Structural prerequisites for G-protein activation by the neurotensin receptor

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We previously determined the structure of neurotensin receptor NTSR1 in an active-like conformation with six thermostabilizing mutations bound to the peptide agonist neurotensin. This receptor was unable to activate G proteins, indicating that the mutations restricted NTSR1 to relate agonist binding to G-protein activation. Here we analyse the effect of three of those mutations (E166A3.49, L310A6.37, F358A7.42) and present two structures of NTSR1 able to catalyse nucleotide exchange at Gα. The presence of F3587.42 causes the conserved W3216.48 to adopt a side chain orientation parallel to the lipid bilayer sealing the collapsed Na+ ion pocket and linking the agonist with residues in the lower receptor part implicated in GPCR activation. In the intracellular receptor half, the bulkier L3106.37 side chain dictates the position of R1673.50 of the highly conserved D/ERY motif. These residues, together with the presence of E1663.49 provide determinants for G-protein activation by NTSR1.

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GPCRs are highly versatile signalling molecules that modulate second messenger responses in the cell. Binding of an extracellular agonist leads to conformational changes in the receptor, triggering activation of associated signalling partners on the intracellular side of the membrane. GPCRs are no longer thought to be two-state switches (inactive or active, although rhodopsin may come close to this definition) but are able to sample many conformational states depending on the bound ligand, associated signalling partner and membrane environment. Recent advances in the structural biology of GPCRs have resulted in high-resolution snapshots of inactive active-like and active receptor conformations.

Our research focuses on understanding the structural and functional requirements for the activation of the neurotensin receptor 1 (NTSR1, ref. 8). Its agonist ligand neurotensin (NTS) is a 13-amino acid peptide that functions as a neurotransmitter and a hormone in the nervous system and in peripheral tissues. NTS has a wide range of biological activities with important aspects in anorexia, cancer cell growth and the pathogenesis of schizophrenia. Most of the known agonist effects of NTS are assumed that the mutations E166A3.49, L310A6.37 and F358A7.42 of the highly conserved D/ERY motif, along with the neighbouring L310A6.37 for G-protein activation (Fig. 1).

Pharmacological characterization of NTSR1-LF and NTSR1-ELF. Ligand-binding experiments (Supplementary Table 2) showed that the apparent affinity of NTSR1-LF and NTSR1-ELF for the agonist NTS was comparable to that of the wild-type receptor and NTSR1-GW5. The agonist-binding experiments using wild-type NTSR1 were conducted at equilibrium. In contrast, binding of [3H]NTS to the NTSR1 mutants did not reach equilibrium within the incubation time because of the slow agonist off-rates. The apparent IC\textsubscript{50} values for the antagonist SR48692 (ref. 14) were 3–5-fold higher than the corresponding wild-type value, but more than 20-fold lower than the value for NTSR1-GW5. The shift in IC\textsubscript{50} values may be caused by substituting the G-proteins with the enhancing Gq activation.

Results

NTSR1 constructs used in this study. Here, we describe structural, biochemical and pharmacological data of several NTSR1 mutants (see Supplementary Tables 1 and 2) with either wild-type intracellular loop 3 (ICL3), or with most of ICL3 replaced by T4 lysozyme (T4L). In the Methods section, we distinguish between NTSR1 constructs containing the wild-type ICL3 sequence or T4L. In the main text, we use only one name for a particular construct for ease of reading. For example, NTSR1-ELF refers interchangeably to NTSR1-ELF-T4L and NTSR1-ELF, the latter containing the wild-type ICL3, not T4L. The identity of the respective construct is evident from the context of writing. Protein crystals were obtained with receptors where most of ICL3 was replaced by T4L. Pharmacological and biochemical experiments were conducted with receptors containing the wild-type ICL3 sequence, but also included T4L variants for comparison. In Figures and Tables relating to biochemical and pharmacological data, the identity of constructs is unambiguously specified.

Active-like NTSR1 mutants which activate Gq protein. We previously reported the crystal structure of NTSR1-GW5 (ref. 3) containing six stabilizing mutations (A86L1.54, E166A3.49, G215A5.41, L310A6.37, F358A7.42 and V360A7.44; ref. 12; superscripts are the Ballesteros–Weinstein numbers). NTSR1-GW5 displayed features of an active-like receptor such as an outward-tilted transmembrane helix (TM) 6 at the cytoplasmic surface and key conserved residues in positions characteristic for active and/or active-like but not for inactive GPCRs. NTSR1-GW5 did not catalyse nucleotide exchange at Gq in response to NTS in G-protein-coupling assays (Fig. 1), suggesting that some of the stabilizing mutations have limited the ability of NTSR1-GW5 to activate G protein. On the basis of their location, we assumed that the mutations E166A3.49, L310A6.37 and F358A7.42 affected the NTSR1 activation state, whereas the involvement of the mutations A86L1.54, G215A5.41 and V360A7.44 was not obvious. We therefore reverted the three mutations E166A3.49, L310A6.37 and F358A7.42 to wild-type residues, alone or in combination (Supplementary Table 1), to analyse their role in G-protein activation. In contrast to NTSR1-GW5, the triple revertant NTSR1-ELF (with E166, L310, F358) was able to stimulate nucleotide exchange at Gq to almost wild-type level (Fig. 1, Supplementary Fig. 1, Supplementary Table 2). The double revertant NTSR1-EL (with E166 and L310) showed reduced activity in nucleotide exchange assays, but highlights the importance of E166A3.49, of the highly conserved D/ERY motif, along with the neighbouring L310A6.37 for G-protein activation (Fig. 1).

Figure 1 | Mutational analysis of NTSR1 for activation of G protein. Agonist-stimulated activation of Gq: GDP/[35S]GTP\textsubscript{S} exchange assays contained purified Gq protein, [35S]GTP\textsubscript{S}, insect cell membranes with NTSR1 and saturating concentrations of NTS (20 \mu M). Fold stimulation of the exchange of GDP for [35S]GTP\textsubscript{S} at Gq in the presence of NTS is compared with the nucleotide exchange in the absence of ligand (number of independent experiments: wild-type NTSR1 n = 7; NTSR1-GW5 n = 1; NTSR1-ELF n = 4; NTSR1-L n = 5; NTSR1-F n = 4; NTSR1-EL n = 5; NTSR1-ELF n = 4; NTSR1-LF n = 7; NTSR1-ELF n = 6). A value of 1 (dotted line) indicates the absence of receptor-catalysed nucleotide exchange. All G-protein activation experiments were conducted with NTSR1 constructs (containing the wild-type ICL3, not T4L) in urea-washed P2 insect cell membranes. The identity of the NTSR1 constructs is given in Supplementary Table 1. Error bars correspond to s.e.m.
partly by a change in affinity of SR48692 to the NTSR1 mutants, and/or because SR48692 and [3H]NTS binding to the NTSR1 mutants did not reach equilibrium under the experimental conditions because of the change in the off-rate of [3H]NTS for the receptor mutants.

NTSR1-GW5, NTSR1-LF and NTSR1-ELF showed reduced sensitivity of agonist binding in the presence of Na+ ions (Supplementary Table 2) possibly indicating a high-affinity agonist conformation of the NTSR1 mutants. In contrast to wild-type NTSR1, the presence of NaCl did not increase the dissociation of [3H]NTS from NTSR1-LF, as was also observed with NTSR1-GW5 (ref. 3). However, NaCl did increase the dissociation of [3H]NTS from NTSR1-ELF, albeit not as pronounced as that seen with the wild-type receptor (Supplementary Fig. 1). The crystal structures of NTSR1-LF-T4L and NTSR1-ELF-T4L cannot explain the observed differences in the Na+ ion-dependent kinetics of NTS dissociation from NTSR1-LF and NTSR1-ELF. However, NTSR1-ELF may be more dynamic than NTSR1-LF, indicated by its lower thermal stability (Supplementary Table 3) and thus higher flexibility, accounting for the observed differences of the kinetic properties of both receptor mutants.

In G-protein-coupling assays, NTSR1-LF had moderate ability to activate the G protein. NTSR1-ELF was able to stimulate nucleotide exchange at Gq to almost wild-type level (Fig. 1, Supplementary Fig. 1, Supplementary Table 2). The dose-response curves for the determination of the half maximal effective concentration of NTS on the exchange of GDP for GTPγS on Gq (EC50 values) were multiphasic (Hill slope < 1) for NTSR1-ELF and wild-type NTSR1, indicating high and low affinity agonist-binding sites at non-saturating G-protein concentrations. In contrast, NTSR1-LF showed monophasic curves accounting for the observed differences of the kinetic properties of both receptor mutants.

Architecture of NTSR1-ELF and NTSR1-LF. To understand the structural implications of E1663.49, L3106.37 and F3587.42 on the receptor core by (A49, G50 and P51) in NTSR1-LF, providing additional contacts to the receptor core by hydrogen bond-mediated interactions with NTSR1-LF and NTSR1-ELF compared with NTSR1-GW5 (Supplementary Tables 4–7). Its R8 side chain is in strong hydrogen bond-mediated contact with D54 and D56 of the receptor N terminus. The R8 side chain is also connected to TM7 through water-mediated hydrogen bonds to D3457.29. The R9 side chain also forms a strong hydrogen bond with the main chain oxygen of I334 of ECL3. Overall, NTS8–13 engages in more hydrogen bond-mediated interactions with NTSR1-LF and NTSR1-ELF compared with NTSR1-GW5 (Supplementary Tables 4–7).

On the intracellular side, the ends of TM3 of NTSR1-LF and NTSR1-ELF differ from NTSR1-GW5 in a number of regions. The amino (N) terminus adopts a short helix (S53–L55) and is, compared with NTSR1-GW5, extended by two residues (G50 and P51) in NTSR1-ELF and three residues (A49, G50 and P51) in NTSR1-LF, providing additional contacts to extracellular loop (ECL) 2. ECL3 (residues I334–T340) of NTSR1-ELF and NTSR1-LF is shifted slightly towards the receptor core by ~2.5 Å (Fig. 2). Subtle, yet distinct differences exist in the NTS8–13 binding mode (Supplementary Tables 4–7). Its R8 side chain is in strong hydrogen bond-mediated contact with D54 and D56 of the receptor N terminus. The R8 side chain in NTSR1-LF (but not in NTSR1-ELF) is also connected to TM7 through water-mediated hydrogen bonds to D3457.29. The R9 side chain also forms a strong hydrogen bond with the main chain oxygen of I334 of ECL3. Overall, NTS8–13 engages in more hydrogen bond-mediated interactions with NTSR1-LF and NTSR1-ELF compared with NTSR1-GW5 (Supplementary Tables 4–7).

**Table 1 | Data collection and refinement statistics.**

|                        | NTSR1-LF-T4L | NTSR1-ELF-T4L |
|------------------------|--------------|---------------|
| **Data collection**     |              |               |
| Space group            | P 222        | P 222         |
| MoI/ASU                | 1            | 1             |
| Cell dimensions        | a, b, c (Å)  | 49.8, 88.4, 161.3 | 491.881.161.3 |
| α, β, γ (°)            | 90, 90, 90   | 90, 90, 90    |
| Resolution (Å)         | 34.0–2.60    | 45.0–2.90     |
| Rmerge (%)             | 0.15 (0.72)  | 0.15 (0.80)   |
| Mean R(fo-fc) (%)      | 10.9 (1.5)   | 9.3 (1.5)     |
| Completeness (%)       | 99.4 (98.6)  | 90.2 (87.4)   |
| Redundancy             | 7.2 (4.0)    | 6.6 (3.7)     |
| **Refinement**         |              |               |
| Resolution (Å)         | 34.0–2.60    | 45.0–2.90     |
| No. of unique reflections | 161,974 (8,709)  | 96,173 (5,081) |
| Rwork/Rfree (%)        | 23.2/28.0    | 23.1/28.1     |
| No. of atoms           | Protein 3,714 | 3,666         |
|                        | Ligand 58    | 58            |
|                        | Water 55     | 10            |
|                        | B-factors (Å²)|              |
| NTSR1-ELF              | NTSR1-4L     |
|                        | 59.8         | 71.8          |
|                        | 66.5         | 78.9          |
| T4L                    | 47.7         | 58.7          |
| NTS8–13                | 56.6         | 65.5          |
| Water                  | 53.4         | 54.1          |
| R.m.s. deviations      | Bond lengths (Å) | 0.007          |
|                        | Bond angles (°) | 1.185          |

R.m.s., root mean squared.

Number of crystals for NTSR1-LF-T4L and NTSR1-ELF-T4L was 6 and 5, respectively.

Highest resolution shell is shown in parenthesis.

lysosome) indicating active-like NTSR1 conformations (Fig. 2). Despite the overall similar architecture of the three NTSR1 structures, NTSR1-ELF and NTSR1-LF differ from NTSR1-GW5 in a number of regions. The amino (N) terminus adopts a short helix (S53–L55) and is, compared with NTSR1-GW5, extended by two residues (G50 and P51) in NTSR1-ELF and three residues (A49, G50 and P51) in NTSR1-LF, providing additional contacts to extracellular loop (ECL) 2. ECL3 (residues I334–T340) of NTSR1-ELF and NTSR1-LF is shifted slightly towards the receptor core by ~2.5 Å (Fig. 2). Subtle, yet distinct differences exist in the NTS8–13 binding mode (Supplementary Tables 4–7). Its R8 side chain is in strong hydrogen bond-mediated contact with D54 and D56 of the receptor N terminus. The R8 side chain in NTSR1-LF (but not in NTSR1-ELF) is also connected to TM7 through water-mediated hydrogen bonds to D3457.29. The R9 side chain also forms a strong hydrogen bond with the main chain oxygen of I334 of ECL3. Overall, NTS8–13 engages in more hydrogen bond-mediated interactions with NTSR1-LF and NTSR1-ELF compared with NTSR1-GW5 (Supplementary Tables 4–7).
ICL1 showed electron density for the main chain atoms in NTSR1-LF but not in NTSR1-ELF. ICL2, thought to be important for G-protein coupling, adopts a two-turn $\alpha$ helix in a slightly different position compared with the $\pi$ helix seen in NTSR1-GW5 (Fig. 2). The structural significance of this observation for the ability of NTSR1 to activate G protein is unclear, as ICL2 adopts an $\alpha$-helical structure in the active $\beta_2$-adrenergic receptor–Gs complex and in the inactive $\beta_2$AR structure but is an extended loop in the inactive $\beta_1$AR structure.

Transmembrane helix 7 and helix 8. In contrast to NTSR1-GW5, TM7 shows partial ‘unwinding’ after the conserved NPxxY motif around N370 (Fig. 3), similar to that seen in the structure of the active muscarinic acetylcholine M2 receptor, but not in the structure of active $\beta_3$AR. This region (L371–N375) is followed by a short helix H8 in both NTSR1-LF and NTSR1-ELF structures. The aromatic ring of F358, a conserved residue of the H8 motif, is well resolved in NTSR1-LF but is only weakly anchored in a hydrophobic pocket between TM1 and TM7. In NTSR1-ELF, F358 is no longer anchored between TM1 and TM7 but is rotated outward forming hydrophobic interactions with L371. These features of NTSR1-LF and NTSR1-ELF are distinct from the NTSR1 mutant TM86V-DIC3A, which adopts an apparent inactive receptor conformation at the inner side in the crystal structure, for example, lacking the outward movement of TM6 (ref. 18). In TM86V-DIC3A, TM7 does not unwind as seen in NTSR1-ELF. The residue F358 is partially inserted into the pocket between TM1, TM2 and TM7, whereas F358 of NTSR1-ELF is not anchored into the receptor core (Fig. 3). In addition, H8 of NTSR1-ELF and NTSR1-LF is shorter than that of TM86V-AIC3A by two and three residues from the carboxy (C) terminus, respectively (Supplementary Table 9). Whether this reflects a general instability of H8, or its dynamic nature in the active-like receptor conformation, remains to be explored. Recent molecular dynamics simulations suggested that unravelling of H8 is related to the agonist-occupied state of NTSR1 (ref. 19).

The conserved residue W321 of the CWxP motif. Of significance is the position of W321 within the CWxP motif, a highly conserved amino acid in class A GPCRs. Spectroscopic evidence suggested changes in the environment of W6.48 upon rhodopsin activation. However, a rotamer change of W6.48 is not observed in any crystal structure of active rhodopsin or the $\beta_2$AR suggesting that changes in the W6.48 rotamer orientation might not be an essential part of the GPCR activation mechanism. In contrast to all the previously determined GPCR structures, the W321 side chain in NTSR1-LF and NTSR1-ELF is oriented parallel to the lipid bilayer (Fig. 4; Supplementary Fig. 2). This orientation results from the presence of F358 whose phenyl side chain prevents W321 from adopting the side chain conformation found in NTSR1-GW5, which contained the stabilizing F358A mutation. Consequently, the W321 indole side chain makes additional van der Waals interactions with residues of TM3, TM5 and TM6 (Supplementary Fig. 3). It is worth noting that 73% of Figure 2 | Overview of NTSR1 structures bound to the peptide agonist NTS8–13. Cartoon representation of NTSR1-ELF-T4L (blue; NTS8–13 in purple), NTSR1-LF-T4L (green) and NTSR1-GW5-T4L (grey; NTS8–13 in orange, PDB code 4GRV). NTS8–13 is depicted as a stick model. (a) Side view of NTSR1-ELF-T4L. Residues E166, L310 and F358 are shown as cyan spheres; residues D113, W321 and R167 are depicted in red. (b) Extracellular view. An arrow indicates ECL3, which is shifted towards the receptor core in NTSR1-ELF-T4L. (c, d) Intracellular view. Arrows indicate the position shift of the intracellular ends of TM3, TM5, TM6 and TM7 of NTSR1-ELF-T4L (c) and NTSR1-LF-T4L (d) compared with NTSR1-GW5-T4L. ICL1 is disordered in NTSR1-ELF-T4L. In contrast to NTSR1-GW5-T4L, NTSR1-ELF-T4L and NTSR1-LF-T4L have a short helix H8. T4L has been omitted from the intracellular view for clarity.
class A GPCRs, including rhodopsin, have small residues (G, A, S) at position 7.42; bulky tyrosine and phenylalanine residues are rare and comprise only 4% of class A GPCRs. For example, neuromedin U receptors and NTSR2 have a phenylalanine at position 7.42 and a tryptophan residue at position 6.48. The luteinizing hormone/choriogonadotropin and thyroid-stimulating hormone receptors have a tyrosine at position 7.42, but a methionine at position 6.48 in lieu of a tryptophan residue.

All class A receptors, for which crystal structures have been determined to date, have a small residue at position 7.42 (muscarinic receptors have a cysteine 7.42 residue, the P2Y12 receptor has a threonine 7.42 residue, the orexin OX2 receptor has a valine 7.42 residue) except NTSR1, possibly explaining in part why the W6.48 rotamer conformation seen here in NTSR1-LF and NTSR1-ELF has not been observed in other receptor structures.

A network of interactions links NTS to the hydrophobic core. NTSR1-LF and NTSR1-ELF structures help explain how the agonist peptide transmits its extracellular signal to the intracellular portion of the receptor. Hydrogen bond and van der Waals interactions link NTS8–13 with residues of the hydrophobic core associated with helical rearrangements seen in active-state structures (Fig. 4). NTS8–13 is connected to Y324 6.51 via a hydrogen bond network from the carboxylate of its L13 residue through R327 6.54. The aromatic ring of Y324 6.51 is engaged in hydrophobic stacking interactions with F358 7.42 that is in contact with W321 6.48, as previously discussed. The hydrophobic network results in the packing of W321 6.48 against the hydrophobic F317 6.44 that has been implicated in the reorganization of transmembrane segments upon agonist binding in β2AR. The rotamer position of F317 6.44 is almost the same in NTSR1-GW5, NTSR1-LF and NTSR1-ELF.
The collapsed Na\(^+\) ion-binding pocket. Na\(^+\) ions have a negative allosteric effect on agonist binding to wild-type NTSR1 and the highly conserved D113\(^{2.50}\) in the middle of TM2 has been assigned a pivotal role in the Na\(^+\) ion sensitivity of agonist binding and G-protein activation. Recent high-resolution structures of GPCRs in the inactive state have revealed a conserved Na\(^+\) ion-binding pocket within the receptor transmembrane bundle, providing a structural explanation for the allosteric effect of Na\(^+\) ions on agonist binding. In each of those inactive structures, the Na\(^+\) ion is coordinated by a salt bridge to the highly conserved D2.50 and by four additional contacts with receptor side chains and water molecules (Fig. 5d).

In the active-like NTSR1-LF and NTSR1-ELF structures (and in NTSR1-GW5), the Na\(^+\) ion-binding pocket has collapsed (Fig. 5), which explains the reduced Na\(^+\) ion sensitivity of agonist binding (Supplementary Table 2). The D113\(^{2.50}\) side chain atoms form an extensive hydrogen bond network with T156\(^{3.39}\), S362\(^{7.46}\) and N365\(^{7.49}\) of the NPxxY motif, preventing the coordination of a Na\(^+\) ion. Absent in the collapsed NTSR1 Na\(^+\) ion pocket are any water molecules, which fill the cavity in inactive-state receptors. In NTSR1-LF and NTSR1-ELF, W321\(^{7.48}\) forms van der Waals interaction with residues of the Na\(^+\) ion-binding pocket, effectively sealing off the top of the collapsed Na\(^+\) ion pocket and disrupting a vertical cavity seen in NTSR1-GW5 (Supplementary Fig. 4).

The structure of the NTSR1 mutant TM86V-ΔIC3A is similar to our active-like NTSR1 structures in the extracellular half, which is responsible for ligand binding, but dissimilar in the intracellular half, adopting an apparent inactive receptor conformation in the crystal structure. The Na\(^+\) ion-binding region is located underneath the ligand binding pocket between the intracellular and extracellular receptor halves. In TM86V-ΔIC3A, the D113\(^{2.50}\) side chain is in contact with neighbouring residues, albeit the interactions are different when compared with NTSR1-GW5, NTSR1-LF and NTSR1-ELF (Fig. 5). In addition, no electron density for water molecules or a Na\(^+\) ion has been reported in the TM86V-ΔIC3A structure. Note that S362\(^{7.46}\), which contacts D113\(^{2.50}\) in NTSR1-LF and NTSR1-ELF, is mutated to an alanine residue in TM86V-ΔIC3A (Fig. 5).

The residue L310\(^{6.37}\) positions the R167\(^{3.50}\) side chain. The residue at position 6.37 (L310\(^{6.37}\) in NTSR1) is highly conserved among class A GPCRs; 80% of receptors have hydrophobic residues (I, L, V) at this position. The significance of this residue becomes apparent in the NTSR1-LF and NTSR1-ELF structures (Fig. 6). L310\(^{6.37}\) is central to the positioning of the R167\(^{3.50}\) side chain such as to allow a bona fide productive interaction with the G protein. In the signalling incompetent, active-like NTSR1-GW5 structure, R167\(^{3.50}\) is linked to the conserved N257\(^{5.58}\), S164\(^{6.47}\) and G306\(^{6.33}\) by a hydrogen bond network, likely facilitated by the decreased side chain size of the L310A\(^{6.37}\) stabilizing mutation. Those interactions stabilize R167\(^{3.50}\) in a position
The presence of the larger L3106.37 side chain in NTSR1-LF-T4L and NTSR1-ELF-T4L is sterically incompatible with such an arrangement and the R1673.50 side chain is stabilized by a hydrogen bond network to N2575.58, S1643.47 and G3066.33, facilitated by the L310A mutation. The moderate nucleotide exchange at G suggested that N582.39 either directly stabilizes the active receptor or engages in direct interactions with G protein or G protein mimetics (Fig. 6).

Discussion

Stabilization of detergent-solubilized receptor–ligand complexes is one of the key factors for successful crystallization and structure determination of membrane proteins. Wild-type NTSR1 is not particularly stable in detergent solution39; thus the use of stabilized NTSR1 mutants has resulted in the successful production of well-diffracting crystals (Supplementary Table 3). Our previously reported active-like NTSR1-GW5 mutant36 was obtained by conformational thermostabilization31 in the presence of the agonist neurotensin12,30. An alternative approach, directed evolution32–34, has resulted in structures of NTSR1 (ref. 18), which are similar to our active-like NTSR1 structures in the intracellular half.

The structures of NTSR1-LF and NTSR1-ELF, presented here, are very similar (root mean squared deviation values of 0.3 Å for Cα atoms, excluding T4 lysozyme), yet their ability to activate the G protein in response to NTS differs: NTSR1-LF mediates moderate nucleotide exchange at Gzq, whereas NTSR1-ELF has almost wild-type receptor properties (Supplementary Table 2). The glutamic acid3.49 of the highly conserved D/ERY motif, absent in NTSR1-LF, but present in NTSR1-ELF, has been deemed critical for G-protein coupling15. Thus the mutation E166A3.49 alone may explain the pharmacological behaviour of NTSR1-LF, highlighting the importance of E1663.49 for G-protein activation. In the active M2 receptor, D1203.49 is stabilized by a hydrogen bond with N582.39 (T682.39 in β3AR); and it has been suggested that N582.39 either directly stabilizes the active receptor conformation, or engages in direct interactions with G protein5. The equivalent residue in NTSR1 is V1022.39, which is hydrogen bonded to the side chain of T1011.38, the main chain amide of V1022.39, and weakly linked to H1052.42 (Supplementary Fig. 5). In the β3AR–Gs complex7, T682.39 and D1303.49 interact with the ICL2 helix via Y141ICL2 positioning the helix such that a phenylalanine docks into a hydrophobic pocket on the G protein surface. ICL2 has been found essential for the G-protein activation pathway, especially for the dissociation of the receptor–G protein complex in the presence of GTP35. Thus E1663.49 may optimally position ICL2 in the presence of G protein allowing efficient G-protein binding and release. In β3AR, Y141ICL2 links the receptor–G protein interactions of ICL2 with the D/EYR motif. The equivalent interaction in NTSR1 may come by M181ICL2 as the NTSR1 residue, equivalent to Y141ICL2 of β3AR, is an alanine (A177).

In conclusion, our current NTSR1 structures provide insight into mechanistic details of an active-like, agonist-occupied peptide GPCR. The conserved W3216.48, oriented parallel to the membrane plane, seals the top of a collapsed Na+ ion-binding pocket; W3216.48 in combination with F3587.42 link the agonist peptide, bound near the receptor surface, with hydrophobic core residues in the inner half of NTSR1. The highly conserved residue L3106.37 in the vicinity of the D/EYR motif is central to the side-chain orientation of R1673.50 to promote the productive interaction with Gq protein. The neighbouring E1666.49 residue is vital for G-protein activation, possibly by coupling receptor–G protein interactions with the D/EYR motif.

Methods

NTSR1 constructs. The baculovirus construct NTSR1-LF-T4L consisted of the hemagglutinin signal peptide and the Flag tag46, followed by the destabilized rat NTSR1 (T43-K396 containing the mutations A86L, E166A, G215A, V360A) with the ICL3 residues H269-E296 replaced by the cysteine-free bacteriophage T4 lysozyme (N2-Y161 with the mutations C54T and C97A) and a GSGS linker. A deca-histidine tag was placed at the C terminus. NTSR1-LF contained the wild-type ICL3 sequence. NTSR1-ELF-T4L and NTSR1-ELF were like NTSR1-LF-T4L and NTSR1-LF, respectively, but had only three mutations (A86L, G215A, V360A). The wild-type NTSR1 used here was like NTSR1-LF but did not have the four mutations. Additional NTSR1 mutants, used for pharmacological analyses, are listed in Supplementary Table 1.

In the Methods sections, we distinguish between NTSR1 constructs containing T4L or the wild-type ICL3 sequence. In the main text, we use only one name for a particular construct; for example, NTSR1-ELF refers interchangeably to NTSR1-ELF-T4L and NTSR1-ELF, the latter containing the wild-type ICL3, not T4L. The identity of the respective construct is evident from the context of writing.

Expression of NTSR1 in insect cells. Recombinant baculoviruses were generated using a modified pFastBac1 transfer plasmid (Invitrogen). Trichoplusia ni cells were infected with recombinant virus, and the temperature was lowered from 27 to 21 °C. Cells were collected by centrifugation 48 h post infection, resuspended in

Figure 6 | Effect of L3106.37 on the positioning of the R1673.50 side chain. (a,b) Comparison of NTSR1-LF-T4L (green) and NTSR1-ELF-T4L (blue) with NTSR1-GW5-T4L (grey, PDB code 4GRV). Hydrogen bonds are indicated by dashed lines, and water molecules are represented as spheres. In NTSR1-GW5-T4L, the R1673.50 side chain is stabilized by a hydrogen bond network to N2575.58, S1643.47 and G3066.33, facilitated by the L310A mutation. The presence of the larger side chain of L3106.27 in NTSR1-LF-T4L and NTSR1-ELF-T4L is sterically incompatible with such an arrangement and the R1673.50 side chain interactions with N2575.58 and S1643.47 are lost. (c) The R167 side chain now adopts a conformation similar to that seen in metarhodopsin II (ref. 4; purple, PDB code 3PQR), the β3AR–Gs complex2 (orange, PDB code 3SN6) and the active M2 receptor2 (red, PDB code 4MQS).
hypotonic buffer (10 mM Hepes pH 7.5, 10 mM MgCl₂, 20 mM KCl), flash frozen in liquid nitrogen and stored at −80 °C until use.

Expression of Gq protein in insect cells. The baculovirus construction His-Tev-Gq was a hexa-histidine tag followed by a tobacco etch virus (TEV) protease recognition site and the human Gq6 sequence (M7-V359). Human Gβ1 was unmodified. Human Gγ1 was preceded by a hexa-histidine tag. We refer to His-Tev-Gq His-Gβ1 as Gq protein. Trichoplusia ni cells were triple-infected with the recombinant viruses at 27 °C. Cells were collected by centrifugation 48 h post infection, resuspended in hypotonic buffer (10 mM Hepes pH 7.5, 10 mM MgCl₂, 20 mM KCl), flash frozen in liquid nitrogen and stored at −80 °C until use.

Preparation of urea-washed P2 insect cell membranes. NTSR1-enriched membranes were obtained as a P2 fraction from insect cells30,37. Before G-protein-coupling assays and ligand binding experiments, the P2 membranes were treated with urea to remove peripherally bound membrane proteins38,39. The receptor density in urea-washed P2 membranes was determined by [3H]NTS saturation binding analysis40.

Ligand-binding experiments. All radioligand binding assays were conducted with urea-washed P2 insect cell membranes containing the isolated NTSR1 constructs. Independent experiments were carried out in single data points. For agonist [3H]NTS (5,11-tetrahydronaphthalene-3H)-progu-Leu-Tyr-Glu-Asn-Pro-Arg (Arg-Arg-Pro-Tyr-Ile-Leu) saturation binding experiments, receptors were incubated on ice for 1 h in 250 μl TEBB buffer (50 mM TrisHCl pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 40 μg/ml bacitracin, 0.1% (w/v) BSA) containing [3H]NTS at a concentration of 0.6–20 nM. Nonspecific binding was determined in the presence of 1 μM unlabeled NTS. Specific binding was calculated as the difference between total and nonspecific binding. Data were analyzed by nonlinear regression using the GraphPad Prism four-parameter dose-response equation (standard slope) using the concentrations of total SR48692 (ref. 14) and bound [3H]NTS. Inhibition constant (K_i) values were derived from IC50 values using the Cheng and Prusoff equation, K_i = IC50/(1 + K_r), where I is the concentration of [3H]NTS (ref. 40).

Competition assays with the nonpeptide antagonist SR48692 (ref. 14) were performed in the presence of [3H]NTS (TEBB buffer, 4.5–5 nM [3H]NTS, NTSR1 concentration 0.5 μM, incubation for 2 h). Data were best fit to a sigmoidal dose-response equation with standard slope using the concentrations of total NTS added versus bound [3H]NTS. Inhibition constant (K_i) values were derived from IC50 values using the Cheng and Prusoff equation, K_i = IC50/(1 + K_r), where I is the concentration of [3H]NTS (ref. 40).

Purification of Gq protein from insect cells. All the steps were performed at 4 °C or on ice. Cells from 2 to 4 l of insect cell culture were thawed and sedimented by centrifugation (45 Ti rotor, 4 °C, 125,000 × g, 20 min). The pellet was resuspended in buffer A (50 mM NaCl, 0.5% (w/v) CHAPS) over 10 column volumes. The pellet was resuspended in buffer B (20 mM Hepes pH 8.0, 300 mM NaCl, 5 mM MgCl₂, 5 mM β-mercaptoethanol, 10 μM GDP, 100 μM AEBSF (4-(2-Aminoethyl)benzen sulfonyl fluoride hydrochloride), 3 μg/ml leupeptin, 3 μg/ml ttpyn inhibitor, 20 μg/ml t-lysol chymotrypsin, 20 μg/ml t-lysol phenylalanyl chymotrypsin, 50 μg/ml deoxyribonuclease, 1% (w/v) CHAPS). The sample was clarified by centrifugation (45 Ti rotor, 125,000 × g, 1 h, Optima L90K, Beckman), diluted 1.5-fold with buffer C (20 mM Hepes pH 8.0, 100 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 3 mM MgSO₄, 0.3% (w/v) BSA, 1 μM GDP, 4–8 nM [35S]GTPγS (PerkinElmer), 40 μM adenylyl imidodiphosphate, 0.4 mM cytidine 5’-monophosphate, 0.1% (w/v) 3’-[3-(cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS; Anacrace). Reactions were terminated by diluting the reaction mixture with 2 ml of ice-cold stop buffer (20 mM TrisHCl pH 8.0, 100 mM NaCl and 25 mM MgCl₂) and were filtered over nitrocellulose membranes on a vacuum manifold. Filters were then washed six times with 2 ml of ice-cold stop buffer. The nitrocellulose membranes were dried, and the radioactivity was quantified by liquid scintillation in a Beckman Counter. All data from dose-response experiments were fit to equations with a Hill slope of 1 or variable slope.

The Gq protein used for exchange assays was purified in buffer containing CHAPS. However, the Gq protein addition in the exchange assays did not exceed one-fifth of the reaction volume, thus limiting the free CHAPS concentration to one-fourth of its critical micelle concentration. The purification of Gq protein was done as above with the following modifications. Duplicate reaction mixes (final volume of 75 μl) contained 1 μM unlabelled GTPγS instead of GDP, 25 nM receptor, a defined amount of the Gq protein preparation and 25 nM NTS or no ligand. Aliquots (10 μl) of the reaction were transferred into 2 ml of ice-cold CHAPS, mixed and incubated 30 min.

[35S]GTPγS binding in the absence of NTS (non-catalysed nucleotide exchange) was subtracted from [35S]GTPγS binding in the presence of NTS (total nucleotide exchange). The resulting data were fit to a one-site binding model in the Prism software. GraphPad Prism software and best fit to a one-site binding equation to determine the dissociation constant (K_d) for the interactions.

Purification of NTS receptor from insect cells. The effect of NaCl on the crystallization of the His-Tev-Gq receptor was studied at 4 °C. From cells to 2 to 4 l of insect cell culture were thawed and sedimented by centrifugation (45 Ti rotor, 4 °C, 125,000 × g, 20 min). The cell supernatant was added versus bound [3H]NTS. Inhibition constant (K_i) values were derived from IC50 values using the Cheng and Prusoff equation, K_i = IC50/(1 + K_r), where I is the concentration of [3H]NTS (ref. 40).
addition of 65 ml of a 3% (w/v) lauryl maltose neopentyl glycol (2,2-didicyclopropyl-1,3-bis-D-maltopyranoside; LMNG; Anatche)18.0/2% (w/v) CHS (cholesterol monomethoxypolyethylene glycol succinate; TM3 salt) solution. After 1 h, NaCl was added and the solution was gently stirred for an additional 15 min. The final volume was 195 ml containing 50 mM TrisHCl pH 7.4, 30% (v/v) glycerol, 200 mM NaCl, 1% (w/v) LMNG/0.1% (w/v) CHS and 10 μM NTS8–13. The sample was clarified by centrifugation (45Ti rotor, 125,000g, 1 h, Optima L90K, Beckman), adjusted with imidazole to a final concentration of 20 mM and batch-incubated overnight with 1.5 ml Talon resin equilibrated with Talon-A+ buffer (50 mM TrisHCl pH 7.4, 30% (v/v) glycerol, 200 mM NaCl, 20 mM imidazole, 1 μM NTS12–13, 0.1% (w/v) LMNG/0.01% (w/v) CHS). After washing the resin with 22.5 ml of buffer Talon-A+ and 1 ml of buffer Talon-A++ (250 mM TrisHCl pH 7.4, 30% (v/v) glycerol, 200 mM NaCl, 20 mM imidazole, 1 μM NTS12–13, 0.05% (w/v) LMNG/0.005% (w/v) CHS), NTS12–13 NSF-T and NTS12–13 ELF-T4L were eluted in 0.5 ml steps with Talon-A++ buffer (50 mM TrisHCl pH 7.4, 30% (v/v) glycerol, 200 mM NaCl, 250 mM imidazole, 10 μM NTS12–13, 0.05% (w/v) LMNG/0.005% (w/v) CHS). Peak fractions were collected (2.5 ml) and desalted using a PD10 column equilibrated in PD10 buffer (50 mM TrisHCl pH 7.4, 200 mM NaCl, 0.003% (w/v) LMNG/0.0003% (w/v) CHS). NTS12–13 NSF-Ts was then added to a concentration of 20 μM and the sample was used for crystallization. Three liters of insect cell culture yielded ~2 mg of purified NTS12–13 NSF-T or NTS12–13 ELF-T4L.

Stability tests in detergent solution. The cell pellets from 10 ml of insect cell cultures were resuspended in 1.8 ml buffer containing LMNG/CHS to give a final buffer composition of 50 mM TrisHCl pH 7.4, 200 mM NaCl, 1% (w/v) LMNG/0.1% (w/v) CHS. The samples were placed on a rotating mixer at 4°C for 1 h. Cell debris and non-solubilized material were removed by ultracentrifugation (TLA 120.1 rotor, 45Ti rotor, 125,000g, 1 h, Optima L90K, Beckman), adjusted with imidazole to a final concentration of 20 mM and batch-incubated overnight with 1.5 ml Talon resin equilibrated with Talon-A+ buffer (50 mM TrisHCl pH 7.4, 30% (v/v) glycerol, 200 mM NaCl, 20 mM imidazole, 1 μM NTS12–13, 0.1% (w/v) LMNG/0.01% (w/v) CHS). After washing the resin with 22.5 ml of buffer Talon-A+ and 1 ml of buffer Talon-A++ (250 mM TrisHCl pH 7.4, 30% (v/v) glycerol, 200 mM NaCl, 20 mM imidazole, 1 μM NTS12–13, 0.05% (w/v) LMNG/0.005% (w/v) CHS), NTS12–13 NSF-T and NTS12–13 ELF-T4L were eluted in 0.5 ml steps with Talon-A++ buffer (50 mM TrisHCl pH 7.4, 30% (v/v) glycerol, 200 mM NaCl, 250 mM imidazole, 10 μM NTS12–13, 0.05% (w/v) LMNG/0.005% (w/v) CHS). Peak fractions were collected (2.5 ml) and desalted using a PD10 column equilibrated in PD10 buffer (50 mM TrisHCl pH 7.4, 200 mM NaCl, 0.003% (w/v) LMNG/0.0003% (w/v) CHS). NTS12–13 NSF-Ts, was then added to a concentration of 20 μM and the sample was used for crystallization. Three liters of insect cell culture yielded ~2 mg of purified NTS12–13 NSF-T or NTS12–13 ELF-T4L.

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Author contributions
B.E.K. expressed and purified NTSR1 crystallization constructs, conducted pharmacological experiments, optimized the purification procedure, performed crystallization trials, collected diffraction data, solved and refined the structures, and performed the experimental work. J.F.W. expressed and purified NTSR1 and G protein, tested NTSR1 mutants for stability and conducted pharmacological experiments. P.S. performed the molecular biology tasks. R.G. performed the crystallographic experiments. B.E.K. expressed and purified NTSR1 and G protein, tested NTSR1 mutants for stability and conducted pharmacological experiments. P.S. performed the experimental work. R.G. performed the crystallographic experiments, assisted with data collection and was responsible for the overall project strategy. The manuscript was written by B.E.K. and R.G.

Additional information
Accession codes. Coordinates and structure factors for NTSR1-LF-T4L and NTSR1-ELF-T4L are deposited in the Protein data Bank under the accession codes 4XEE and 4XEE, respectively.

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