ChemBioChem

Supporting Information

Probing the Conformation States of Neurotensin Receptor 1 Variants by NMR Site-Directed Methyl Labeling

Inguna Goba, David Goricanec, Dominik Schum, Matthias Hillenbrand, Andreas Plückthun, and Franz Hagn*

Supporting Information Methods

Construct design and mutagenesis

rNTR1

Stabilized rNTR1 variant constructs – HTGH4 ΔIC3, HTGH4-L167R (BM) ΔIC3 and TM86V-L167R (BM) ΔIC3 – have been reported previously[1]. Site-directed mutagenesis (QuikChange Lightning Mutagenesis Kit, Agilent Technologies) was used to generate rNTR1 constructs with a cysteine residue at position 300 (V300C) (located at the cytoplasmic end of TM6) in the HTGH4-BM and TM86V-BM variants, the mutations R101T, D113S, D124E, E150D, A260I, R262N-R263K in the HTGH4-BM V300C variant and a single C386S mutation within HTGH4-BM variant for resonance assignment (**Tab. S1**).

Table S1. Primers used for mutagensis of HTGH4-BM

Primer	Sequence (5' → 3')	Length (bp)	Application
HTGH4-C386S-1	CTTTCTGTCCACGCTGGCCAGCCTTTGTCCTGGGACCC	38	Mutagenesis HTGH4-BM-C386S
HTGH4-C386S-2	GGGTCCCAGGACAAAGGCTGGCCAGCGTGGACAGAAAG	38	
NTR1-V300C-1	GACCATCGAGCCGGGTCGTTGCCAGGCCCTGCGCCGCG	38	Mutagenesis HTGH4-BM-V300C and TM86V-BM -V300C
NTR1-V300C-2	CGCGGCGCAGGGCCTGGCAACGACCCGGCTCGATGGTC	38	
HTGH4-R101T-1	GCAGAGCCTGCAGAGCACGGTGGATTACTACCTGGGC	37	Mutagenesis HTGH4-BM-V300C-R101T
HTGH4-R101T-2	GCCCAGGTAGTAATCCACCGTGCTCTGCAGGCTCTGC	37	
HTGH4-D113S-1	GGCAGCCTGGCACTGTCTTCTCTGCTTATCCTTCTGTTTGCC	42	Mutagenesis HTGH4-BM-V300C-D113S
HTGH4-D113S-2	GGCAAACAGAAGGATAAGCAGAGAAGACAGTGCCAGGCTGCC	42	
HTGH4-D124E-1	CTGTTTGCCCTGCCCGTGGAACTATACAACTTCATCTGG	39	Mutagenesis HTGH4-BM-V300C-D124E
HTGH4-D124E-2	CCAGATGAAGTTGTATAGTTCCACGGGCAGGGCAAACAG	39	
HTGH4-E150D-1	GGCTACTATTTCCTGCGTGATGCCTGCACCTATGCC	36	Mutagenesis HTGH4-BM-V300C-E150D
HTGH4-E150D-2	GGCATAGGTGCAGGCATCACGCAGGAAATAGTAGCC	36	
HTGH4-A260I-1	CCTCCATCCTAAACACCGTGATTGCCAGAAGACTGACAGTCATG	44	Mutagenesis HTGH4-BM-V300C-A260I
HTGH4-A260I-2	CATGACTGTCAGTCTTCTGGCAATCACGGTGTTTAGGATGGAGG	44	
HTGH4-R262N-R263K-1	CCTAAACACCGTGGCAGCCAATAAACTGACAGTCATGGTGCACC	44	Mutagenesis HTGH4-BM-V300C-R262N-R263K
HTGH4-R262N-R263K-2	GGTGCACCATGACTGTCAGTTTATTGGCTGCCACGGTGTTTAGG	44	

Gαi1 helix 5

The GB1 fusion construct of Gαi1 helix 5 was designed harboring an N-terminal His_6-tag, GB1 followed by a tobacco-etch-virus (TEV) protease cleavage site in a pET25b plasmid. The final amino acid sequence of the GB1-TEV-fusion construct of Gαi1 helix 5 is:
MSYYHHHHHHDYDIPTTAMEYKLILNGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDD
AKTFTVTEIPTTENLYFQGGTDAVTDVIIKNNLKDCGLF.

Protein production and purification

rNTR1

The expression and purification of all rNTR1 variants were performed as described previously with minor modifications.[1a, 2] Briefly, the expression of rNTR1 was performed in NEB® Express I^q competent *E. coli* cells (New England Biolabs) in 2xYT medium with 100 µg/mL ampicilin overnight at 20 ºC, 130 rpm and induced at an OD_{600} of 0.75 with 1 mM isopropyl thiogalactopyranoside (IPTG). Cells were harvested after 20 h expression by centrifugation (6,000 × g, 20 min, 4 °C). Cells were then lysed and solubilized by sonication in 2x solubilization buffer containing 100 mM HEPES pH 8.0, 20% glycerol (w/v), 400 mM NaCl, 2.5 mM $MgCl_2$, 0.6/0.12% CHAPS/CHS, 1.7% DM (w/v), 100 mg lysozyme, one tablet of Complete Protease Inhibitor Cocktail (Roche), and 250 U benzonase. All subsequent steps were carried out at 4°C or on ice. After sonication 10 mM EDTA was added and further stirred at 1,000 rpm for 30 min. To separate cell debris from the lysate centrifugation was performed (50,000 x g, 30 min, 4 °C). The supernatant was mixed with the corresponding pD-NT resin (wild type, mutant P10A or Y11A)[2] pre-equilibrated in 5 mL NT wash buffer 1 (25 mM HEPES pH 8.0, 10% glycerol (w/v), 600 mM NaCl, 0.5% DM (w/v)) and incubated overnight at 4 °C on a nutating shaker. The flow-through from the pD-NT resin was then discarded, and the resin was washed with 20 mL NT wash buffer 1 and 10 mL NT wash buffer 2 (25 mM HEPES pH 7.0, 10% glycerol (w/v), 150 mM NaCl, 2 mM DTT, 0.3% DM (w/v)). The pD-NT resin was resuspended in 2 mL of NT wash buffer 2 within the column, containing 0.7 mg of HRV 3C protease (produced in house), followed by incubation for 2 h at 4 °C on a roller mixer. For the preparation of antagonist bound NTR1 with mutant NT1 resin without a 3C cleavage site (P10A or Y11A), 4.3 mM SR142948[3] was added at this step for elution together with the 3C protease. The column was drained and further eluted by NT wash buffer 2 to give a total of 10 ml elution. Subsequently, 1 ml of 10% DDM was added to the eluted rNTR1, diluted threefold by SP binding buffer (10 mM HEPES pH 7.0, 10% glycerol (w/v), 2 mM DTT, 0.05% DDM (w/v), 0.3 µM SR142948 (for antagonist-bound receptor preparations) or 0.1 µM NT1 peptide (for agonist-bound receptor preparations) and subjected to 5 mL SP Sepharose (bed volume) resin, which had been pre-equilibrated with SP binding buffer. The SP column was washed with 20 mL SP binding buffer, 25 mL SP wash buffer (10 mM HEPES pH 7.7, 10% glycerol (w/v), 35 mM NaCl, 2 mM DTT, 0.05% DDM (w/v), 0.3 µM SR142948 or 0.1 µM NT1 peptide, respectively), followed by another 5 mL SP binding buffer. Elution was carried out by 15 mL SP elution buffer (10 mM HEPES pH 7.0, 10% glycerol (w/v), 350 mM NaCl, 2 mM DTT, 0.05% DDM (w/v), 0.5 µM NT1 peptide or 0.3 µM SR142948). The eluate was concentrated by an Amicon-15 Ultra spin concentrator with 50 kDa MWCO to ~1 mL and loaded on a Superdex 200 10/300 Increase (S200a) column (GE Healthcare) that was pre-equilibrated with running buffer (10 mM HEPES pH 8.0, 150 mM NaCl, 2 mM DTT, 0.02% DDM (w/v), 0.1 µM NT1 peptide or 0.3 µM SR142948). Fractions containing pure and homogenous proteins were collected and stored at 4 °C for further use, i.e. verification of elution fractions by SDS-PAGE analysis or pooling of desired elution fractions for further ^{13}C methyl labeling experiments. Typically expression yields of rNTR1 variants are between 0.5-2.0 mg/L.

$G\alpha_{i,1}$ helix 5

The plasmid containg the described $G\alpha_{i,1}$ helix 5 GB1 fusion protein construct was introduced into *E. coli* BL21(DE3) (New England Biolabs) and the cells were grown in 1L LB medium supplemented with 100 µg/mL ampicilin to an OD_{600} of 0.8 at 37 °C followed by induction of protein production by the addition of 1 mM IPTG. The bacterial culture was grown at 37 °C for a further 3 h and harvesting by

centrifugation (6,000 × g, 20 min, 4 °C). Cell pellets were solubilized in 50 mM Tris/HCl pH 8.0, 500 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol containing one tablet of Complete protease inhibitor cocktail (Roche), and lysed by the addition of 2 mg/mL lysozyme followed by sonication and digestion of high-molecular weight DNA by the additon of 5 U/mL benzonase (+5 mM $MgCl_2$). Cell debris was removed by centrifugation (50,000 x g, 30 min, 4 °C) and the cell extract was loaded on a Ni-NTA affinity column of 5 mL bed volume equilibrated with 25 mL buffer A (50 mM Tris/HCl pH 8.0, 500 mM NaCl, 10 mM β-mercaptoethanol). After washing with 50 mL buffer A and buffer A + 10 mM imidazole, Gαi1 helix 5 was eluted with buffer A supplemented with 350 mM imidazole. The elution fraction was dialyzed against 20 mM NaPi pH 7.5, 20 mM NaCl, 1 mM EDTA, 2 mM DTT overnight at 4 °C in a 3.5 kD MWCO dialysis membrane (Carl Roth, Karlsruhe, D). After dialysis, the GB1 tag was removed by proteolytic digestion with TEV protease (1 A_{280} TEV for 100 A_{280} protein) for 48 h at 4 °C follwed by buffer exchange to 20 mM NaPi pH 7.8, 200 mM NaCl, 10 mM β-mercaptoethanol in a 500 Da MWCO dialysis membrane (SpectraPor) overnight at 4 °C. This solution was then subjected to a reverse Ni-NTA precedure where the desired peptide product is now in the flow-through since the His_6-tag is attached to the fusion protein GB1. This fraction was lyophilized and then dissolved in 350 µL of NMR buffer (20 mM NaPi pH 7.0, 50 mM NaCl, 2 mM DTT). Due to a lack of sufficient UV absorption at 280 nm, the concentration of Gαi1 helix 5 was determined by UV absorption of the peptide backbone at 205 nm according to a published procedure[4]. The final yield of the $G\alpha_{i,1}$ helix 5 peptide is typically around 1 mg L^{-1}.

G-protein [^{35}S]GTPγS exchange stimulation assay

The [^{35}S]GTPγS assay was performed according to a previously described protocol[1, 5] with slight modifications. Briefly, the expression of rNTR1 was performed in Sf9 insect cells and rNTR1-containing membranes were isolated and urea-washed to remove endogenous G proteins. The amount of rNTR1 in the isolated membranes was determined by ligand binding experiment using [^{3}H]NT. G protein ($\alpha_{i1}\beta_1\gamma_1$) was also expressed in Sf9 insect cells and purified as described before[1, 5]. For the GTP exchange assay, urea-washed membranes containing 1 nM of GPCR and 100 nM purified G protein ($\alpha_{i1}\beta_1\gamma_1$) were stimulated with either no ligand, 200 µM NT or 200 µM SR142948. The reaction was stopped after 5 min at 25°C by filtration over nitrocellulose filters (MultiscreenHTS-HA plates).

S-methyl-^{13}C methanethiosulfonate (^{13}C-MMTS) labeling

MMTS labeling was done according to a published protocol[6] with specific modifications required for a membrane protein. After chromatographic separation by a semi-preparative S200 (300/10) column the pooled fractions of each rNTR1 variant were concentrated in Amicon 15 centrifugal devices (30,000 MWCO) to approx. 250 µL and washed with 3 x 12.5 mL ^{13}C methyl labelling buffer (50 mM NaPi pH 7.5, 1 mM EDTA and 0.02% DDM (w/v), 0.1 µM NT1 peptide or 0.3 µM SR142948) to remove the reducing agent DTT. After buffer exchange, 1 mL of protein sample was transferred to a reaction tube and the exact concentration of the protein sample was determined by UV/Vis spectroscopy for subsequent S-methyl-^{13}C-methanethiosulfonate (^{13}C-MMTS) labeling[6]. The concentration of (^{13}C)-MMTS (Sigma-Aldrich, 100 mM stock in DMSO) was adjusted to a 5-fold molar excess per cysteine residue in the GPCR and the mixture was incubated overnight at 4°C on a rotating mixer.

Subsequently ^{13}C-methyl labeled rNTR1 was washed with 3 x 12.5 mL NMR buffer (20 mM NaPi pH 7.0, 50 mM NaCl, 0.5 mM EDTA, 0.02% DDM (w/v), 0.1 µM NT1 peptide or 0.3 µM SR142948) to remove the remaining free ^{13}C-MMTS reagent. To verify labeling success 1-3 mg/mL of the unlabeled or ^{13}C methyl labeled rNTR1 variant was used for ESI-MS analysis.

ESI-mass spectrometry (ESI-MS)

The MS experiments were conducted on an LCQ-FLEET (Thermo Scientific) system equipped with a 3D ion trap and using electro spray ionization (ESI). The instrument is connected to a high-performance liquid chromatography (HPLC) system (Thermo UltiMate 3000, column: Dionex with a Retain PEP, Drop-in, 10×2.1 mm).

NMR experiments

All 2D-[^{1}H,^{13}C]-HMQC correlation spectra were recorded at 313 K on a 800 MHz Bruker Avance III spectrometer equipped with a triple resonance TCI cryoprobe with z-axis gradient. Each sample was prepared containing 5% D_2O in a 5 mm diameter Shigemi NMR tube. The NT1 peptide was dissolved in H_2O (5 mM stock), SR142948 in d_6-DMSO (50 mM stock) and was directly added to the protein sample before data acquisition at a final concentration ratio of 1:1. For all experiments, the spectral widths were set to 11,160.7 Hz (14 ppm) and 603.6 Hz (3 ppm) for the ^{1}H and ^{13}C dimensions, respectively. The recycle delay was set to 1 s. The ^{13}C carrier frequency was set to 22.5 ppm, and the ^{1}H carrier to 4.7 ppm. All 2D-[^{1}H,^{13}C]-HMQC experiments were conducted with 256 increments (128 complex points) in the indirect ^{13}C dimension and 128 to 512 transients, depending on the final sample concentration. NMR data were processed (2k x 1k data points in the direct and indirect dimension, respectively) in Topspin version 3.5.6 (Bruker Biospin) (apodization with a Gauss window function: LB=-10 Hz; GB=0.1) and analyzed in NMRFAM-Sparky 1.414[7], based on Sparky4 (Goddard and Kneller, UCSF).

Circular dichroism (CD) spectrophotometry

The Far-UV CD spectra of rNTR1 variants were recorded using a Jasco J-715 CD spectropolarimeter (Groß-Umstadt, Germany) at a scanning speed of 100 nm/min and a data pitch of 0.5 nm from 260 nm to 195 nm in a 0.1 cm path length cuvette. Thermal stability scans were monitored at a wavelength of 222 nm from 20 to 100°C, a resolution of 0.5°C and a heating rate of 60°C/h. Thermal transitions were fitted with a Boltzmann equation[8]. The concentration of the rNTR1 variant was set to 10 µM in 10 mM NaPi pH 7.0, 2 mM DTT, 0.02% DDM (w/v), 0.1 µM NT1 peptide or 0.3 µM SR142948.

Supporting Information References

[1] aP. Egloff, M. Hillenbrand, C. Klenk, A. Batyuk, P. Heine, S. Balada, K. M. Schlinkmann, D. J. Scott, M. Schutz, A. Plückthun, *Proc Natl Acad Sci U S A* **2014**, *111*, E655-662; bK. M. Schlinkmann, M.

Hillenbrand, A. Rittner, M. Kunz, R. Strohner, A. Plückthun, *J Mol Biol* **2012**, *422*, 414-428; cD. J. Scott, L. Kummer, P. Egloff, R. A. Bathgate, A. Plückthun, *Biochim Biophys Acta* **2014**, *1838*, 2817-2824.

[2] P. Egloff, M. Deluigi, P. Heine, S. Balada, A. Plückthun, *Protein Expr Purif* **2015**, *108*, 106-114.

[3] D. Gully, B. Labeeuw, R. Boigegrain, F. Oury-Donat, A. Bachy, M. Poncelet, R. Steinberg, M. F. Suaud-Chagny, V. Santucci, N. Vita, F. Pecceu, C. Labbé-Jullié, P. Kitabgi, P. Soubrié, G. Le Fur, J. P. Maffrand, *J Pharmacol Exp Ther* **1997**, *280*, 802-812.

[4] N. J. Anthis, G. M. Clore, *Protein Sci* **2013**, *22*, 851-858.

[5] Y. Shibata, J. F. White, M. J. Serrano-Vega, F. Magnani, A. L. Aloia, R. Grisshammer, C. G. Tate, *J Mol Biol* **2009**, *390*, 262-277.

[6] T. L. Religa, A. M. Ruschak, R. Rosenzweig, L. E. Kay, *Journal of the American Chemical Society* **2011**, *133*, 9063-9068.

[7] W. Lee, M. Tonelli, J. L. Markley, *Bioinformatics* **2015**, *31*, 1325-1327.

[8] P. L. Privalov, *Adv Protein Chem* **1979**, *33*, 167-241.

Supporting Information Figures

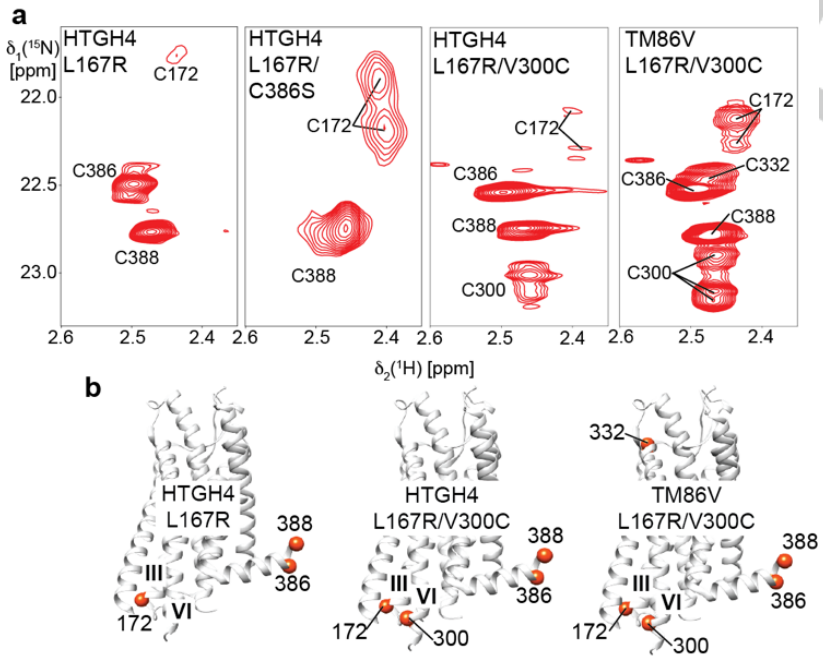

Figure S1. Mutagenesis-based assignment strategy of methyl resonances in MMTS-labelled HTGH4 and TM86V NTR1 variants. (a) C-terminal C386 and C388 in HTGH4 are flexible and give rise to a sharp peak whereas C172, located at the G-protein binding interface, experiences line broadening. The assignment can be resolved by a single C386S mutation leading to the disappearance of one of the strong signals. Addition of an additional cysteine in V300C clearly leads to new peaks, as well as C332 present in TM86V. (b) Location of the cysteine residues in HTGH4 and TM86V and their variants. Helices III and VI that are involved in G-protein binding are labeled.

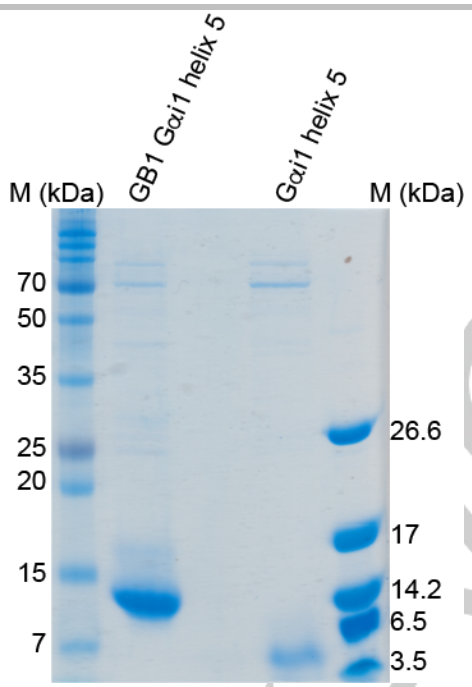

Figure S2. Purification of C-terminal helix 5 of the inhibitory G-protein alpha subunit type 1 ($G\alpha_{i,1}$) as a fusion protein with GB1 containing a TEV protease cleavage site. The fusion protein appears on the SDS-PAGE gel at ~13 kDa (calculated MW = 11.7 kDa) and the G-alpha C-terminal helix at ~4 kDa (calculated MW = 2.2 kDa).

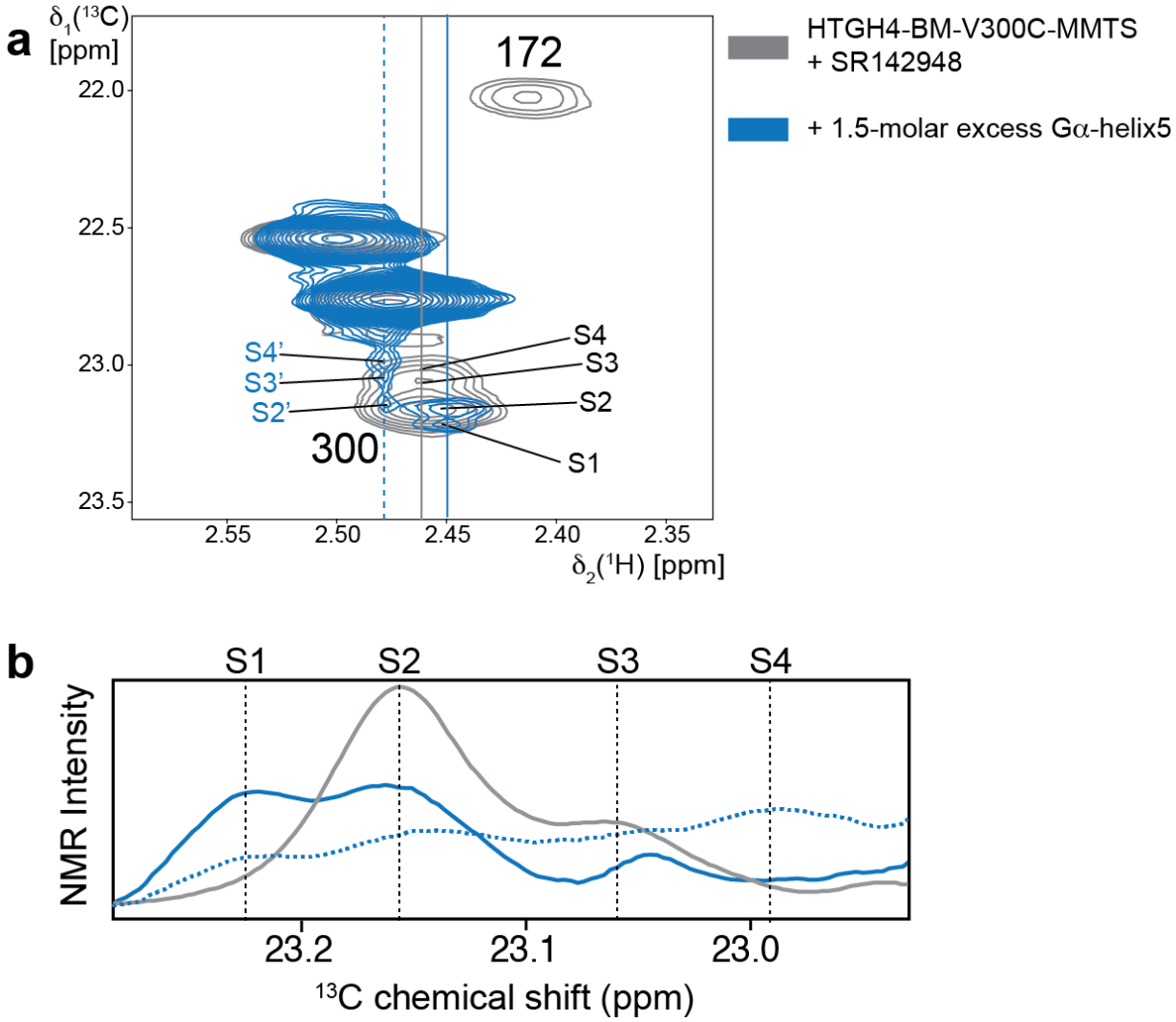

Figure S3. NMR titration of ^{13}C-MMTS-labeled HTGH4-BM-V300C in the antagonist bound form (SR142948) with a peptide derived from the C-terminal helix 5 of a $G\alpha_{i,1}$ subunit. (a) 2D-[^{13}C,^{1}H]-HMQC experiments of both states reveals marked differences in signal intensity and chemical shifts for amino acid residues close to the cytoplasmic G-protein coupling interface (residues 172 and 300). HTGH4 binds to the G-protein even in the antagonist-bound form leading to complete line broadening at position 172 and chemical shift perturbations as well as changes in signal intensity at position 300. (b) Slices along the axes in the ^{13}C dimension indicated in panel (a) show that mostly the S2 state and to some extent the S3 state is populated in complex with the antagonist (grey line). Upon addition of the G-protein peptide we observe a splitting of the NMR resonances according to the indicated states. One set of signals corresponding to the S1 and S2 states remain at a similar position as observed without the peptide (blue line). In addition, a second set of signals appears at a different ^{1}H frequency (broken blue line). There, the active states S3 and S4 are populated to a higher extent, suggesting that these signals originate from the G-protein-bound species. In order to distinguish between the two sets of signals, we denote these as S1-S4 and S1'-S4'. These data indicate that there is an equilibrium between free and G-protein bound receptor in slow exchange giving rise to two sets of NMR signals. The free form adopts mostly the inactive states, whereas the G-protein-bound form is predominantly present in the active conformation.