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Supplementary Materials for

Conformational dynamics of a G protein–coupled receptor helix 8 in lipid membranes

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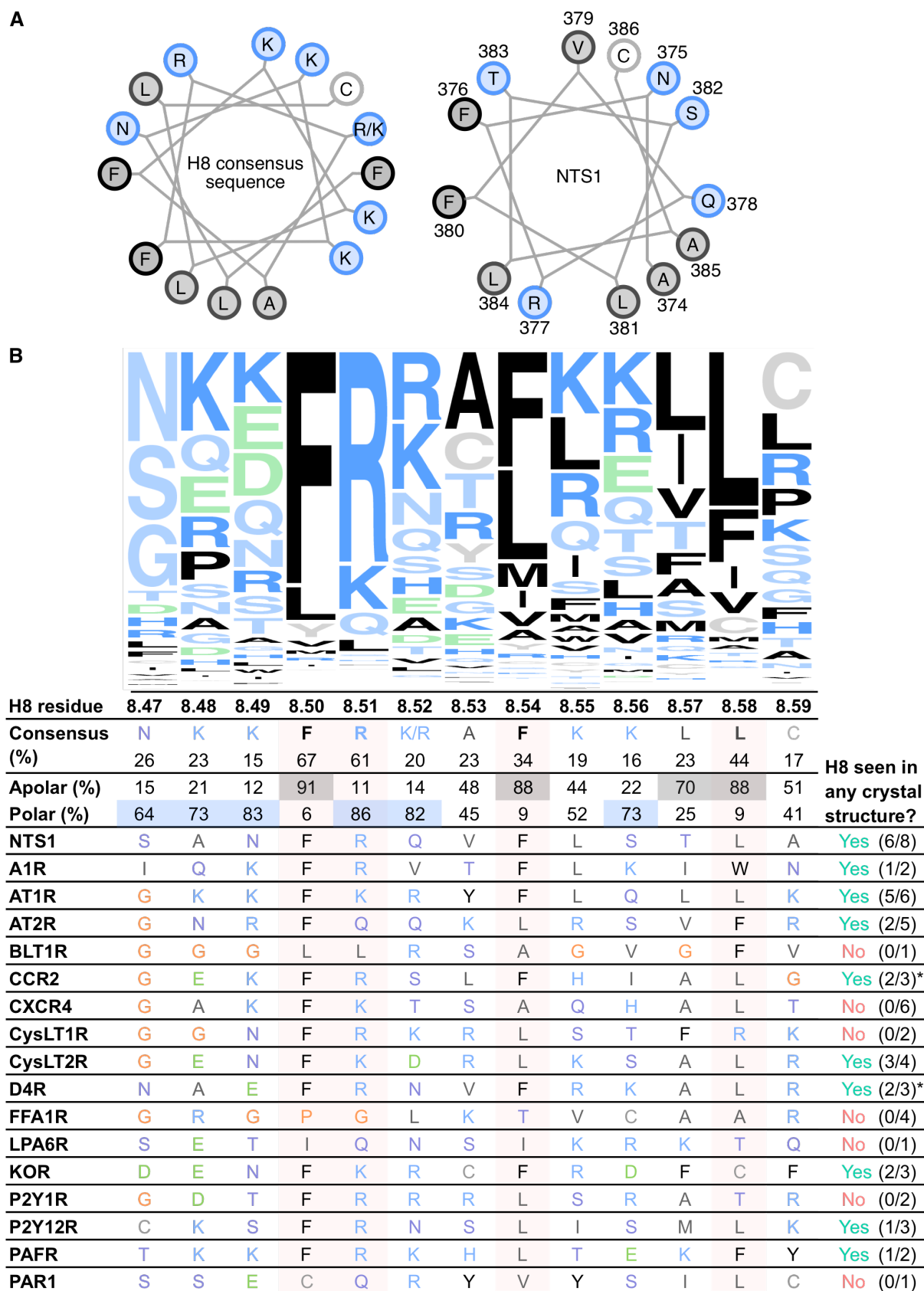


Fig. S1. H8 sequence analysis. (A) The consensus and the rat neurotensin receptor 1 (NTS1) H8 sequences are shown in a helical wheel diagram where polar, apolar and aromatic residues are colored blue, grey, and black, respectively. Clustering of polar/apolar residues suggests H8 forms an amphipathic helix. (B) The consensus sequence of H8 derived from alignment of all class A GPCR

sequences available from the GPCR database (GPCRDB) (41), and a more detailed sequence analysis generated by WebLogo (42) (top) are shown. The table shows the percentage identity for each of the amino acid in the consensus sequence over all of the aligned sequences for each residue position, as well as the percentage of apolar or polar residues found in the alignment at each residue position. As evident from the WebLogo frequency plot, for most positions the exact amino acid identity is not conserved, but the pattern of polar (blue/green letters) and apolar (black letters) residues is. Percentages do not add up to 100%, as some residues are not found in all receptors. Cells are shaded grey or light blue, when a clear consensus for apolar or polar residue, respectively, was found. The sequence of the corresponding H8 region is also given for NTS1 (13, 16) and all other GPCRs for which a structure has previously been solved lacking a structured H8, or where H8 was found in an unusual conformation (43–62). Red shading highlights residue positions corresponding to the semi-conserved F(R/K)xx(F/L)xxx(L/F) motif. The last column details the number of structures that have been solved for each receptor type and whether any of the solved structures did show a structured H8. For the angiotensin II receptor type 2, AT2R, H8 was structured, but was located in the intracellular cleft, interacting with TM3, 5 and 6, rather than lying parallel to the membrane (55). A non-standard inward-facing H8 conformation was also seen for one of the published platelet-activating-factor receptor (PAFR) structures, where H8 was wedged in between TM2 and 4 (48). For the angiotensin II receptor type 1, AT1R, the authors suggested that the destabilization of H8 may have been due to a 4-residue C-terminal truncation (44). *(For the CC chemokine receptor type 2 (CC2R) and for the D₄ dopamine receptor (D4R) H8 was unstructured in only one of the protomers in the resolved asymmetric unit, and (partially) structured in the other.)

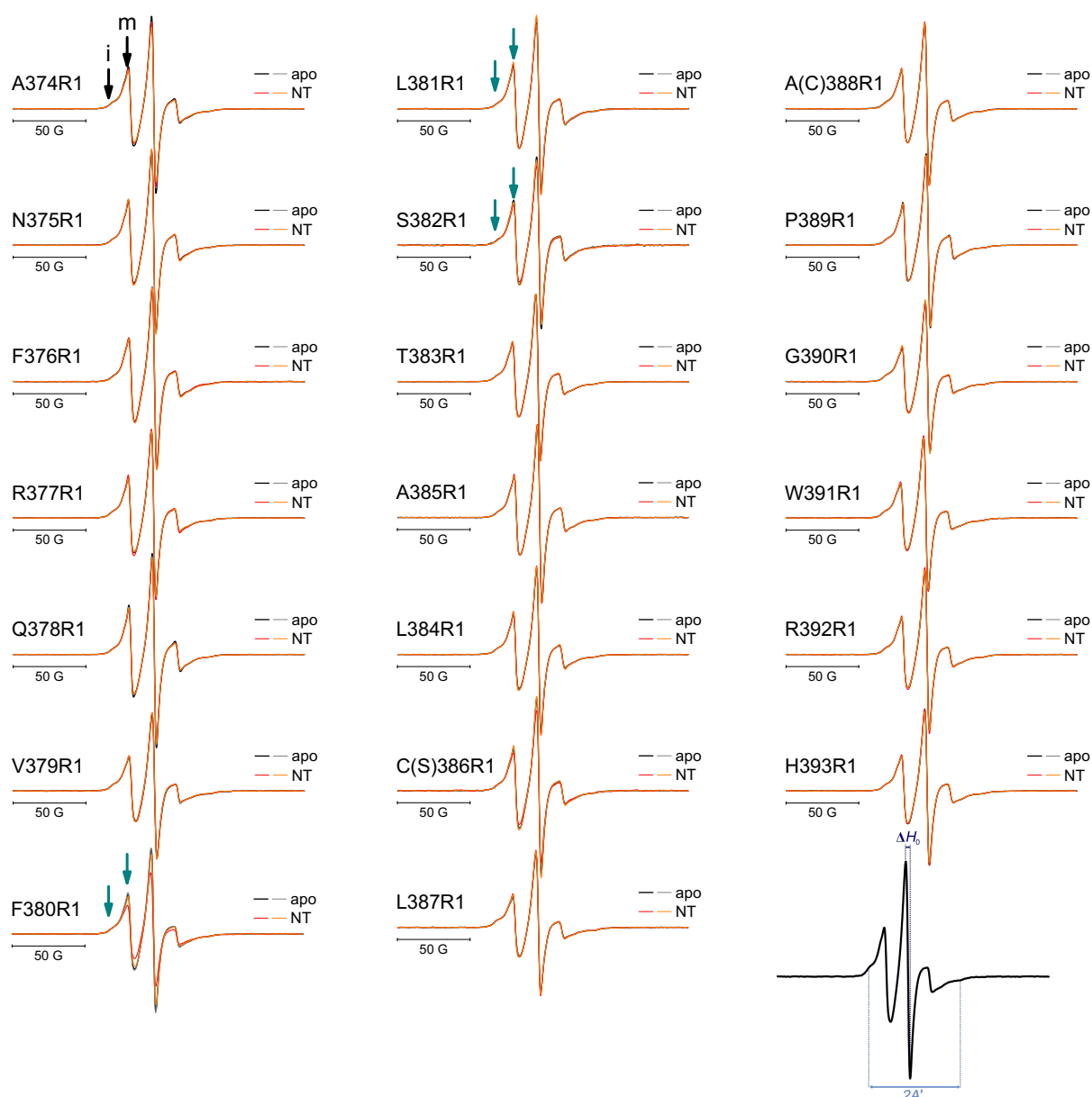


Fig. S2. EPR spectra of H8 and C-terminus recorded at 295 K. Sequential single cysteine mutants of NTS1 were labeled with MTSL, generating the R1 side chain. EPR spectra were recorded at 295 K for residues along the C-terminal stretch after TM7, encompassing the putative H8. Spectra were recorded in the absence (black and grey lines) and presence of agonist neurotensin (NT, red and orange lines); note that in most cases the spectra of the duplicates overlap and cannot be distinguished from one another. The spectra are multi-component, containing a more and less motionally restricted component labeled "i" and "m", respectively, on the spectra of A374R1. Cyan arrows indicate these spectral components for a number of spectra showing the largest changes in the presence of ligand relative to the apo state (F380R1, L381R1, and S382R1). The spectral parameters ΔH_0 (central peak width) and $2A'_{zz}$ (maximum apparent splitting) as defined in the bottom right panel can be used to assess spin label mobility. Spectra have been normalized to the integrated intensity of the absorption spectra.

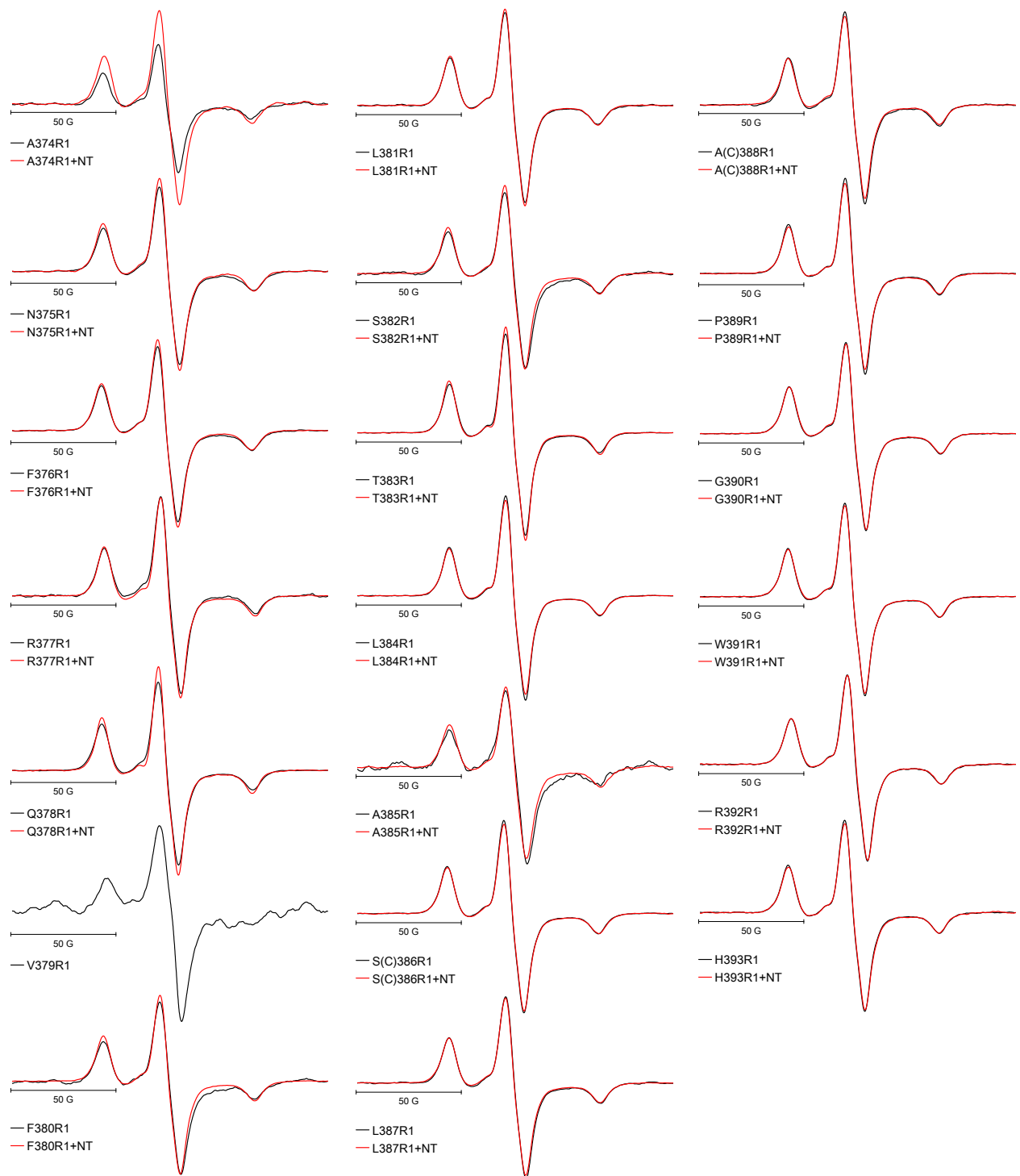


Fig. S3. EPR spectra of H8 and C-terminus recorded at 170 K. EPR spectra were recorded at 170 K, in the absence and presence of agonist neurotensin (+NT) for spin-labeled residues along the C-terminal stretch after TM7 as described in fig. S2 (different samples were used for the data in the two figures). Spectra have been normalized to the integrated intensity of the absorption spectra. These spectra were used to determine the rigid limit $2A_{zz}^R$ (rigid limit maximum outer splittings) which was subsequently used to estimate the rotational correlation times (τ_c) from the maximum outer splitting $2A_{zz}'$ of the spectra recorded at 295 K using the method of Freed and co-workers (21).

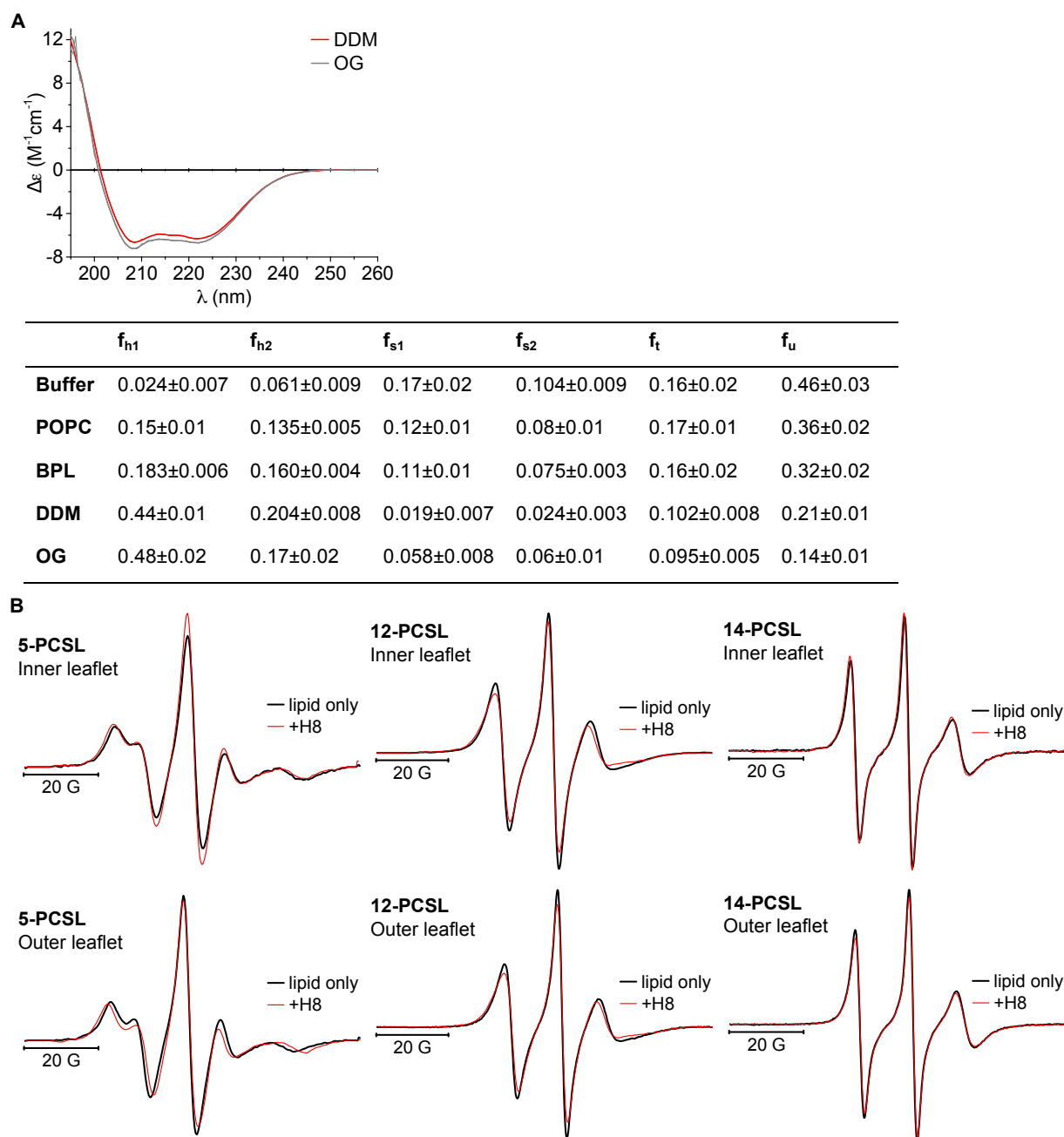


Fig. S4. Spectroscopic analysis of influence of a membrane (mimetic) on H8 and vice versa. (A)

The molar circular dichroism ($\Delta\epsilon$) was measured for a peptide (0.1 mg/mL) corresponding to a short NTS1 C-terminal stretch (S373-C388) including the H8 sequence. The peptide was dissolved in saline buffer supplemented with 1% octyl β -D glucoside (OG, grey line), or 0.1% dodecyl β -D maltoside (DDM, red line), both supplemented with 0.01% cholesteryl hemisuccinate (all percentages are in w/v). Smoothed data (fat line) are plotted on top of the raw data. The table gives the fraction of ordered helices (f_{h1}), disordered helices (f_{h2}), ordered strands (f_{s1}), disordered strands (f_{s2}), turns (f_t), and unordered sequences (f_u) contributing to the total secondary structure as determined by spectral analysis of the CD data (63). The data were smoothed in OriginPro 8.5 (OriginLab) using Savitzky-Golay filtering (10 nm window, 2nd order polynomial) and the DichroWeb server (64) was used to estimate secondary structure; data were fitted using multiple algorithms (K2D, CONTIN, CDSSTR, and SELCON3 (65–68)) and reference data sets (sets 4, 7, SP175 and SMP180 (69–71)), and results giving a normalized root-

mean-square deviation (NRMSD) below 0.1 were averaged (72). Contributions for f_{h1} and f_{h2} were added to get the total fraction of helix (63). **(B)** EPR spectra were recorded of large unilamellar POPC vesicles (100 nm) with DOXYL-PC spin-labeled lipid (PCSL) specifically installed in either the outer or inner leaflet of the vesicle. Spectra were recorded at room temperature at X-band in the absence (lipid only, black lines) and presence (+H8, red lines) of the S373-C388 H8 peptide. Three types of DOXYL-PC spin labeled lipids were used with the nitroxide located either at the 5-position of the carbon chain (5-PCSL), closest to the external headgroup region, or closer to the center of the bilayer at position 12 (12-PCSL) or 14 (14-PCSL) of the carbon chain. Spectra have been normalized to the integrated intensity of the absorption spectra.

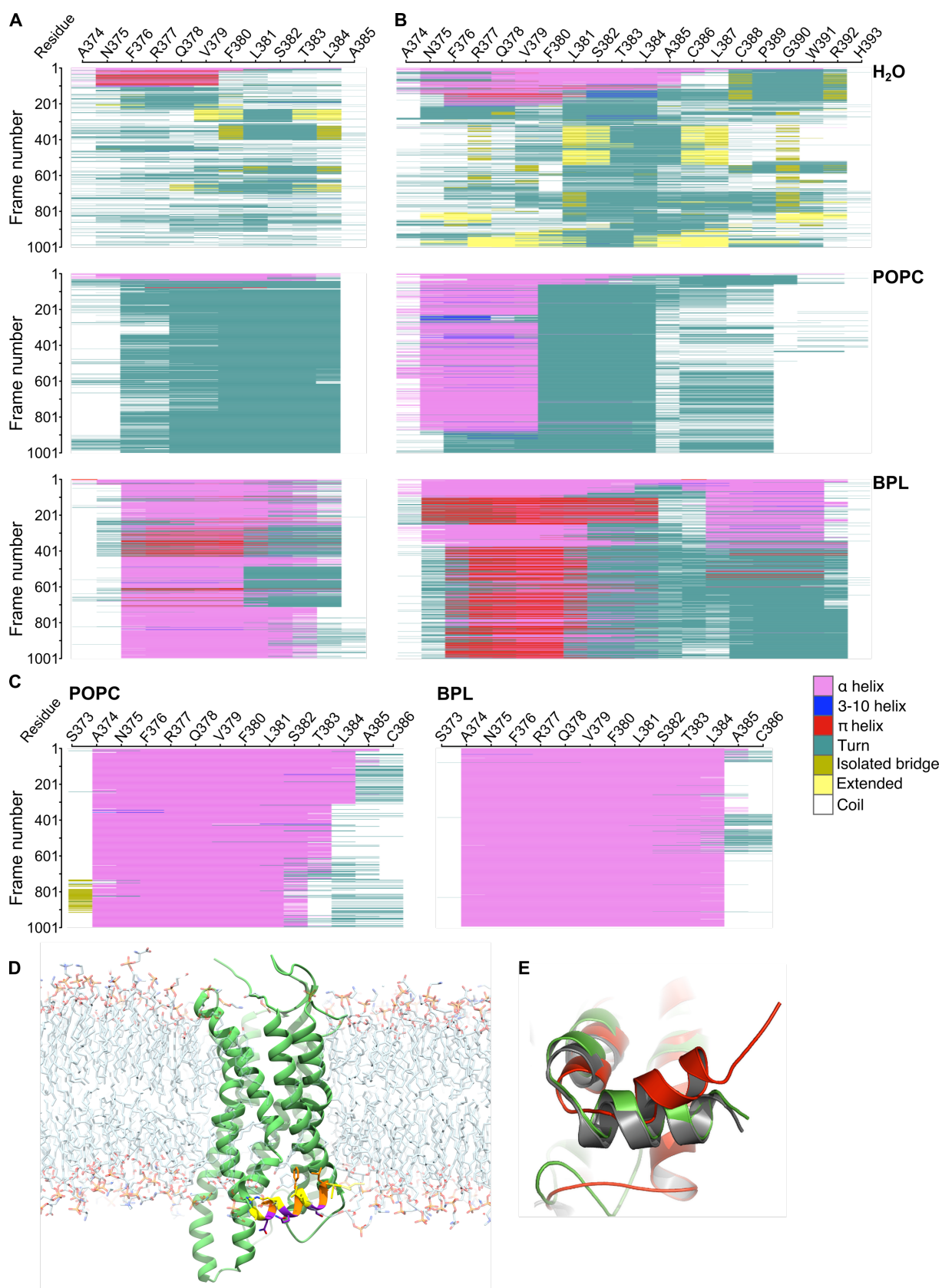
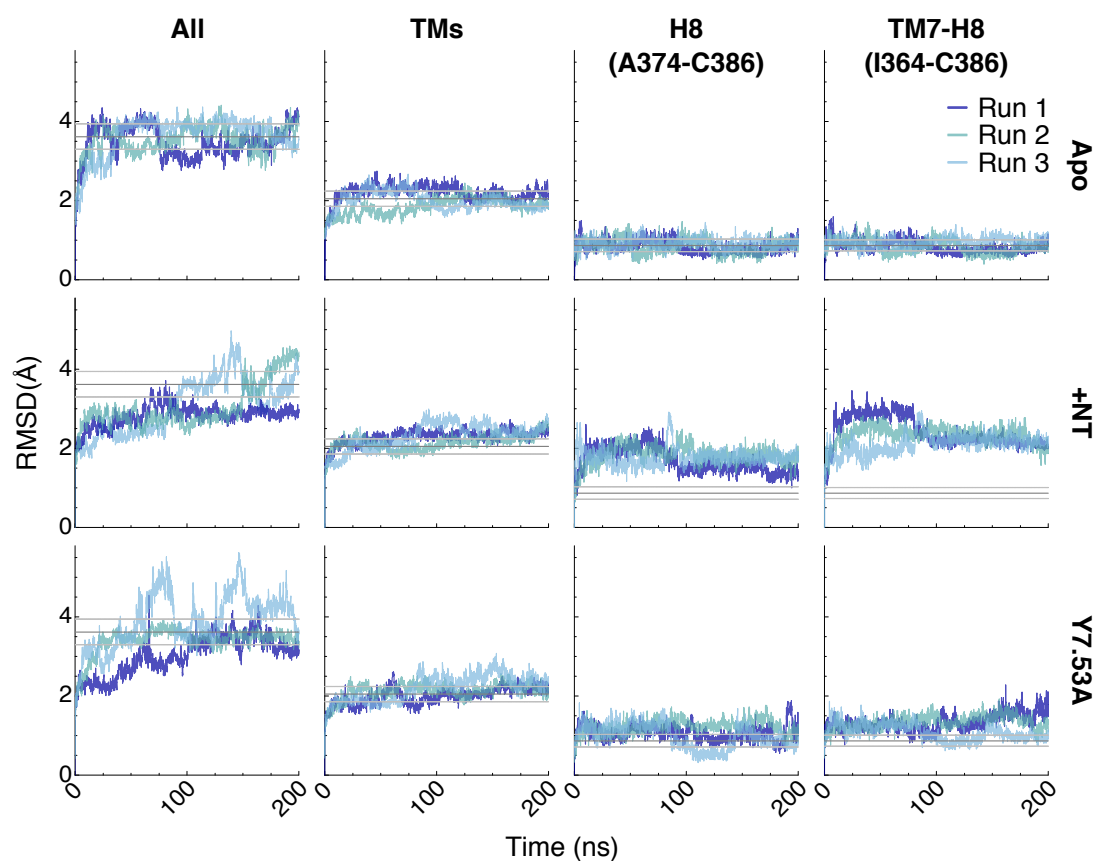


Fig. S5. MD simulations of H8 in water, POPC, and BPL. Timeline representation of the evolution of the secondary structure of a peptide corresponding to H8 residues (**A**) A374-A385 (H8p) and (**B**) A374-H393 (eH8p) in explicit water (top panel), on top of a POPC membrane (middle panel), or on top of a BPL-like membrane (bottom panel); and of (**C**) H8 within NTS1 embedded in a POPC (left panel) or

BPL-like (right panel) membrane. The NTS1 model used was based on the 4BUO structure (14) with thermostabilizing mutations reverted back to the corresponding wild-type residues. Time between frames is 200 ps. The different colors represent the secondary structure adopted by the corresponding residues in each frame as indicated in panel (C). (A) In water and in the presence of POPC lipids only, the H8p peptide becomes fully unstructured (turn and coil conformations), while in the presence of a BPL-like membrane the α -helical structure is maintained for the F376-T383 fragment. (B) In water, the eH8p peptide becomes fully unstructured (turns and coils predominantly). In the presence of POPC lipids only, the peptide becomes fully unstructured (turns and coils) after 150 ns. In the presence of a BPL-like membrane the eH8p peptide keeps a helical structure (α and π helices) within the F376-L381 fragment, while the rest of it becomes unstructured (turns and coils). (C) For NTS1 embedded in a POPC only membrane, the A374-S382 fragment is mostly α -helical, whereas the rest of the fragment becomes unstructured. For NTS1 embedded in a BPL-like membrane, the A374-L384 fragment keeps an α -helical structure throughout the AT-MD simulation, whereas the rest of the fragment becomes unstructured. The average RMSD for the H8 backbone was 1.9 ± 0.8 and 0.9 ± 0.2 Å for NTS1 within a POPC and BPL-like membrane, respectively. The average overall protein backbone (TM domains) was 3.6 ± 0.5 and 1.7 ± 0.2 Å for the POPC and BPL-like membrane systems, respectively. (D) The orientation of H8 in the MD simulations of apo NTS1 with respect to the BPL lipid bilayer is shown; H8 is nearly parallel to the bilayer, with some degree of insertion into the hydrophobic membrane. H8 residues are shown as sticks, with solvent-exposed residues, residues at the solvent-phospholipids polar heads interface, and membrane-buried residues colored purple, yellow, and orange, respectively. (E) Comparison of the H8 conformation in the 4BUO crystal structure (14) (grey) with the last frame of the AT-MD simulations of NTS1 embedded in a POPC (red) and a BPL-like (green) membrane.



	RMSD (Å)			
	All	TM	H8(374-386)	TM7-H8(364-386)
Apo	3.6 ± 0.3	2.1 ± 0.2	0.9 ± 0.2	0.9 ± 0.2
+NT	3.1 ± 0.5	2.3 ± 0.2	1.8 ± 0.3	2.3 ± 0.3
Y7.53A	3.6 ± 0.4	2.2 ± 0.2	1.1 ± 0.3	1.3 ± 0.2

Fig. S6. Structural fluctuations during MD simulations of NTS1 under different conditions. The root-mean-square displacement (RMSD) of the C α backbone residues during the triplicate simulations performed in BPL-like membranes are shown here. RMSD is reported for the whole receptor (All), the TM helices (TMs), H8, and TM7-H8 during three individual MD simulations (runs 1-3) for apo WT NTS1 (Apo, top), NT-bound WT NTS1 (+NT, middle), and apo mutant NTS1 where Tyr369 is mutated to Ala (Y7.53A, bottom). The average RMSD during the simulations of apo WT NTS1 is shown as a dark grey line in each panel, while light grey lines indicate the average RMSD $\pm 1\sigma$ (standard deviation). The table shows the average RMSD for the three simulations and the error represents the standard deviation.

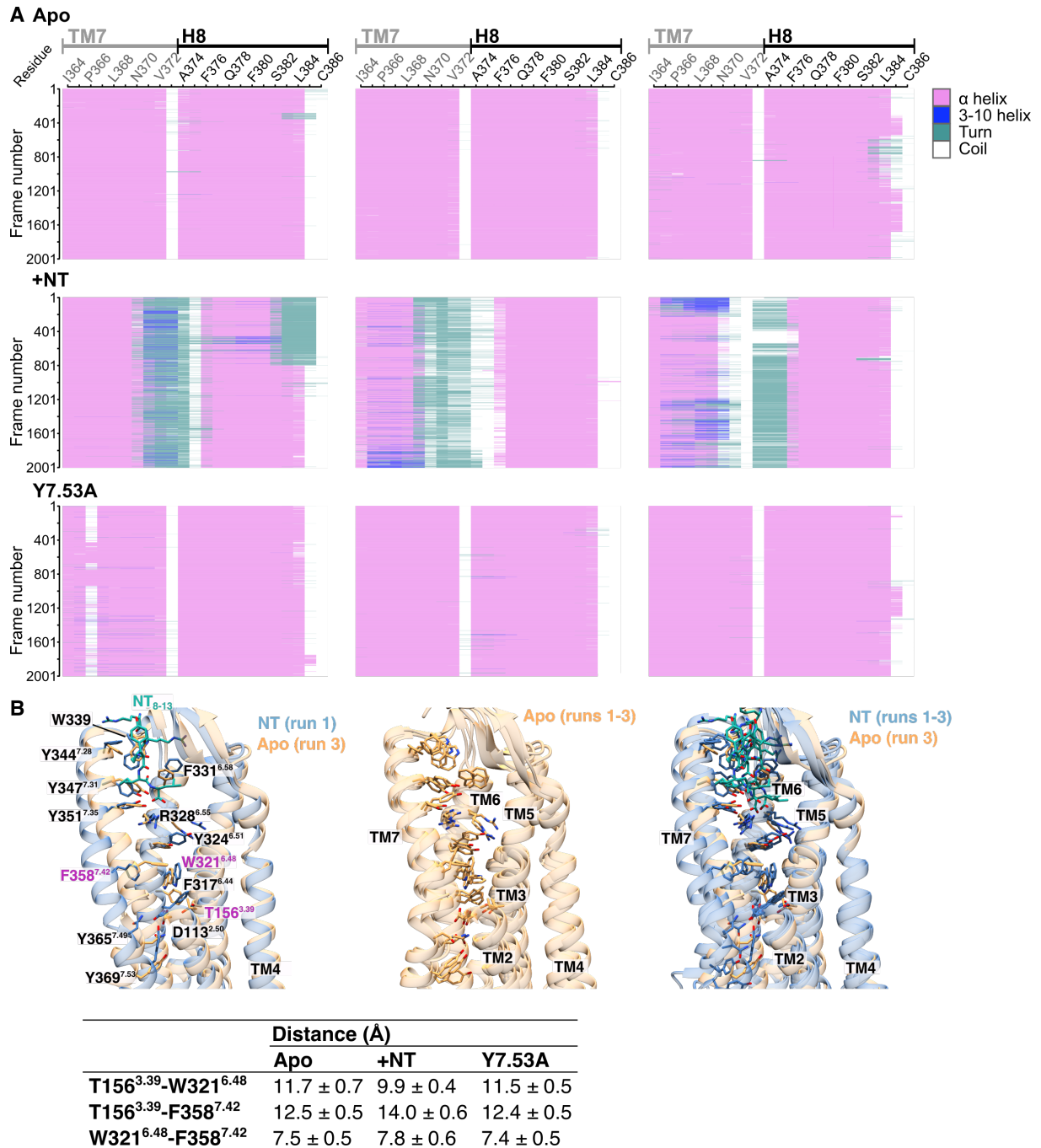


Fig. S7. Structure of NTS1 in BPL during MD simulations. (A) Timeline representation of the evolution of the secondary structure of the intracellular end of TM7 and H8 within NTS1 embedded in a BPL-like membrane. Results are shown for three individual simulations per conditions: apo WT NTS1 (top), NT-bound WT NTS1 (middle), and apo mutant NTS1 where Tyr369 is mutated to Ala (Y7.53A, bottom). The NTS1 model used was based on the 4BUO structure (14) with thermostabilizing mutations reverted back to the corresponding wild-type residues. Time between frames is 100 ps. The different colors represent the secondary structure adopted by the corresponding residues in each frame as indicated. (B) Superposition of last frames of individual MD simulations are shown for WT NTS1 in the apo and NT-bound state. A cascade of mostly aromatic residues (shown here) links the ligand binding pocket to the intracellular side of the membrane (16). In simulations of the NT-bound receptor, on

average, the EC ends of TM6 (from residue W321^{6.48} onwards) and TM7 are found closer and further from the receptor core, respectively, compared to their positions in the apo receptor. The average distance between the C α atoms of T156^{3.39} on TM3, W321^{6.48} on TM6, and F358^{7.42} on TM7 (residues labeled in magenta) during the three simulations are given in the table. The error represents the standard deviation.

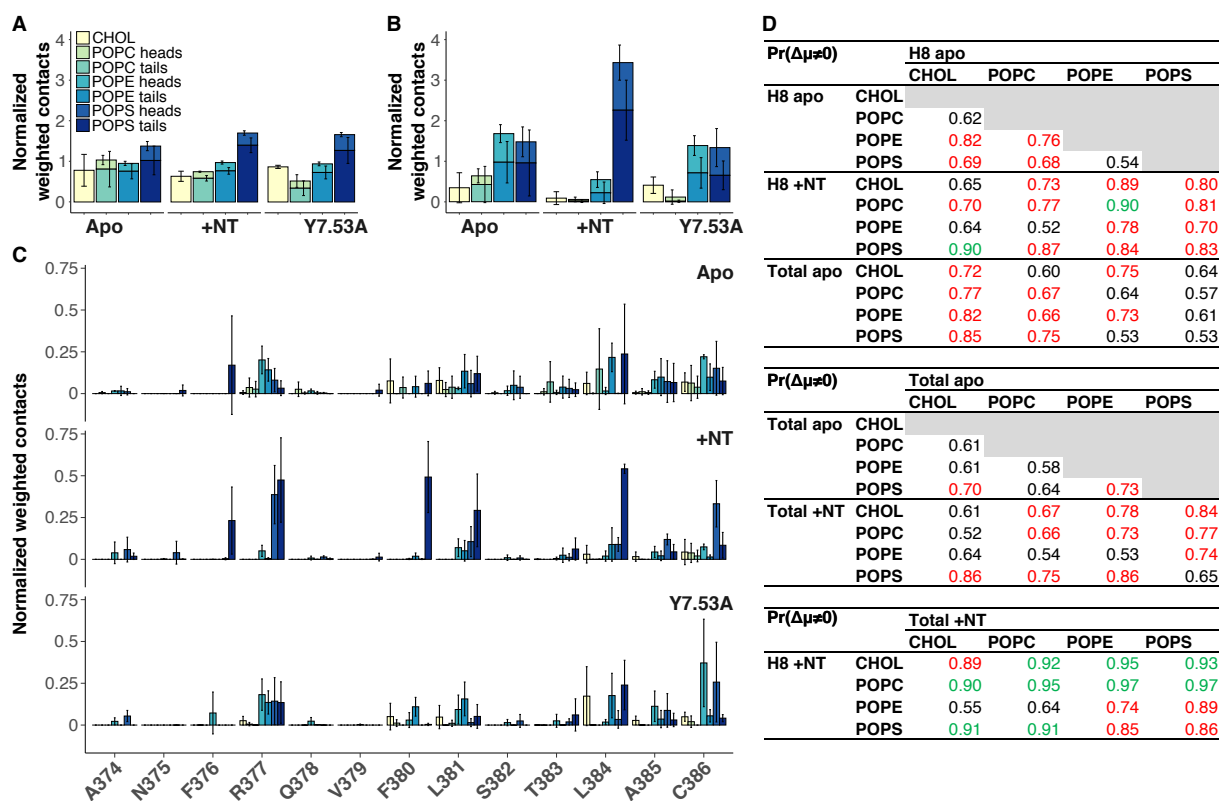


Fig. S8. Lipid-protein interactions observed in MD simulations. Number of lipid-protein contacts (≤ 3.5 Å) per lipid species observed during MD simulations of NTS1 in a BPL-like bilayer normalized by the total number of interactions during each simulation run and weighted by the percentage of each lipid type found in the mixed bilayer. Results are shown for simulations of apo WT NTS1 (Apo), NT-bound WT NTS1 (+NT), and apo mutant NTS1 where Tyr369 is mutated to Ala (Y7.53A). Averages of three simulation runs for each condition for the timeframe in equilibrium (20-200 ns) are shown with error bars representing the standard deviation. Contacts for POPC, POPE, and POPS are shown for the lipid tails and lipid headgroups separately. Contacts are shown **(A)** for the whole receptor, **(B)** for H8 only (residues A374-C386), and **(C)** for the individual H8 residues. **(D)** Statistical analysis of the number of lipid-protein contacts observed with the whole receptor ("Total") or with H8 during simulations run in triplicates for the WT apo or NT-bound (+NT) receptor. The probability (Pr) that the difference between the average number of contacts ($\Delta\mu$) for all possible "pairs" is non-zero (i.e. that the analyzed "pairs" can reasonably said to have differing number of average lipid-protein contacts) was analyzed by Bayesian methods. Probabilities are binned as being <0.66 (black), and thus not likely distinguishable, at least $0.66-0.9$ (red) and thus likely distinguishable, or >0.9 (green), and thus very likely distinguishable. No likely differences were observed between apo WT and Y7.53A (probabilities not shown), except for the number of contacts with POPC (probability ~ 0.7).

Table S1. H8 sequence of GPCRs of known structure. (See next page).

	8.47	8.48	8.49	8.50	8.51	8.52	8.53	8.54	8.55	8.56	8.57	8.58	8.59
Consensus	N	K	K	F	R/K	R/K	A	F/L	K/R	K/R	L	L/F	C
[Human] A1 receptor	I	Q	K	F	R	V	T	F	L	K	I	W	N
[Human] A2A receptor	I	R	E	F	R	Q	T	F	R	K	I	I	R
[Human] apelin receptor	D	P	R	F	R	Q	A	C	T	S	M	L	C
[Human] AT1 receptor	G	K	K	F	K	R	Y	F	L	Q	L	L	K
[Human] AT2 receptor	G	N	R	F	Q	Q	K	L	R	S	V	F	R
[Common turkey] β1-adrenoceptor	S	P	D	F	R	K	A	F	K	R	L	L	C
[Human] β2-adrenoceptor	S	P	D	F	R	I	A	F	Q	E	L	L	C
[Guinea pig] BLT1 receptor	G	G	G	L	L	R	S	A	G	V	G	F	V
[Human] C5a1 receptor	S	L	P	S	L	L	R	N	V	L	T	E	E
[Human] CB1 receptor	S	K	D	L	R	H	A	F	R	S	M	F	P
[human] CB1 receptor	S	K	D	L	R	H	A	R	R	S	M	F	S
[human] CB2 receptor	S	G	E	I	R	S	S	A	H	H	C	L	A
[Human] CCR2	G	E	K	F	R	S	L	F	H	I	A	L	G
[Human] CCR5	G	E	K	F	R	N	Y	L	L	V	F	F	Q
[Human] CCR9	G	E	R	F	R	R	D	L	V	K	T	L	K
[Human] CXCR4	G	A	K	F	K	T	S	A	Q	H	A	L	T
[Human] CysLT1 receptor	G	G	N	F	R	K	R	L	S	T	F	R	K
[Human] CysLT2 receptor	G	E	N	F	K	D	R	L	K	S	A	L	R
[Human] D2 receptor	N	I	E	F	R	K	A	F	L	K	I	L	H
[Human] D3 receptor	N	I	E	F	R	K	A	F	L	K	I	L	S
[Human] D4 receptor	N	A	E	F	R	N	V	F	R	K	A	L	R
[Human] ETB receptor	S	K	R	F	K	N	C	F	K	S	C	L	C
[Human] FFA1 receptor	G	R	G	P	G	L	K	T	V	C	A	A	R
[human] FP receptor 2	G	Q	D	F	R	E	R	L	I	H	S	L	P
[human] GPR52	N	S	V	F	R	L	G	L	R	R	L	S	E
[Human] H1 receptor	N	E	N	F	K	K	T	F	K	R	I	L	H
[Human] 5-HT1B receptor	N	E	D	F	K	Q	A	F	H	K	L	I	R
[Human] 5-HT2B receptor	N	K	T	F	R	D	A	F	G	R	Y	I	T
[Human] 5-HT2C receptor	N	K	I	Y	R	R	A	F	S	N	Y	L	R
[Human] LPA1 receptor	D	K	E	M	S	A	T	F	R	Q	I	L	C
[Zebrafish] LPA6 receptor	-	-	-	I	Q	N	S	I	K	R	K	T	Q
[Human] M1 receptor	N	K	A	F	R	D	T	F	R	L	L	L	L
[Human] M2 receptor	N	A	T	F	K	K	T	F	K	H	L	L	M
[Rat] M3 receptor	N	K	T	F	R	T	T	F	K	T	L	L	L
[Human] M4 receptor	N	A	T	F	K	K	T	F	R	H	L	L	L
[Human] M5 receptor	N	R	T	F	R	K	T	F	K	M	L	L	L
[Human] MT1 receptor	N	Q	N	F	R	K	E	Y	R	R	I	I	V
[Human] NOP receptor	D	E	N	F	K	A	C	F	R	K	F	C	C
[human] NTS1 receptor	S	A	N	F	R	H	I	F	L	A	A	L	A
[Rat] NTS1 receptor	S	A	N	F	R	Q	V	F	L	S	T	L	A
[Human] δ opioid receptor	D	E	N	F	K	R	C	F	R	Q	L	C	R
[Mouse] δ opioid receptor	D	E	N	F	K	R	C	F	R	Q	L	C	R
[Human] κ opioid receptor	D	E	N	F	K	R	C	F	R	D	F	C	F
[Mouse] μ opioid receptor	D	E	N	F	K	R	C	F	R	E	F	C	I
[Human] OX1 receptor	S	G	K	F	R	E	Q	F	K	A	A	F	S
[Human] OX2 receptor	S	G	K	F	R	E	E	F	K	A	A	F	S
[Human] P2Y1 receptor	G	D	T	F	R	R	R	L	S	R	A	T	R
[Human] P2Y12 receptor	C	K	S	F	R	N	S	L	I	S	M	L	K
[Human] PAF receptor	T	K	K	F	R	K	H	L	T	E	K	F	Y
[Human] PAR1	S	S	E	C	Q	R	Y	V	Y	S	I	L	C
[Human] PAR2	S	H	D	F	R	D	H	A	K	N	A	L	L
[Bovine] Rhodopsin	N	K	Q	F	R	N	C	M	V	T	T	L	C
[Human] Rhodopsin	N	K	Q	F	R	N	C	M	L	T	T	I	C
[Japanese flying squid] Rhodopsin	H	P	K	F	R	E	A	I	S	Q	T	F	P
[Human] S1P1 receptor	N	K	E	M	R	R	A	F	I	R	I	M	S
[rat] Succinate receptor 1	G	D	H	F	R	D	M	L	F	S	K	L	R
[strain AD169] US28	G	T	K	F	R	Q	E	L	H	C	L	L	A

Table S1. H8 sequence of GPCRs of known structure. (Continued). Alignment of H8 sequences of class A GPCRs of known structure available from the GPCR database (GPCRDB (41)) is shown, as well as the consensus sequence (see fig. S1B for a more detailed sequence analysis generated by WebLogo (42)). GPCR subtypes that have been crystallized without a structured H8 are highlighted in red. Residues corresponding to F(R/K)xx(F/L)xx(F/L) motif positions are highlighted in blue (in agreement with the motif) or orange (deviating from the motif).

Table S2. H8 in published NTS1 crystal structures. The sequence corresponding to the helical portion of H8 for all published rat NTS1 structures is reported in the table. Where two chains were present, the helical H8 sequence is given for each of the chains. For structures without a helical H8 (n.r., not resolved) the last resolved residue of TM7 is listed instead. The activation state of the structures as defined in each of the studies is listed as well. In all cases NTS1 was crystallized in the agonist-bound state.

PDB ID	NTS1 construct	Modifications	Sequence helical H8	State	Ref.
4GRV	GW5-T4L	Truncations: 1-42, 397-424, 269-299 (IC3, replaced with T4L+GSGS linker) A86L, E166A, G215A, L310A, F358A, V360A	n.r. (TM7 ends at A374)	Active-like	(13)
3ZEV	TM86V-ΔIC3A	Truncations: 280-295 (IC3) A86L, H103D, H105Y, A161V, R167L, R213L, V234L, I253A, H305R, F358V, S362A	A374-L384 (chain A) A374-C386 (chain B)	Inactive	(14)
4BUO	TM86V-ΔIC3B	Truncations: 273-290 (IC3) Mutations as TM86V-ΔIC3A	A374-L384 (chain A) A374-A385 (chain B)	Inactive	
4BV0	OGG7- ΔIC3A	Truncations: as TM86V-ΔIC3A S83G, A86L, T101R, H103D, H105Y, L119F, M121L, E124D, R143K, D150E, A161V, R167L, R213L, V234L, K235R, V240L, I253A, I260A, N262R, K263R, H305R, C332V, F342A, T354S, F358V, S362A	A374-C386 (chain A) A374-L387 (chain B)	Inactive	
4BWB	HTGH4-ΔIC3A	Truncations: as TM86V-ΔIC3A S83G, A86L, T101R, H103D, H105Y, L119F, M121L, E124D, L125V, R143K, D150E, A161V, R167L, C172R, A177H, M208V, R213L, V234L, V240L, I253A, N262R, K263R, H305R, V313M, C332V, F342A, T354S, F358V, S362A	A374-C388 (chain A) A374-L384 (chain B)	Inactive	
4XES	LF-T4L	Truncations: as GW5-T4L A86L, E166A, G215A, V360A	N375-F380	Active-like	(15)
4XEE	ELF-T4L	Truncations: as GW5-T4L A86L, G215A, V360A	N375-S382	Active-like	
5T04	EL-T4L	Truncations: as GW5-T4L A86L, G215A, F358A, V360A	n.r. (TM7 ends at V372)	Active-intermediate	(16)

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