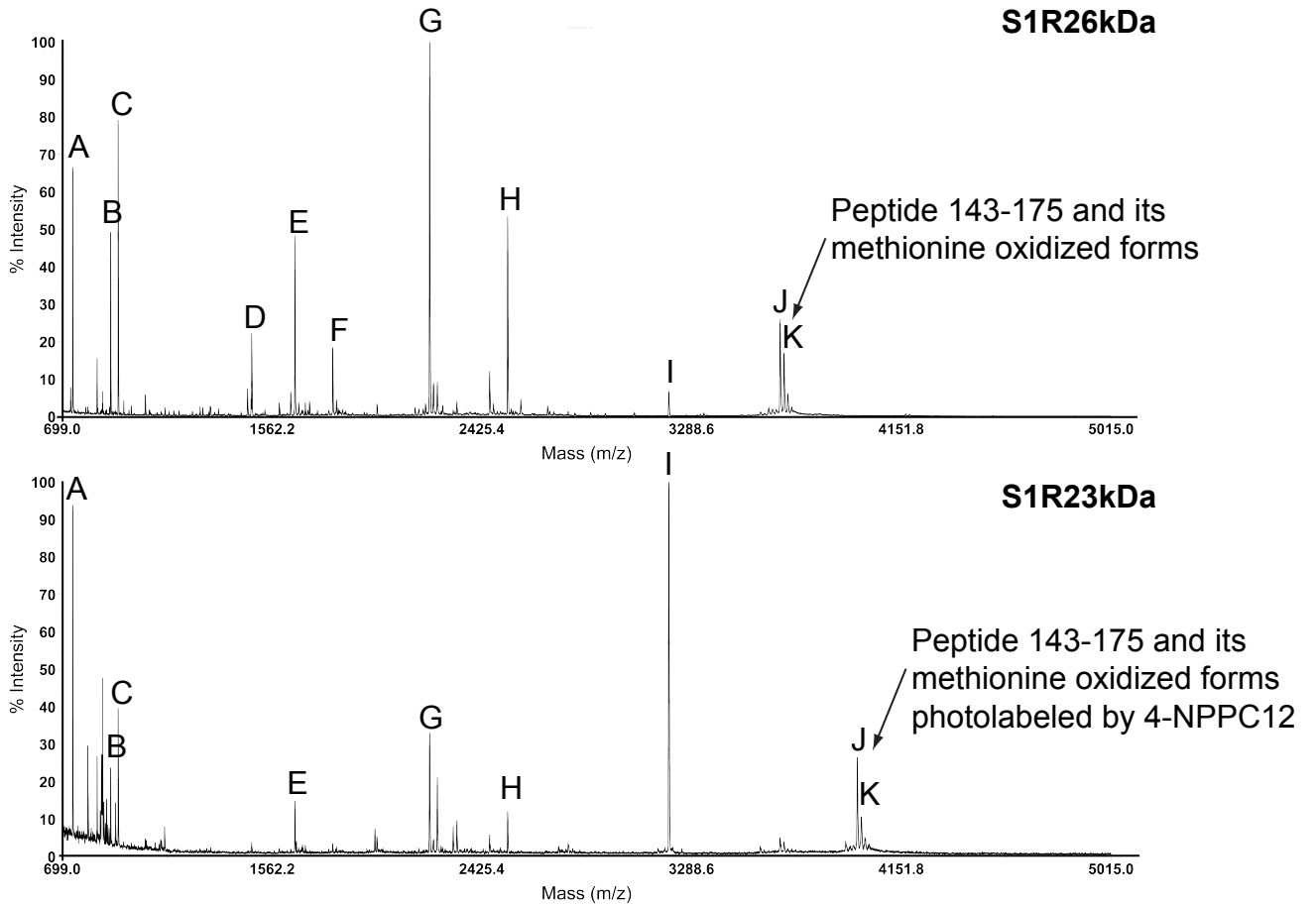

Figure S1: MALDI-TOF-TOF spectra from analyses of trypsin digested S1R26kDa and S1R23kDa species. Only peptides between 699 and 5015 Da were detected due to the limitation in the methodology. Letter notation of each peak corresponds to the peptides identified and organized by molecular weight in Tables S1 and S2.

Figure S2

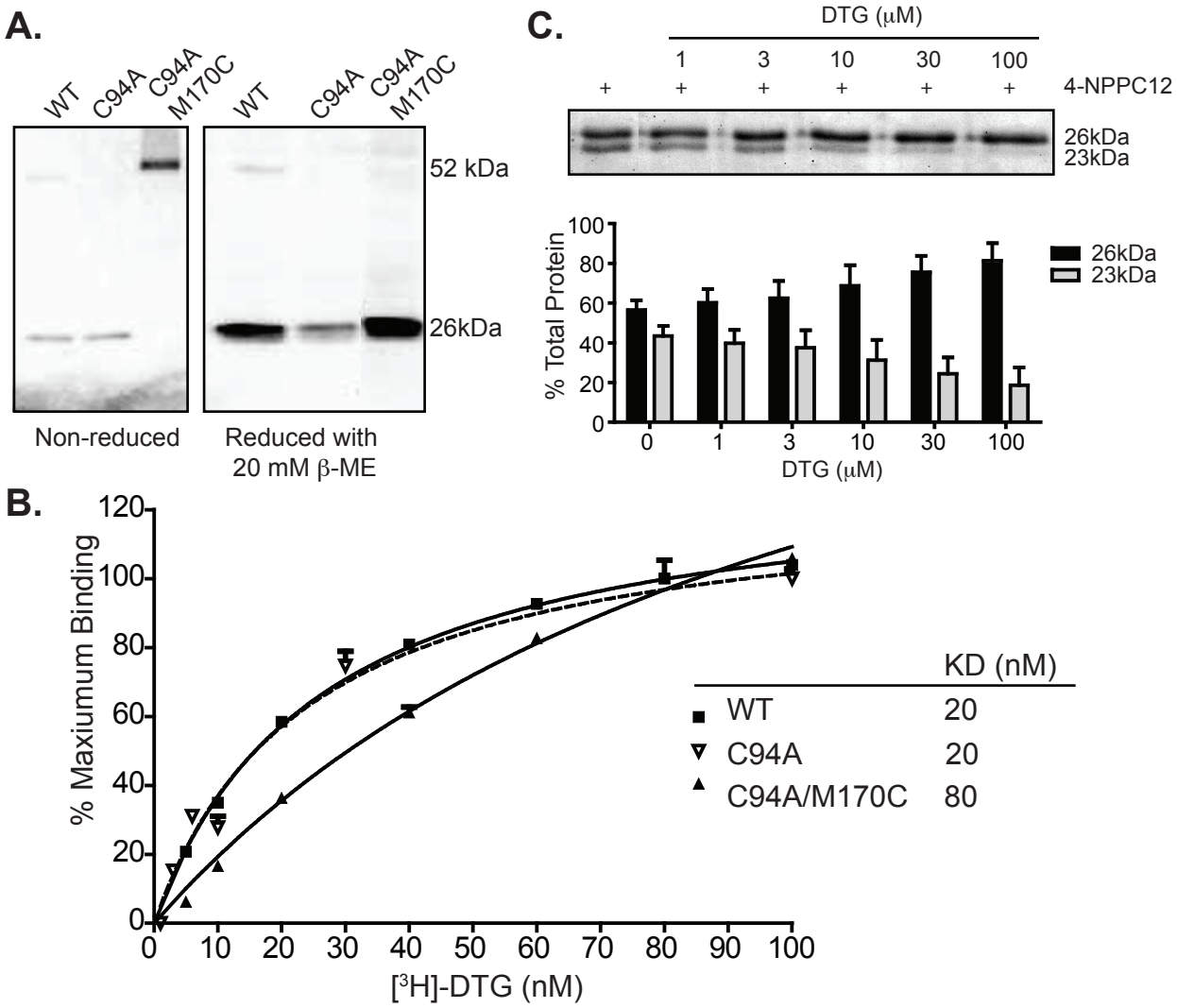

Figure S2: Evidence for sigma-1 receptor dimer formation. A. A point mutation at position 170 to cysteine in the sigma-1 receptor sequence results in a SDS-resistent β-mercaptoethanol sensitive band at the dimer position. B. Binding of [^{3}H]-DTG to the wild-type, C94A and C94A/M170C indicates comparable ligand binding affinities between these mutants. The C94A/M170C mutation was made in the 94A cysteine-less background while the wild-type has a single cysteine at position 94. The transfection for the WT, C94A and C94A/M170C were performed on separate days. The dimer maintains binding activity for [^{3}H]-DTG. C. DTG on the pure protein protected against the generation of the sigma-1 receptor 23kDa form by 4-NPPC12. The reduction in the apparent mobility shift of the 23 kDa as shown from a coomassie stained gel (upper panel) is quantified by densitometry and normalized to total protein (lower panel). The molar concentration of the pure sigma-1 receptor was considerably higher in these experiments (10 μM) as compared to the membrane samples used in the binding assays which contributed to the requirement for higher DTG concentrations to fully inhibit the formation of the S1R23kDa form.

Table S1. Peptide Mapping of the S1R26kDa form after Trypsin Digestion

Peptide Sequence	Residue Nos.[a] (Recombinant Protein)	Residue Nos.	Peak	Predicted Mass[b,c]	Observed Mass[c]
				Da	Da
[ISEFGSAT][d]MQWAVGR (Met Ox)	1 - 15	1 - 7	E	1655.8	1655.8
[ISEFGSAT][d]MQWAVGRR (Met Ox)	1 - 16	1 - 8	F	1811.9	1811.9
EEIAQLAR	48 - 55	40 - 47	C	929.5	929.5
QYAGLDHELAFSK	56 - 68	48 - 60	D	1478.7	1478.8
LIVELR	69 - 74	61 - 66	A	742.5	742.5
LIVELRR	69 - 75	61 - 67	B	898.6	898.6
YWAEISDTIISGTFHQWR	128 - 145	120 - 137	G	2210.1	2211.1
SEVFYPGETVVHGPGEATAVEWGPNTWMVEYGR	151 - 183	143 - 175	J	3651.7	3653.8
SEVFYPGETVVHGPGEATAVEWGPNTWMVEYGR (Met Ox)	151 - 183	143 - 175	K	3667.7	3669.8
GVIPSTLGFALADTVFSTQDFLTLFYTLR	184 - 212	176 - 204	I	3193.7	3194.7
ALQLELTTYLFGQDP[HHHHHH][d]	217 - 237	209 - 223	H	2531.2	2532.3

Table S2. Peptide Mapping of the S1R23kDa form after Trypsin Digestion

Peptide Sequence	Residue Nos.[a] (Recombinant Protein)	Residue Nos.	Peak	Predicted Mass[b,c]	Observed Mass[c]
				Da	Da
[ISEFGSAT][d]MQWAVGR (Met Ox)	1 - 15	1 - 7	E	1655.8	1655.8
EEIAQLAR	48 - 55	40 - 47	C	929.5	929.5
LIVELR	69 - 74	61 - 66	A	742.5	742.5
LIVELRR	69 - 75	67 - 67	B	898.6	898.6
YWAEISDTIISGTFHQWR	128 - 145	120 - 137	G	2210.1	2211.1
SEVFYPGETVVHGPGEATAVEWGPNTWMVEYGR	151 - 183	143 - 175	L	3651.7	3971.0 (3653.8+317.2*)
SEVFYPGETVVHGPGEATAVEWGPNTWMVEYGR (Met Ox)	151 - 183	143 - 175	M	3667.7	3987.0 (3669.8+317.2*)
GVIPSTLGFALADTVFSTQDFLTLFYTLR	184 - 212	176 - 204	I	3193.7	3194.7
ALQLELTTYLFGQDP[HHHHHH][d]	217 - 237	209 - 223	H	2531.2	2532.3

[a] Residue numbers based on the recombinant sigma-1 receptor which contains 8 extraneous N-term amino acids and 6 histidine tag

[b] Predicted peptide mass were obtained from Proteinprospector, MS-product, http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct.

[c] Predicted mass and observed mass values are shown for peptides generated from the recombinant protein

[d] Extraneous amino acid sequence engineered into the sigma-1 receptor as method of purification

* 317.2 Da mass addition is 31.1 Da smaller than the mass of 4-NPPC12 of 348.29 Da

Table S3: MS/MS sequencing of peptide 151 - 183 from the S1R26kDa form

Peptide Sequence	Residue Nos.[a]		Predicted Mass[b]	Observed Mass
		Y	*Da*	*Da*
R	175	1	175.1	175.1
YGR	173 - 175	3	395.2	395.1
MVEYGR	170 - 175	6	754.3	754.3
WMVEYGR	169 - 175	7	940.4	940.3
TWMVEYGR	168 - 175	8	1041.5	1041.4
PNTWMVEYGR	166 - 175	10	1252.6	1252.5
WGPNTWMVEYGR	164 - 175	12	1495.9	1495.7
EWGPNTWMVEYGR	163 - 175	13	1624.7	1625.2
AVEWGPNTWMVEYGR	161 - 175	15	1794.8	1795.1
ATAVEWGPNTWMVEYGR	159 - 175	17	1966.9	1967.8
EATAVEWGPNTWMVEYGR	158 - 175	18	2095.9	2096.0
PGEATAVEWGPNTWMVEYGR	156 - 175	20	2250.0	2250.5
GPGEATAVEWGPNTWMVEYGR	155 - 175	21	2307.0	2307.8
HGPGEATAVEWGPNTWMVEYGR	154 - 175	22	2444.1	2444.7
VHGPGEATAVEWGPNTWMVEYGR	153 - 175	23	2543.2	2543.0
TVVHGPGEATAVEWGPNTWMVEYGR	151 - 175	25	2743.3	2744.4
		B		
SEVFYPGETVVHGPGEATAVEWGPNTWM	143 - 170	28	3029.4	3029.2
SEVFYPGETVVHGPGEATAVEWGPNTWMVE	143 - 172	30	3257.5	3258.7

Table S4: MS/MS sequencing of peptide 151 - 183 from the S1R23kDa form

Peptide Sequence	Residue Nos.[a]		Predicted Mass[b]	Observed Mass
		Y	*Da*	*Da*
TWMVEYGR	168 - 175	8	1041.5	1041.5
GPNTWMVEYGR	165 - 175	11	1309.6	1309.7
WGPNTWMVEYGR	164 - 175	12	1495.7	1496.6
ATAVEWGPNTWMVEYGR	158 - 175	17	1966.9	1968.2
EATAVEWGPNTWMVEYGR	157 - 175	18	2095.9	2096.5
GPGEATAVEWGPNTWMVEYGR	155 - 175	21	2307.0	2308.1
H*GPGEATAVEWGPNTWMVEYGR	154 - 175	22	2444.1	2761.9 (2444.7 + 317.25)
TVVH*GPGEATAVEWGPNTWMVEYGR	151 - 175	25	2743.3	3061.6 (2744.4 + 317.25)
		B		
SEVFYPGETVVH*	143 - 154	12	1345.6	1663.2 (1345.6 + 317.25)

[a] Residue numbers are based on the true sigma-1 receptor sequence and does not contain the N-terminal 8 amino acids.

[b] Predicted peptide mass were obtained from Proteinprospector, MS-product, http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct.

*Residues containing 317.25 Da addition