

The dynamic basis of G-protein recognition and activation by a GPCR

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Abstract

G protein-coupled receptor (GPCR) signaling occurs through heterotrimeric G proteins, whose selective activation leads to distinct cellular outcomes. Although over 200 GPCR-G protein complex structures have been determined, these static snapshots provide limited insight into the dynamics of G-protein association and dissociation. Here, we present cryogenic electron microscopy (cryo-EM) structures of human neurotensin receptor 1 (NTSR1) with minimally modified Go and Gq, revealing how the receptor's intracellular surface dynamically rearranges to accommodate each G-protein subtype. Furthermore, time-resolved cryo-EM analyses of NTSR1-Gi visualized G-protein dissociation processes upon GDP/GTP binding. Characterization of over 20 intermediates, complemented by mutational and computational analyses, identifies four key mechanistic features. First, GDP/GTP induces Gi release from both canonical and non-canonical active conformations with distinct kinetics. Second, NTSR1 uses common intracellular rearrangements to recognize different G-protein subtypes and to promote activation of a single subtype. Third, separation from Gβγ involves stepwise remodeling of the Gα switch I-III. Finally, Gi dissociates from the receptor through a pathway that is distinct from that of Gs, and the canonical and non-canonical NTSR1-Gi complexes further diverge in their dissociation trajectories. These findings provide a comprehensive framework for understanding GPCR signaling dynamics and guiding signal-targeted therapeutic development.

Introduction

G protein-coupled receptors (GPCRs) respond to diverse extracellular stimuli and transduce signals across the cell membrane by activating intracellular effector proteins, notably heterotrimeric G proteins. Heterotrimeric G proteins fall into four subfamilies (Gs, Gi/o, Gq/11, and G12/13), each mediating distinct physiological responses¹.

Structural studies of GPCRs in complex with G proteins have provided important insights into their coupling and activation mechanisms, yielding more than 200 complex structures determined predominantly by cryo-EM². However, these static snapshots offer limited insight into the highly dynamic processes of G-protein recruitment, nucleotide exchange, and dissociation. Especially, most represent nucleotide-free states and provide little information on the conformation and dynamics of the α -helical domain (AHD). Moreover, many previous structural studies have relied on engineered constructs, such as mini-G proteins, dominant-negative G α mutants, or HiBiT-fusion construct²⁻⁶, to stabilize these complexes. While such modified systems have been invaluable for structure determination, they may not capture the receptor's full native conformational landscape⁷.

To overcome these limitations, we focused on the neurotensin receptor 1 (NTSR1), a prototypical class A GPCR involved in thermoregulation, blood pressure control, analgesia, and appetite modulation^{8,9}. NTSR1 is strikingly promiscuous, signaling through all four G-protein subfamilies as well as arrestins, and it is a promising therapeutic target for various disorders⁹⁻¹¹. Crystal and cryo-EM structures have captured NTSR1 in both inactive and active states, including complexes with Gi, β -arrestin 1, and GPCR kinase 2¹²⁻¹⁶. However, the structural basis of NTSR1's broad G-protein promiscuity and the dynamics of partner protein activation remain obscure. Notably, a recent cryo-EM study of the NTSR1-Gi complex revealed two distinct coupling conformations: a canonical (C) state and a non-canonical (NC) state in which the G protein is rotated by ~45 degrees relative to the receptor^{16,17}. However, the functional relevance of this NC state remains unclear.

Results

G-protein promiscuity and specificity by NTSR1

To elucidate the mechanisms underlying NTSR1 promiscuity, we first performed conventional cryo-EM analysis of NTSR1-Go and NTSR1-Gq complexes in nucleotide-free states. Unlike previous studies using ICL3-truncated receptors or engineered mini-G proteins¹⁶⁻¹⁸, we utilized human NTSR1 with an intact ICL3, wild-type G α o, and minimally modified G α q (Methods).

NTSR1-Go adopted three distinct states, whereas NTSR1-Gq adopted a single state (Fig. 1a, Supplementary Table 1, and Supplementary Figs. 1-3). In both complexes, one state corresponds to a C-state-like conformation, whereas the other two NTSR1-Go states resemble NC conformations (NC1 and NC2; Fig. 1a,b). Strikingly, the population of NC states is significantly higher than that of the C state in the NTSR1-Go sample (C:NC = 35:65; Supplementary Fig. 1), contrasting with typical GPCR-G protein complexes where the C state predominates. Note that although NTSR1-Gq was prepared with delipidated Gq to enhance coupling, the absence of NC states is not due to this modification, since NTSR1 with delipidated Gi still shows both C and NC states (Supplementary Fig. 4).

Comparison of NTSR1-Go, NTSR1-Gq, and previously determined NTSR1-Gi C-state structures showed overall similarity, but the G α o α 5 helix is rotated by ~5 degrees relative to Gi and Gq (Extended Data Fig. 1a). This rotation is likely caused by a unique salt bridge between NTSR1

R294^{6,26} (superscripts denote Ballesteros–Weinstein numbering¹⁹) and Gαo’s D341^{G.H5.13} (superscripts denote Common Gα Numbering (CGN)²⁰) on the α5 helix. Consequently, the interaction between the receptor’s F174^{34,51} and the hydrophobic pocket of Gα is markedly weaker in NTSR1–Go, potentially explaining the lower C-state population (Fig. 1c and Extended Data Fig. 1b).

As the interaction between F^{34,51} and the Gα hydrophobic pocket is important for stabilizing the GPCR–G protein complex in a canonically active state and facilitating GDP release²¹, and F^{34,51} does not interact with this pocket in the NC states of Gi/o complexes (Fig. 1d and Extended Data Fig. 1c), we hypothesized that the F174^{34,51} mutation would differentially affect G-protein subtypes. In NanoBiT G-protein dissociation assays, the F174^{34,51}A mutation abolished Gq activation but only partially reduced Gi and Go activation ($E_{max} = 28.0 \pm 8.9\%$ and $37.4 \pm 2.4\%$, respectively) (Fig. 1e, f, and Extended Data Fig. 1d). This indicates that the F174^{34,51}A mutation confers altered G-protein selectivity and suggests that the NC state is a functional, Gi/o-specific intermediate, and that nucleotide-induced G-protein dissociation can be triggered directly from the NC state.

Further comparison revealed three intriguing features on the intracellular side of the receptor (Extended Data Fig. 1e–h). First, the C-terminus of ICL3 is disordered in both Gi and Go complexes, but it makes extensive van der Waals interactions with Gαq (Extended Data Fig. 1f). This is consistent with a previous study demonstrating that ICL3 deletion significantly affects intracellular levels of inositol trisphosphate (IP3), but not cyclic adenosine monophosphate (cAMP)²². Second, the TM2 N-terminus adopts different secondary structures between the Gi/o and the Gq complexes. In the Gi and Go complexes, the N-terminal TM2 forms an α helix until Q98^{2,36}, and Q98^{2,36} packs against D350^{G.H5.22} and G350^{G.H5.22}, respectively, stabilizing α5-helix binding. In contrast, in the Gq complex, the final helical turn is unwound, creating space to accommodate the bulkier E355^{G.H5.22}, which would otherwise cause significant steric clash (Extended Data Fig. 1g). Third, R184^{4,41} on TM4 flips orientation: it points toward the intracellular side in Gi/o complexes but toward α5 in the Gq complex, where it forms a hydrogen-bonding network that stabilizes binding (Extended Data Fig. 1h). Notably, R184^{4,41} is reported to interact with E28^{G.HN.52} of Gαi (R28^{G.HN.52} in Gαq) in lipid nanodiscs¹⁷, suggesting that R184^{4,41} stabilizes G-protein binding through two different modes. Collectively, these results strongly suggest that NTSR1 exploits intracellular plasticity, particularly in TM2, TM4, and ICL3, to accommodate diverse G-protein subtypes.

Heterogeneity of G-protein binding states in a lipid bilayer

To verify conformational heterogeneity in a more physiologically relevant environment, we performed high-speed atomic force microscopy (HS-AFM) on NTSR1 reconstituted in lipid bilayers using delipidated G proteins (Extended Data Fig. 2 and Methods). We immobilized NTSR1 and measured Gi dwell times under equilibrium conditions in the presence of GDP (Extended Data Fig. 2a–d,g and Supplementary Movies 1 and 2). Dwell-time distribution analysis revealed four time constants: $t_1 = 0.077$ s, $t_2 = 1.70$ s, $t_3 = 10.3$ s, and $t_4 = 129.8$ s (Extended Data Fig. 2d). While the very short (t_1) and long (t_4) components likely represent nonspecific binding, the presence of two distinct intermediate components (t_2 and t_3) suggests that NTSR1–Gi populates two distinct states, consistent with a previous bio-layer interferometry study reporting two kinetic dissociation rates of Gi from rat NTSR1¹⁷.

We next performed identical HS-AFM experiments with NTSR1–Gq (Extended Data Fig. 2e,f,h and Supplementary Movie 3). After excluding artifactual short (t_1) and long (t_3) components, Gq exhibited only one specific component ($t_2 = 2.15$ s). This comparison reveals three key features.

First, the heterogeneity mirrors cryo-EM findings: NTSR1–Gi exhibited two states (t_2 , t_3), whereas NTSR1–Gq showed only one (t_2). Second, the amplitude-derived population ratio for Gi (46% t_2 : 54% t_3) moderately matches the cryo-EM particle distribution of the delipidated Gi complex (36% NC : 64% C; Supplementary Fig. 4), suggesting that the short-lived t_2 component corresponds to the NC state and the long-lived t_3 component to the C state. Third, relative dwell times align with in-cell measurements: Gq dwell time ($t_{Gq} = 2.15$ s) was 2.95-fold shorter than the Gi weighted-average ($t_{Gi} = 6.34$ s), consistent with in-cell BRET data showing Gq activation is 2.79-fold faster than Gi activation²³. Together, these findings suggest that the structural heterogeneity observed in conventional cryo-EM largely matches that observed by HS-AFM, and that G-protein association/dissociation from the NC state is faster than from the C state.

Time-resolved cryo-EM analysis of the NTSR1–Gi complex with GDP

To gain deeper insight into G-protein recognition and activation dynamics, we next performed time-resolved (TR) cryo-EM analysis of the NTSR1–Gi complex. Since GDP addition to the nucleotide-free complex reverses the association reaction and this process occurs more slowly than GTP-driven dissociation²⁴, we reasoned that this would facilitate trapping early intermediates. We accordingly initiated TR-cryo-EM experiments in the presence of GDP at effective reaction times of ~8 and 15 seconds (Extended Data Fig. 3 and Methods).

From the 8-second incubation sample (GDP_{8s}), extensive classification yielded seven distinct high-resolution structures: four C-state-like (GDP_{8s}-C1 to C4) and three NC-state-like (GDP_{8s}-NC1 to NC3) (Fig. 2a,b, Supplementary Table 1, and Supplementary Figs. 3, 5–8). The C-state and NC-state structures each exhibited high internal similarity, with subtle differences primarily in G-protein orientation (Fig. 2c). However, clear distinctions emerged between the GDP_{8s}-C1/C2 and GDP_{8s}-C3/C4 states. In GDP_{8s}-C1 and C2, GDP was absent, the AHD was open and detached from the RLD, and the $\alpha 5$ helix was deeply inserted into the receptor core. In contrast, in GDP_{8s}-C3 and C4, GDP occupied the nucleotide-binding pocket, the AHD was closed and attached to the RLD, and the $\alpha 5$ helix had withdrawn (Fig. 2a and Extended Data Fig. 3b). Despite extensive classification, we did not observe intermediate states such as (1) GDP-unbound with a closed AHD, (2) GDP-bound with an open AHD, or (3) a closed AHD with an inserted $\alpha 5$ helix (Supplementary Table 2). These observations imply two critical points. First, AHD closure stabilizes GDP binding, and vice versa; intermediate states lacking this coupling appear inherently unstable. This contrasts with Gs, where a nucleotide-free, AHD-closed state has been observed in a previous TR-cryo-EM study²⁵. Second, both GDP binding and AHD closure are prerequisites for $\alpha 5$ -helix withdrawal from the receptor core, aligning with previous studies showing that GDP binding alone is insufficient to disengage the $\alpha 5$ helix^{26,27}.

We also performed the same analysis for the 15-second incubation sample (Extended Data Fig. 4a, Supplementary Table 1, and Supplementary Figs. 3, 9, 10). Surprisingly, from this sample, only two states were reconstructed, which were essentially identical to GDP_{8s}-C3 and C4 (designated GDP_{15s}-C1 and C2) (Extended Data Fig. 4a). To confirm that the absence of NC-state maps was not a data-processing artifact, we performed various analyses, including heterogeneous refinements using the 8-second NC maps as input; however, no high-resolution NC-state maps emerged (Supplementary Fig. 9 and Methods). This suggests that GDP binds directly to the NC state, inducing dissociation significantly faster than from the C state, consistent with our HS-AFM analysis (Extended Data Fig.

2).

As the 15-second sample yielded no additional states, we examined whether the dissociation reaction had reached equilibrium by this time point. To test this, we collected data from a 60-second sample, merged particles from the 8-, 15-, and 60-second conditions, and classified them into four distinct states: the AHD-open NC state (NC-open), the AHD-open C state (C-open), the AHD-closed C state (C-closed), and free G protein (free G protein) (Methods). The populations of the NC-open and C-open states consistently decreased, whereas the free Gi fraction consistently increased from 8 to 60 seconds (Extended Data Fig. 4b,c). These observations suggest that dissociation continues beyond 15 seconds, supporting the idea that the 8- and 15-second time windows are suitable for capturing reaction intermediates.

Structural transitions in the GDP-bound NTSR1-Gi complexes

Next, to understand conformational transitions in more detail, we focused on the GDP_{8s}-C1 to -C4 states, as the GDP_{15s} states were essentially identical to GDP_{8s}-C3/4 (Extended Data Fig. 4a and Supplementary Fig. 11). Structural superposition of the receptor confirmed that the entire G protein undergoes a clockwise rotation of ~8 degrees from GDP_{8s}-C1 to -C4 (Fig. 2d,e). This is consistent with the anticlockwise rotation observed during Gs association²⁸.

Intriguingly, this Gi rotation is coupled to receptor conformational changes (Extended Data Fig. 3c-f). From GDP_{8s}-C1 to -C4, the intracellular sides of TM5 and TM6 move inward, partially reverting toward inactive conformations (Extended Data Fig. 3d). Moreover, ICL3 and the intracellular ends of TM1, TM2, and ICL1 continuously change conformations to provide an optimal receptor surface for accommodating the rotating G α i (Extended Data Fig. 3e, f). Remarkably, the TM2 and ICL3 conformations in GDP_{8s}-C4 closely resemble those in the C-state NTSR1-Gq complex (Extended Data Fig. 1f,g); ICL3 interacts with G α , and the TM2 N-terminus unwinds to avoid steric clashes. These observations suggest that NTSR1 intracellular plasticity plays dual roles: accommodating multiple G-protein subtypes (i.e., G-protein promiscuity) and facilitating state transitions during G-protein association and dissociation.

From GDP_{8s}-C2 to -C3, GDP binding and AHD closure trigger a significant α 5-helix conformational shift: it rotates ~60° and translates ~5.8 Å downward toward GDP (Fig. 2f,g). This is accompanied by unwinding of the last α 5 helical turn and rearrangement of the TCAT motif on the β 6- α 5 loop (Fig. 2g,h). Notably, the α 5 helix also shifts ~3.6 Å toward ICL2, forming a new contact with F174^{34,51} (Fig. 2h). The F174^{34,51} rotamer slightly changes to avoid a steric clash with I344^{G.H5.16} (Fig. 2i and Extended Data Fig. 3g), establishing additional interactions with D341^{G.H5.13}, I344^{G.H5.16}, and K345^{G.H5.17} (Extended Data Fig. 3h).

To assess the functional importance of these interactions, we performed NanoBiT G-protein dissociation assays using alanine mutants of these residues and found that all three mutants exhibited significantly reduced NTSR1-mediated Gi activation (Fig. 2j, left, and Extended Data Fig. 3i). Notably, a similar trend was observed in BLT1 (conserved F^{34,51}) (Extended Data Fig. 3j, k), whereas the μ -opioid receptor (MOR; containing V^{34,51}) showed minimal effects (Fig. 2j, right, k and Extended Data Fig. 3l). These findings suggest that (1) the basic architecture and functionality of these G-protein mutants remain intact, and (2) specific interactions between F^{34,51} and these residues are important for efficient Gi activation by GPCRs harboring F^{34,51}, possibly by stabilizing intermediate conformations and lowering energy barriers during G-protein association and dissociation.

228

229 **Step-by-step transitions during GDP-induced G-protein dissociation**

230 To dissect the simultaneous events of GDP binding, AHD closure, and $\alpha 5$ -helix withdrawal during the
 231 conformational transition from GDP_{8s}-C1/C2 to GDP_{8s}-C3/C4, we performed a 3D variability analysis
 232 (3D-VA) (Fig. 3, Extended Data Fig. 5, Supplementary Figs. 12, 13, and Supplementary Movie 4).
 233 Briefly, we merged particles from GDP_{8s}-C1 to -C4 and classified them into 20 subsets (frames 1–20)
 234 based on the $\alpha 5$ -helix extraction motion, enabling atomic modeling of each step (Methods, Fig. 3a,
 235 Extended Data Fig. 5, and Supplementary Figs. 12, 13).

236 In frames 1–5, structures exhibited no clear density for GDP or the AHD (Fig. 3b–d; frame 1).
 237 The $\alpha 5$ helix remained deeply inserted, forming extensive interactions, including a hydrogen bond
 238 between R166^{3.50} of NTSR1 and C351^{G.H5.23} of G α i (Fig. 3e; frame 1). Thus, these structures were
 239 essentially identical to GDP_{8s}-C1. In contrast, GDP phosphate density became visible near the P-loop
 240 in frame 6 (Fig. 3b–d; frame 6). Concurrently, the $\alpha 1$ helix rotated slightly away from the $\alpha 5$ helix,
 241 creating space for GDP binding (Fig. 3c; frame 6). Due to this movement, Q52^{G.H1.07} on $\alpha 1$ oriented
 242 toward $\alpha 5$ and formed a hydrogen bond with the main-chain carbonyl of D328^{G.s6h5.05} (Fig. 3d; frame
 243 6). In the next frame (frame 8), the guanine moiety of GDP appeared (Fig. 3b–d; frame 8), and the $\beta 6$ –
 244 $\alpha 5$ loop underwent slight conformational changes (Fig. 3c; frame 8). Subsequently, in frame 9, the
 245 AHD approached the RLD, sandwiching GDP (Fig. 3b; frame 9). This closure and tight GDP binding
 246 triggered a downward shift of the $\alpha 5$ helix (Fig. 3c; frame 9). Notably, instead of moving as a rigid
 247 body, the last three residues (C351^{G.H5.23}, G352^{G.H5.24}, and L353^{G.H5.25}) of G α i maintained interactions
 248 with the receptor core, and the C-terminal single helical turn (residues L348^{G.H5.20}–C351^{G.H5.23}) of the
 249 $\alpha 5$ helix unwound to facilitate its withdrawal (Fig. 3c,e; frame 9). While the last helical turn was
 250 unwinding, the rest of the $\alpha 5$ helix maintained its helical structure (Fig. 3c; frame 9). This contrasts
 251 markedly with Gs withdrawal, in which the middle region of $\alpha 5$ unwinds²⁵. These distinct mechanisms
 252 might contribute to the kinetic differences between Gs and Gi²⁹.

253 Intriguingly, concomitant with withdrawal, Q52^{G.H1.07} flipped its side chain while maintaining
 254 interaction with $\alpha 5$, as if supporting its withdrawal movement (Fig. 3d; frame 9). This idea aligns with
 255 previous mutational analyses of the Q52^{G.H1.07}R mutant of G α i, which binds GDP/GTP but fails in
 256 receptor activation³⁰. After $\alpha 5$ -helix withdrawal and the structural rearrangement of the TCAT motif,
 257 the structure adopted a conformation nearly identical to the GDP_{8s}-C4 state (Fig. 3a–e; frame 20).

258

259 **Time-resolved cryo-EM analysis of the NTSR1–Gi complex with GTP**

260 Next, to explore GTP-induced G-protein dissociation dynamics, we performed TR-cryo-EM analysis
 261 using GTP with 8-second and “0–5-second” incubation conditions (Methods). Note that we verified
 262 that the 8-second incubation had not reached equilibrium (Extended Data Fig. 4 and Supplementary
 263 Fig. 14).

264 Analysis of the 8-second condition revealed no NC states, consistent with the 15-second GDP
 265 experiment (Supplementary Figs. 9, 15). This strongly suggests that the NC state can be directly
 266 activated by GDP/GTP and dissociates faster than the C state. The three complexes reconstructed
 267 represent C-state-like structures; thus, we designated them as GTP_{8s}-C1 to GTP_{8s}-C3 (Fig. 4a,
 268 Supplementary Table 1, and Supplementary Figs. 3, 15, 16).

269 The overall structure of the GTP_{8s}-C1 state closely resembles that of GDP_{8s}-C1/C2, while the

structures of GTP_{8s}-C2 and -C3 are similar to those of GDP_{8s}-C3 and -C4, respectively. Moreover, structural changes driving the transition from GTP_{8s}-C1 to -C3, such as α N-helix rotation, intracellular receptor plasticity, and α 5-helix shifts, were similar to those in GDP-bound structures (Supplementary Fig. 17). However, further analysis revealed notable differences between the GDP_{8s} and GTP_{8s} structures.

First, clear GTP density was observed in the AHD-open GTP_{8s}-C1 state, and no nucleotide-free states were reconstructed in the GTP sample (Supplementary Table 2 and Supplementary Fig. 15). This contrasts with the 8-second GDP sample, wherein AHD-open structures were nucleotide-free (Supplementary Table 2). These observations suggest that GTP binds more stably to the RLD of the C state, likely due to the γ -phosphate group. Comparison between GDP_{8s}-C1 and GTP_{8s}-C1 indicates that GTP binding slightly relocates the α 1 helix and TCAT motif and influences the dynamics (Supplementary Fig. 17a), but it does not trigger α 5-helix withdrawal, confirming that both nucleotide binding and AHD closure are required for withdrawal from the C state.

Second, although GTP binds to the RLD in both GTP_{8s}-C1 and -C2 states, the guanine moiety position is significantly different. While the phosphate group's position remains constant, the guanine moiety binds deeply in GTP_{8s}-C2 (closed AHD), likely because the closed AHD pushes it toward the α 5 helix. In contrast, in GTP_{8s}-C1 (open AHD), it binds shallowly and is solvent-exposed (Fig. 4b and Supplementary Fig. 17b,c). To test whether the guanine moiety, unlike the phosphate group, requires AHD closure to stably occupy the deep pocket, we performed unbiased, all-atom MD simulations starting from GTP_{8s}-C1 with GTP placed at either shallow or deep positions (Methods and Extended Data Fig. 6). Simulations confirmed that the phosphate group binds significantly more stably than the guanine moiety (Fig. 4c and Extended Data Fig. 6a,b). Even when initialized at the deep site, the guanine ring is unstable and frequently migrates to the shallow site or exits the pocket entirely (Fig. 4c and Extended Data Fig. 6c, d). This result aligns with our 3D-VA analysis, which indicated that phosphate binding precedes guanine binding (Fig. 3b–d). These findings could also explain why both nucleotide binding and AHD closure are necessary for α 5-helix withdrawal; only upon AHD closure does the guanine moiety move sufficiently close to the α 5 helix to form interactions robust enough to generate the force required to withdraw it from the receptor core.

Sequential conformational changes in switch regions during G-protein dissociation

The fundamental distinction between GDP- and GTP-induced dissociation is whether the G α subunit disengages from G $\beta\gamma$. To elucidate this mechanism, we compared Gi heterotrimer structures in the GDP_{8s}-C3/C4 and GTP_{8s}-C2/C3 states (Fig. 4d–h). While GDP_{8s}-C3/C4 were nearly identical (Fig. 4d, left and Fig. 4e, left), GTP_{8s}-C2/C3 were markedly different in the G α i–G $\beta\gamma$ orientation (Fig. 4d, right; Fig. 4e, right). Since GDP_{8s}-C3 and GTP_{8s}-C2 are virtually indistinguishable, the principal rearrangements must occur during the transition from GTP_{8s}-C2 to -C3, immediately prior to heterotrimer dissociation.

The main G α i–G β interfaces involve the α N helix and the switch II region of G α i³¹. Superimposition of GTP_{8s}-C2 and GTP_{8s}-C3 Gi heterotrimers based on G β revealed no notable differences in α N, indicating that structural changes concentrate in switch II (Fig. 4e). Indeed, in GTP_{8s}-C3, the switch II helix adopts a conformation that significantly weakens interactions with the β -propeller of G β (Fig. 4f–h).

To clarify how GTP binding triggers the switch II rearrangement, we compared our structures

(GDP_{8s}-C1 and GTP_{8s}-C1/C2/C3) with known crystal structures (GDP-bound Gi heterotrimers and GppNHp-bound G α i)^{31,32}. This comparison revealed sequential conformational changes in the switch regions (Extended Data Figs. 7 and 8). In the inactive GDP-bound Gi, R178^{G.hfs2.02} (switch I) forms a salt bridge with E43^{G.s1h1.04} (P-loop), creating the “seatbelt” conformation and stabilizing GDP³³ (Extended Data Figs. 7a, 8a). Upon receptor engagement, α 5-helix insertion and AHD opening disorder the switch I and III loops and disrupt this salt bridge (Extended Data Figs. 7a, 8a, 8f, 8g). GTP binding partially stabilizes the switch III loop (Extended Data Figs. 7b, 8b), but it remains flexible (Extended Data Fig. 8h). Subsequent AHD closure reorganizes the switch I loop (Extended Data Figs. 7c, 8c); however, the γ -phosphate group induces a slightly altered conformation that clashes with G202^{G.s3h2.01} and G203^{G.s3h2.02}, disordering the switch II– β 3 loop (Extended Data Fig. 8c, top). Notably, corresponding Go mutations (G203 and G204, respectively) impair receptor-mediated G α –G β γ dissociation and are linked to neurodevelopmental disorders^{34–36}.

In contrast, R178^{G.hfs2.02} re-establishes the salt bridge with E43^{G.s1h1.04}, which also forms a new salt bridge with R242^{G.H3.01} (switch III), shifting the entire switch III loop toward E43^{G.s1h1.04} (Extended Data Fig. 8c, bottom). Extensive polar interactions involving positively and negatively charged residues, including R205^{G.H2.01}, R208^{G.H2.04}, D237^{G.s4h3.11}, and E245^{G.H3.04} on the switch II– β 3 and III loops, pull the switch II helix toward switch III, weakening its interaction with G β (Extended Data Figs. 7d, 8d, 8i, 8j). R208 and E245 belong to the conserved G–R–E triad, which is critical for coupling GTP binding to G β γ release and is known to harbor disease-associated mutations^{37,38}. The importance of this network is supported by previous MD simulations³⁷ and biochemical studies showing that corresponding Go mutations (R209 and E246) impair receptor-mediated G α –G β γ dissociation³⁰.

The conformations of switches I–III in GTP_{8s}-C3 converge with the GppNHp-bound crystal structure (Extended Data Figs. 7e, 8e, 8k), indicating that our cryo-EM snapshots capture the initial, decisive steps that culminate in complete heterotrimer dissociation. Collectively, these observations demonstrate how GTP binding orchestrates the concerted reshaping of switches I–III to prime the Gi heterotrimer for dissociation.

339

340 **Fast and distinct Gi dissociation from the NC state complex**

341 While we elucidated the C-state dissociation pathway, the NC-state dissociation pathway has remained
 342 elusive due to its rapid kinetics. To visualize early intermediates, we analyzed the 0–5-second GTP
 343 sample (GTP_{0–5s}) (Fig. 5).

344 The dataset was highly heterogeneous, but classification identified seven distinct structural
 345 states (Supplementary Figs. 18, 19). Five matched previously observed GDP_{8s} structures (GDP_{8s}-C1,
 346 -C2, -NC1, -NC2, and -NC3) (Supplementary Fig. 18), but we also identified two additional states not
 347 detected under the GDP_{8s}, GDP_{15s}, or GTP_{8s} conditions (Fig. 5a, Supplementary Fig. 19). Because
 348 these states appeared only under the short-incubation condition, and given that Gi dissociates from the
 349 NC state much faster than from the C state, we interpret them as intermediates along the NC-state
 350 dissociation pathway and designate them as GTP_{0–5s}-NC4 and GTP_{0–5s}-NC5.

351 GTP_{0–5s}-NC4 resembled other NC states but exhibited additional rotations relative to the
 352 membrane plane: the G protein was rotated $\sim$ 10 degrees horizontally and $\sim$ 8 degrees vertically,
 353 weakening α 5-helix interactions with TM5/6 (Fig. 5b). Remarkably, this state also displayed a distinct
 354 GTP binding mode. While the phosphate group was coordinated by the P-loop, the guanine ring faced

the switch III loop rather than the TCAT motif (Fig. 5c, d), suggesting a dissociation mechanism distinct from the C state.

In GTP_{0-5s}-NC5, these rotations became more pronounced. Horizontal rotation increased to ~60 degrees and vertical rotation to ~30 degrees (Fig. 5b). When the lipid bilayer position was calculated based on the cryo-EM map, this excessive vertical tilt placed the N-terminus of the α N helix within the membrane (Fig. 5e). In a cellular context, this would create severe steric clashes.

Based on these observations, we propose a model for NC-state dissociation. GTP phosphate binding increases heterotrimer flexibility, inducing pronounced horizontal and vertical rotations. The vertical rotation lifts the α N helix toward the membrane. Although our structures were obtained in detergent, in cells, this upward motion would likely create steric pressure against the bilayer, generating a lever-like force that pulls the α 5 helix out of the receptor core, pivoting at the α N C-terminus. Consistent with increased mobility, the local resolution of Gi in the NC4/NC5 states is substantially lower (~7 Å) despite high overall resolution (3.1 Å) (Extended Data Fig. 9a). Intriguingly, this lever-like mechanism resembles a model proposed for the D1R-Gs complex²⁷.

Notably, in both GTP_{0-5s}-NC4 and -NC5 states, Gi progressively detaches from the receptor while the AHD remains fully open and separated from the RLD, maintaining contact with G β (Extended Data Fig. 9b). Importantly, we observed free Gi heterotrimers with an open AHD and an extended α 5 helix in NC4/NC5 samples (Extended Data Fig. 9c), confirming that Gi can dissociate from the NC state without AHD closure or α 5 retraction. This contrasts sharply with the C-state dissociation pathway, where both nucleotide binding and AHD closure are prerequisites for α 5-helix withdrawal. For the NC state, GTP binding and the lever-like motion driven by α N tilting appear sufficient for α 5 extraction. These findings explain the rapid dissociation kinetics and demonstrate that the NC-state dissociation mechanism is fundamentally distinct from that of the C state.

Discussion

In this study, we demonstrated how NTSR1 accommodates multiple G-protein subtypes and elucidated the mechanisms of Gi association and dissociation using equilibrium and non-equilibrium cryo-EM analyses complemented by biophysical and computational approaches (Extended Data Fig. 10).

Our findings highlight the structural plasticity of receptor intracellular segments, which is crucial for G-protein recognition and activation. Similar flexibility was observed in the cholecystokinin A receptor (CKAR) study using wild-type Gq³⁹, emphasizing the need for minimally modified G proteins to accurately capture receptor dynamics. Indeed, such plasticity was absent in a previous NTSR1 analysis utilizing engineered mini-G proteins¹⁸.

Recent GDP/GTP-bound structures (e.g., D1R-mini-Gs and GPR139-mini-Gs/q) also utilized mini-G proteins carrying the I372A mutation, which prevents α 5 withdrawal from the receptor^{27,40}. Consequently, these structures likely represent early intermediate-like states with modest changes in the α 1 helix and β 6- α 5 loop (Supplementary Fig. 20). In contrast, our study uses the WT Gi heterotrimer and TR-cryo-EM to capture a broader set of intermediates, offering a more comprehensive view of Gi dissociation.

Our analysis of the C-state dissociation pathway clarifies the functional roles of nucleotide binding. The guanine moiety acts as an AHD-dependent switch, while the phosphate group facilitates initial binding. Notably, R178^{G.hfs2.02} and Q204^{G.s3h2.03} transiently interact with the phosphate group, possibly stabilizing the conformations of the switch I loop and switch II helix in active forms,

respectively (Extended Data Fig. 8e, j), and facilitating $G\alpha$ dissociation from $G\beta\gamma$. Given their known roles in accelerating GTP hydrolysis⁴¹, our findings suggest that $G\alpha$ dissociation from $G\beta\gamma$ and subsequent GTP hydrolysis are functionally coupled processes, with R178^{G.hfs2.02} and Q204^{G.s3h2.03} playing crucial roles.

Crucially, this work also characterizes the enigmatic NC state as a G_i/o -specific intermediate, contributing to the G-protein selectivity of NTSR1. The NC state can be activated considerably faster than the C state, likely because GDP/GTP binding alone is sufficient to induce $\alpha 5$ -helix withdrawal. This reflects weaker receptor- G_i interaction in the NC state, specifically the absence of hydrogen bonds between the $\alpha 5$ helix and the receptor, and the lack of extensive hydrophobic interactions between F174^{34.51} and the G_i hydrophobic pocket. In contrast, $\alpha 5$ -helix withdrawal in the C state requires both GDP/GTP binding and AHD closure, highlighting a mechanistic difference between the two states.

Comparison with TR-cryo-EM of the $\beta 2AR$ - G_s complex reveals a key mechanistic difference between G_i and G_s dissociation, centered on the dynamics of $\alpha 5$ withdrawal (Extended Data Fig. 11). In G_i , $\alpha 5$ remains helical during extraction except for unwinding of its terminal turn, and it is pulled largely straight toward the bound nucleotide (Extended Data Fig. 11a,b). In G_s , $\alpha 5$ follows a curved trajectory away from the $\alpha 1$ helix and becomes unwound near its midpoint (Extended Data Fig. 11c,d). Our 3D-VA trajectory, together with previously reported $\beta 2AR$ - G_s 3D-VA structures, suggests that this divergence stems from distinct initial interactions at the $\alpha 5$ N-terminus. Specifically, R373^{G.H5.05} (G_s) and Q333^{G.H5.05} (G_i) interact differentially with the receptor $\beta 2$ - $\beta 3$ loop (Extended Data Fig. 11e,f), resulting in a substantial angular difference of ~ 27 degrees in the initial $\alpha 5$ position (Extended Data Fig. 11g). This angular difference implies that straight extraction of the G_s $\alpha 5$ helix would cause severe steric clashes with the $\alpha 1$ helix (Extended Data Fig. 11h). To avoid this, G_s follows an arc-like trajectory, creating mechanical stress that leads to unwinding (Extended Data Fig. 11i). This model is further supported by GDP-bound crystal structures: G_i retains a stable $\alpha 5$ helix even in isolation (Extended Data Fig. 11b,i), whereas G_s exhibits intrinsic instability with a kinked and partially unwound central region (Extended Data Fig. 11c,j). Interestingly, the terminal helical turn of G_i is disordered, aligning with our observations during the extraction process (Extended Data Fig. 11b,i). These differences in structural stability and extraction dynamics likely contribute to subtype-specific activation kinetics and coupling specificity⁴². Future TR-cryo-EM studies of additional subtypes will further clarify these mechanisms.

To fully understand the reaction coordinates and verify the similarity between GDP-induced G_i dissociation and GDP-bound G_i association, it is essential to obtain structural information on unstable, low-population intermediates under more physiologically relevant conditions. This will require more extensive TR-cryo-EM studies incorporating additional time points using systems such as nanodiscs or liposomes, complemented by biophysical and computational analyses. Nevertheless, our findings provide comprehensive insights into NTSR1-mediated G-protein promiscuity, selectivity, and activation dynamics, as well as general principles of G_i activation. We expect these results will facilitate the future design of GPCR therapeutics aimed at targeting specific intermediate states and selectively modulating distinct G-protein signaling pathways.

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METHODS

Expression and purification of NTSR1

For NTSR1–Gq complex, N-terminally truncated WT NTSR1 (UniProtKB, P30989; residues 20–418) was modified to include an N-terminal FLAG-tag epitope, and a C-terminal enhanced green fluorescent protein (eGFP) and 10 × histidine tag; the N-terminal and C-terminal tags are removable by tobacco etch virus (TEV) protease and human rhinovirus 3C (HRV3C) protease cleavages, respectively. The A85^{1.54}L mutation was introduced to increase expression. For NTSR1–Go complex, the NTSR1 construct described above was used, except the FLAG tag was replaced by an Avi tag.

These constructs were expressed in *Sf9* insect cells using the pFastBac baculovirus system. *Sf9* insect cells were grown in suspension in ESF921 medium (Expression system) to a density of 3.0–3.5 × 10⁶ cells ml⁻¹, infected with baculovirus, and shaken at 27.5 °C for 44–78 h. The cell pellets were collected by centrifugation at 6,000 *g* for 12 min, lysed with a hypotonic lysis buffer (20 mM HEPES–NaOH pH 7.5, 20 mM NaCl, 10 mM MgCl₂, 1 mM benzamidine, 1 µg ml⁻¹ leupeptin), collected again by centrifugation at 6,000 *g* for 15 min, flash frozen in liquid nitrogen, and stored at –80 °C until use. The cell pellets were lysed with the hypotonic lysis buffer and collected by centrifugation at 9,000 *g* for 10 min. The cell pellets were disrupted by homogenizing with a glass dounce homogenizer in a hypertonic lysis buffer (20 mM HEPES–NaOH pH 7.5, 1 M NaCl, 10 mM MgCl₂, 1 mM benzamidine, 1 µg ml⁻¹ leupeptin), and crude membrane fraction was collected by ultracentrifugation (45Ti rotor, 125,000 *g* for 1 h). The above process was repeated twice; then, the membrane fraction was homogenized with a glass douncer in a membrane storage buffer (20 mM HEPES–NaOH pH 7.5, 500 mM NaCl, 10 mM imidazole, 20% glycerol, 1 mM benzamidine, 1 µg ml⁻¹ leupeptin), flash frozen in liquid nitrogen, and stored at –80 °C until use.

The membrane fraction was lysed with a solubilization buffer (1% lauryl maltose neopentyl glycol (LMNG, Anatrace), 0.1% cholesteryl hemisuccinate tris salt (CHS), 20 mM HEPES–NaOH pH 7.5, 500 mM NaCl, 20% glycerol, 10 mM imidazole, 2 µM NTS₈₋₁₃ (GenScript), 1 mM benzamidine, 1 µg ml⁻¹ leupeptin) and solubilized at 4 °C for 2 h. The insoluble cell debris was removed by ultracentrifugation (45Ti rotor, 125,000 *g*, 45 min), and the supernatant was mixed with the Ni-NTA superflow resin (QIAGEN) at 4 °C for 1.5 h. The Ni-NTA resin was loaded onto an open chromatography column, washed with 5 column volumes of wash1 buffer (0.005% LMNG, 0.0005% CHS, 20 mM HEPES–NaOH pH 7.5, 500 mM NaCl, 5% glycerol, and 40 mM imidazole, 0.4 µM NTS₈₋₁₃) and with 5 column volumes of wash2 buffer (0.005% LMNG, 0.0005% CHS, 20 mM HEPES–NaOH pH 7.5, 300 mM NaCl, 5% glycerol, and 40 mM imidazole, 0.4 µM NTS₈₋₁₃), and eluted by elution buffer (0.005% LMNG, 0.0005% CHS, 20 mM HEPES–NaOH pH 7.5, 300 mM NaCl, 5% glycerol, and 400 mM imidazole, 2 µM NTS₈₋₁₃). After dialysis against dialysis buffer (0.005% LMNG, 0.0005% CHS, 20 mM HEPES–NaOH pH 7.5, 100 mM NaCl, 10% glycerol, 0.5 mM β-ME, 0.5 µM NTS₈₋₁₃) and tag cleavage by His-tagged HRV3C protease, the sample was reapplied onto the Ni-NTA open column to trap the cleaved eGFP–His-tag and His-tagged HRV3C protease. The flow-through fraction was collected and concentrated to approximately 5 mg ml⁻¹ using an Amicon Ultra 50 kDa molecular weight cutoff centrifugal filter unit (Merck Millipore). The concentrated samples were centrifuged (20,000 *g*, 20 min) before size-exclusion chromatography on a Superdex 200 Increase 10/300 GL column (Cytiva), equilibrated in SEC buffer (0.005% LMNG, 0.0005% CHS, 20 mM HEPES–NaOH pH 7.5, 100 mM NaCl, 1 µM JMV449 (Tocris), 100 µM TCEP).

The peak fractions of the protein were collected and concentrated to approximately 10 mg ml⁻¹, flash-frozen in liquid nitrogen, and stored at -80 °C until use.

Expression and purification of heterotrimeric Gq

The heterotrimeric Gq construct was expressed in High Five™ insect cells using the pFastBac baculovirus system. We expressed the heterotrimeric G protein using three separate viruses: the first virus delivered the sequence for the human Gα_q subunit (UniProtKB, P50148), the second expressed the Gβ₁ (UniProtKB, P62873) and Gγ₂ (UniProtKB, P59768) subunits, and the last carried the sequence for the human Ric8A (UniProtKB, Q9NPQ8). Note that the N-terminal 18 residues of Gα_q were replaced with those of Gα_i to enable binding to scFv16, and the C3S mutation in Gα_q and the C68S mutation in Gγ₂ were introduced to prevent lipid modifications. These mutations, which prevent lipidation, are critical for facilitating purification by size-exclusion chromatography, especially given the relatively low formation efficiency of the NTSR1-Gq complex. The sequence for the Gβ₁ subunit contains an amino-terminal 6x histidine tag followed by an HRV3C sequence. The K236E mutation in Ric8A and the T159I mutation in the Gβ₁ subunit occurred accidentally. High Five™ cells were infected at a density of 2.5-3.0 × 10⁶ cells/ml and incubated at 27.5 °C for 47 h. The cell pellets were collected by centrifugation at 6,000 g for 12 min, lysed with a hypotonic lysis buffer, collected again by centrifugation at 6,000 g for 15 min, flash-frozen in liquid nitrogen, and stored at -80 °C until use.

The cell pellet from 6 L of insect cell culture was resuspended to a lysis buffer (30 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 5 mM imidazole, 5 mM β-ME, 50 μM GDP). Cells were broken by sonication and clarified by centrifugation (125,000 g for 1 h). The supernatant was loaded onto a Ni-NTA column. The column was washed sequentially with 10 column volumes of wash1 buffer (20 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 1 mM MgCl₂, 10% glycerol, 10 mM imidazole, 50 μM GDP, 5 mM β-ME) and 10 column volumes of wash2 buffer (20 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 1 mM MgCl₂, 10% glycerol, 30 mM imidazole, 50 μM GDP, 5 mM β-ME). The column was eluted with elution buffer (20 mM HEPES-NaOH pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 10% glycerol, 300 mM imidazole, 50 μM GDP, 5 mM β-ME). The sample was dialyzed overnight against a dialysis buffer (20 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 1 mM MgCl₂, 10% glycerol, 10 μM GDP, and 0.1 mM TCEP). The sample was concentrated to approximately 5 mg ml⁻¹ using an Amicon Ultra 10 kDa molecular weight cutoff centrifugal filter unit (Merck Millipore), and centrifuged (20,000 g, 30 min) before size-exclusion chromatography on a Superdex 200 10/300 GL column (Cytiva), equilibrated in SEC buffer (20 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 1 mM MgCl₂, 10% glycerol, 0.1 mM TCEP, 10 μM GDP). The peak fractions of the protein were collected and concentrated to approximately 20 mg ml⁻¹, flash-frozen in liquid nitrogen, and stored at -80 °C until use.

Expression and purification of heterotrimeric Gi and Go

Gi1/Go heterotrimer was expressed and purified as previously described⁴³. In brief, we expressed the heterotrimeric G proteins using two separate viruses; one virus delivers the sequence for the human native Gα_{i1} or Gα_{oA} subunit (UniProtKB, P63096-1 or P09471), and the other expresses the Gβ₁ and Gγ₂ subunits. Note that the Gγ₂ subunit is wild-type and does not contain the C68S mutation. The sequence for the Gβ₁ subunit contains an amino-terminal 6x histidine tag followed by an HRV3C sequence. High Five™ cells were infected at a density of 2.5-3.0 × 10⁶ cells/ml and incubated at 27.5 °C for 46 h. The cell pellets were collected by centrifugation at 6,000 g for 12 min, lysed with a

hypotonic lysis buffer, collected again by centrifugation at 6,000 *g* for 15 min, flash frozen in liquid nitrogen, and stored at -80°C until use.

Cells were lysed in a lysis buffer (10 mM HEPES-NaOH pH 7.5, 100 μM MgCl_2 , 5 mM β -ME, 10 μM GDP, 1 mM benzamidine, and 1 $\mu\text{g ml}^{-1}$ leupeptin). The membrane fraction was collected by centrifugation at 10,000 *g* for 15 min and solubilized by a Dounce homogenizer with a solubilization buffer (20 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 1% sodium cholate hydrate, 0.05% n-dodecyl- β -D-maltoside (DDM, Millipore), 5 mM MgCl_2 , 1 mM MnCl_2 , 5 mM β -ME, 10 μM GDP, 1 mM benzamidine, and 1 $\mu\text{g ml}^{-1}$ leupeptin). The solubilization reaction was allowed to proceed with 1,000 units of λ -phosphatase (NEB) for 1 h at 4°C . The soluble fraction was incubated in a batch with Ni-NTA resin for 1.5 h at 4°C . The resin was loaded onto an open chromatography column, and the detergent was gradually exchanged from sodium cholate to 0.1% DDM. Heterotrimeric G protein was eluted in an elution buffer (20 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 0.05% DDM, 5 mM MgCl_2 , 100 μM TCEP, 10 μM GDP, 250 mM imidazole). The heterotrimer was concentrated to approximately 5 mg ml^{-1} , and centrifuged (20,000 *g*, 30 min) before size-exclusion chromatography on a HiLoad 16/600 GL column (Cytiva), equilibrated in SEC buffer (20 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 0.05% DDM, 1 mM MgCl_2 , 100 μM TCEP, 10 μM GDP). The peak fractions of the protein were collected and concentrated to approximately 20 mg ml^{-1} , flash-frozen in liquid nitrogen, and stored at -80°C until use.

Expression and purification of scFv16

The single-chain construct of Fab16 (scFv16) was expressed and purified as described⁴⁴. In brief, a C-terminal 8 $\times$ histidine-tagged scFv16 was expressed in secreted form from High FiveTM insect cells using the baculovirus method (Expression Systems) and purified by Ni-NTA (Qiagen) chromatography. Supernatant from baculovirus-infected cells was pH-balanced by the addition of Tris pH 8.0. The Ni-NTA eluent was concentrated and purified through gel-filtration chromatography in a final buffer (20 mM HEPES-NaOH pH 7.5 and 100 mM NaCl). Monomeric fractions were pooled, concentrated, flash-frozen, and stored at -80°C until use.

Formation and purification of NTSR1-Gq complex

Purified NTSR1 was mixed with a 2-molar excess of Gq heterotrimer in a coupling buffer (0.001% LMNG, 0.0001% CHS, 20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 1 mM MgCl_2 , 10% glycerol, 10 μM JMV449, 100 μM TCEP, and 10 μM GDP). The coupling reaction was allowed to proceed with λ -phosphatase at 4°C overnight. Apyrase (New England Biolabs) was added to catalyze unbound GDP hydrolysis. After one more hour of incubation at 4°C , a 3-molar excess of scFv16 was added to the sample, and the complex was further incubated at 4°C for 3 h. The complexing mixture was mixed with a Ni-NTA resin at 4°C for 0.5 h. The bound complex was washed in a buffer containing 0.00375% LMNG and 0.00125% glyco diosgenin (GDN). The complex was then eluted in 0.00375% LMNG, 0.0005% CHS, 0.00125% GDN, 20 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 10% glycerol, 10 μM JMV449, and 300 mM imidazole. The NTSR1-Gq-scFv16 complex was purified by size-exclusion chromatography on a Superdex 200 Increase 10/300 GL column (Cytiva) in 0.00075% LMNG, 0.0001% CHS, 0.00025% GDN, 20 mM HEPES-NaOH pH 7.5, 150 mM NaCl and 5 μM JMV449. Peak fractions were concentrated to about 10 mg ml^{-1} for electron microscopy studies.

Formation and purification of NTSR1–Go complex

Purified NTSR1 was mixed with a 1.2 molar excess of Go heterotrimer, and the coupling reaction was allowed to proceed at 4 °C for 6 h. Apyrase and λ -phosphatase were added to catalyze unbound GDP hydrolysis and remove phosphorylation from proteins, respectively. After one more hour of incubation at 4 °C, a 1.25 molar excess of scFv16 was added to the sample, and the complex was further incubated at 4 °C overnight. The complexing mixture in a coupling buffer (0.25% LMNG, 0.025% CHS, 0.0125% DDM, 20 mM HEPES-NaOH pH 7.5, 50 mM NaCl, 0.5 mM MgCl₂, 7.5 μ M TCEP, 5 μ M GDP, 50 μ M JMV449, 5% glycerol, 5 mM CaCl₂) was loaded onto M1 anti-Flag immunoaffinity resin. The bound complex was first washed in a buffer containing 0.375% LMNG and 0.125% GDN, followed by washes in gradually decreasing the detergent concentrations. The complex was then eluted in 0.00375% LMNG, 0.0005% CHS, 0.00125% GDN, 20 mM HEPES-NaOH pH 7.5, 50 mM NaCl, 5% glycerol, 5 μ M JMV449, 5 mM EDTA and 200 μ g/ml Flag peptide. The NTSR1–Go–scFv16 complex was purified by size-exclusion chromatography on a Superdex 200 Increase 10/300 GL column (Cytiva) in 0.00375 % LMNG, 0.0005% CHS, 0.00125 % GDN, 20 mM HEPES-NaOH pH 7.5, 50 mM NaCl and 1 μ M JMV449. Peak fractions were concentrated to about 10 mg ml⁻¹ for electron microscopy studies.

Cryo-EM Grid Preparation

Prior to grid preparation, the sample was centrifuged at 20,380 *g* for 30 min at 4 °C. The grids were glow-discharged with a PIB-10 plasma ion bombarder (Vacuum Device) at 10 mA current with a dial setting of 2 min for both sides.

For nucleotide-free NTSR1-G protein complexes, 3 μ L of protein solution was applied to freshly glow-discharged Quantifoil R1.2/1.3 Au 300 mesh holey carbon grids. Samples were vitrified by plunging into liquid ethane cooled by liquid nitrogen using an FEI Vitrobot Mark IV (Thermo Fisher Scientific) at 4 °C with 100% humidity. The blotting force was set to 10, and the waiting and blotting times were 10 s and 4 s, respectively.

For time-resolved experiments, the nucleotide-free complex was mixed with nucleotides directly on the cryo-EM grid to capture dissociation intermediates (Extended Data Fig. 3a). Specifically, for GDP_{8s} and GTP_{8s} samples, 0.3 μ L of 10 mM nucleotide was added to the grid, followed by 2.7 μ L of the NTSR1-Gi complex. The solutions were mixed by pipetting for 1 s and subsequently blotted for 4 s using the FEI Vitrobot Mark IV (Thermo Fisher Scientific) at 4 °C and 100% humidity. Given that pipetting requires $\sim$ 1 s and the blotting/plunging sequence takes $\sim$ 7 s ($\sim$ 4 s and $\sim$ 3 s, respectively), this procedure resulted in an effective reaction time of approximately 8 s. For GDP_{15s}, GTP_{15s}, and GDP_{60s} samples, additional incubation times of 7 s, 7 s, and 52 s were included, yielding effective reaction times of $\sim$ 15 s and $\sim$ 60 s, respectively. Since initial screening with a Talos microscope suggested substantial dissociation after incubations longer than 15 s, detailed data collection was mainly focused on the 8 s and 15 s conditions.

For the GTP 0–5 s sample, 0.3 μ L of 6.7 mM GTP was mounted on a grid and 1.7 μ L of protein solution was added, and the grid was promptly blotted for 2 s and frozen without pipetting or incubation. Pipetting was intentionally omitted so that the effective reaction time would vary depending on the particle's position: complexes closer to the GTP droplet could react for up to $\sim$ 5 s, whereas complexes farther away encountered GTP only just before freezing, resulting in reaction times approaching 0 s.

Cryo-EM data acquisition and image processing of NTSR1–Gq complex

The first cryo-EM image set was acquired on a Krios G3i 300 keV microscope (Thermo Fisher Scientific) equipped with a Gatan BioQuantum energy filter and a K3 direct detection camera in the electron counting mode. The movie dataset was collected in CDS mode, using a nine-hole image shift strategy in the SerialEM software⁴⁵. In total, 3,375 movies were acquired with a calibrated pixel size of 0.83 Å pix⁻¹ and with a defocus range of -0.8 to -1.6 µm. Each movie was acquired for 5.0 s and split into 64 frames, resulting in an accumulated exposure of about 55 e⁻ Å⁻² at the grid.

The second cryo-EM image set was acquired at 300 kV on a Krios G3i microscope equipped with a Gatan BioQuantum energy filter and a K3 direct detection camera in the electron counting mode. The movie dataset was collected in CDS mode, using a nine-hole image shift strategy in the SerialEM software. In total, 5,103 movies were acquired with a calibrated pixel size of 0.83 Å pix⁻¹ and with a defocus range of -0.8 to -1.6 µm. Each movie was acquired for 5.0 s and split into 64 frames, resulting in an accumulated exposure of about 55 e⁻ Å⁻² at the grid.

The data processing was performed using RELION 3.1⁴⁶. Each movie set was subjected to Motion correction and CTFIND4⁴⁷. For each dataset, template-based Auto picking in all micrographs was performed, yielding 2,892,870 and 4,424,959 particles, respectively. After 3D classification, selected 659,554 and 1,286,184 particles from two datasets were merged and then subjected to 3D refinement, Bayesian polishing⁴⁸, and 3D refinement. The particles were further aligned to the receptor and classified with a mask covering the entire complex and no-alignment 3D classification. Selected 1,092,577 particles were subjected to 3D refinement, CTF refinement⁴⁹, 3D refinement, Bayesian polishing, CTF refinement, 3D refinement, and particle subtraction for detergent micelle and α-helical domain (AHD). After CTF refinement, the final 3D refinement enabled us to obtain a 2.24 Å map.

Cryo-EM data acquisition and image processing of NTSR1–Go complex

The first cryo-EM image set was acquired on a Krios G3i 300 keV microscope (Thermo Fisher Scientific) equipped with a Gatan BioQuantum energy filter and a K3 direct detection camera in the electron counting mode. The movie dataset was collected in standard mode, using the fringe-free imaging (FFI) and aberration-free image shift (AFIS) strategy in the EPU software (Thermo Fisher Scientific). In total, 5,846 movies were acquired with a calibrated pixel size of 0.83 Å pix⁻¹ and with a defocus range of -0.8 to -1.6 µm. Each movie was acquired for 2.3 s and split into 48 frames, resulting in an accumulated exposure of about 50 e⁻ Å⁻² at the grid.

The second cryo-EM image set was acquired at 300 kV on a Krios G3i microscope (Thermo Fisher Scientific) equipped with a Gatan BioQuantum energy filter and a K3 direct detection camera in the electron counting mode. The movie dataset was collected in standard mode, using the fringe-free imaging (FFI) and aberration-free image shift (AFIS) strategy in the EPU software (Thermo Fisher Scientific). In total, 11,900 movies were acquired with a calibrated pixel size of 0.83 Å pix⁻¹ and with a defocus range of -0.6 to -1.6 µm. Each movie was acquired for 2.1 s and split into 48 frames, resulting in an accumulated exposure of about 50 e⁻ Å⁻² at the grid.

The data processing was performed using the cryoSPARC v3.3.1⁵⁰ and RELION 4.0⁵¹. The collected 17,746 movies were subjected to Patch motion correction and Patch CTF refinement in cryoSPARC. Particles were picked from all micrographs using Blob picker, Template picker, and

Topaz picker⁵². After various classifications including 2D classification and Heterogeneous refinement, a total of 3,136,780 particles were selected and merged for NC state and 1,570,730 for C state. These particles were transferred to RELION 4.0 and were subjected to 3D refinement.

For NC state, detergent micelle and AHD were subtracted from particles first. Subtracted particles were then subjected to focused 3D classification without alignment using a mask covering TMs, resulting in two classes with different F174^{34,51} orientations, named NC1 state and NC2 state. After 3D refinement and CTF refinement, particles were subjected to focused 3D classification without alignment using a mask excluding detergent micelle and AHD for NC1 state, and a mask covering TMs for NC2 state. The final 3D refinements for selected 392,815 and 632,482 particles enable us to obtain 2.95 and 2.99 Å maps for NC1 and NC2 states, respectively.

For C state, focused 3D classification without alignment using mask-covering TMs yielded a map with more distinct TMs. Selected 371,296 particles were then subjected to particle subtraction for detergent micelle and AHD, CTF refinement, and the final 3D refinement, resulting in a 3.37 Å map.

Cryo-EM data acquisition of NTSR1–Gi complexes bound to GDP or GTP

The first cryo-EM image set was acquired on a Talos Arctica 200 keV microscope (Thermo Fisher Scientific) equipped with a Falcon3 direct detection camera. The movie dataset was collected in linear mode. In total, ~1,000 movies for each GDP/GTP time-course sample were acquired with 1.251 Å pix⁻¹ with a defocus range of -0.8 to -1.6 µm. Each movie was acquired for 1.84 s and split into 48 frames, resulting in an accumulated exposure of about 48 e⁻ Å⁻² at the grid.

The additional three cryo-EM image sets were acquired on a Krios 300 kV G4 microscope (Thermo Fisher Scientific) equipped with a Gatan BioQuantum energy filter and a K3 direct detection camera in electron-counting mode. These movie datasets were collected in CDS mode, using the fringe-free imaging (FFI) and aberration-free image shift (AFIS) strategy in the EPU software (Thermo Fisher Scientific). In total, 14,900, 12,172, and 12,073 movies were acquired with a calibrated pixel size of 0.83 Å pix⁻¹ and with a defocus range of -0.6 to -1.6 µm. Each movie was acquired for 2.3 s and split into 48 frames, resulting in an accumulated exposure of about 50 e⁻ Å⁻² at the grid.

Cryo-EM image processing of NTSR1–Gi complexes bound to GDP or GTP

Data processing was performed using cryoSPARC v4.3 and RELION 5.0⁵³. The collected 14,214, 12,172, 12,073, and 17,834 movies were subjected to Patch motion correction and Patch CTF refinement in cryoSPARC.

For the first dataset, GDP_{8s}, particles were picked from all micrographs using a blob picker, extracted, and subjected to several rounds of 2D classification. The averaged classes representing obvious overall GPCR–G-protein structures (Supplementary Fig. 5b) were used as a template for subsequent template-based picking on 14,214 micrographs. 10,752,095 particles were picked by template-based picking, extracted, and subjected to several rounds of 2D classification. The selected 1,528,365 particles were subjected to ab-initio reconstruction (K = 5) and class 1, 4, and 5 maps were used for heterogeneous refinement with all picked particles (Supplementary Fig. 5c). The consensus NC state map (699,612 particles, resolution 3.04 Å) was loaded into no-align 3D classification with 5 classes and resolution-gradient 3D classification with 3 classes, which provided the three representative NC state conformers through NU-refinement. The consensus C state map (1,027,049 particles, resolution 2.93 Å) was expanded with 3D-VA in cluster mode with 20 classes, which

provided the four different conformers and a decoy map. These five maps-guided heterogeneous refinement, 3D-VA, and NU-refinement, provided the two AHD-open and AHD-closed conformers.

For the second dataset, GDP_{15s}, we first performed the same strategy as for GDP_{8s}, but it only showed C state maps and did not represent any NC state map. Then, taking into account that NC state particles had become a very minor population, we performed iterative 2D classification, NU-refinement, no-align classification, and reference-guided 3D classification with a decoy, one NC state AHD-open, one C state AHD-open, and the two C state AHD-closed maps. All these analyses only showed C state AHD-closed maps, and thus we loaded the 2D-classified 1,827,389 particles into a heterogeneous refinement with one decoy and the two C state AHD-closed maps. The obtained particles were subjected to a resolution-gradient classification and NU-refinement.

For the third dataset, GTP_{8s}, we employed the same strategy as for the GDP_{15s} dataset, but similarly no NC state map was obtained. Then, we generated a new reference map from this dataset through blob picking, particle extraction, several rounds of 2D classification, ab-initio reconstruction, NU-refinement, and no-align 3D classification. Subsequently, we performed template-based picking, TOPAZ picking, particle extraction, and several rounds of 2D classification, providing C state consensus particles (1,389,327 particles, resolution 3.12 Å). The consensus particles were subjected to a heterogeneous refinement, and the resulting particles were loaded into resolution-gradient classification and NU-refinement.

For the fourth dataset, GTP_{0-5s}, we used blob picking, particle extraction, several rounds of 2D classification, and reference-guided classification using GTP_{8s} classes to compare the condition of this grid with the others (Supplementary Fig. 18). In the first round of reference-guided classification, a decoy, GDP_{8s}-NC2, GDP_{8s}-C1, GDP_{8s}-C3, and free G protein were used as references, allowing us to isolate junk particles. In the second round, particles assigned to GDP_{8s}-C1 (1,830,213 particles), GDP_{8s}-C3 (472,045 particles), and GDP_{8s}-NC2 (1,022,016 particles) in the first round were further classified based on minor conformational differences by using 2–3 conformers in the same state as references (i.e., GDP_{8s}-NC1–3, GDP_{8s}-C1–2, and GDP_{8s}-C3–4). Notably, the GDP_{8s}-NC2-guided class showed a noisy map after the second round of reference-guided classification; therefore, these particles were further subjected to 3D classification, and 57,597 particles were used for reconstruction of the GDP_{8s}-NC2 state map. Reference-guided classification with the AHD-closed, GDP_{8s}-C3 state as a reference resulted in an AHD-open, GDP_{8s}-C1-like map, suggesting that the population of particles with a closed AHD is very small in this sample, which is consistent with the very short incubation time. These results also suggest that this sample may allow visualization of novel, early intermediate states. Motivated by this, we tested several ab-initio reconstructions through blob picking, particle extraction, and several rounds of 2D classification (Supplementary Fig. 19). As expected, these ab-initio reconstructions revealed two types of free G-protein heterotrimer classes with open and closed AHD, and a unique receptor–G-protein complex map in which the G protein is rotated approximately perpendicular to the receptor. We then performed template-based picking, particle extraction, and several rounds of 2D classification and auto 2D select jobs using this unique complex map. The resulting particles were subjected to two rounds of ab-initio reconstruction, yielding the NC4 and NC5 conformers. These maps and particles were subjected to local refinement of the receptor and G protein, respectively, and the resulting maps were used for model building.

3D-VA using GDP_{8s}-C states

To visualize sequential movement with 3D-VA, we loaded the consensus C state particles from GDP_{8s} into 3D-VA intermediate mode, showing entirely nice movie frames and a reconstructed map. However, high-resolution maps could not be reconstructed through global NU-refinement with particles belonging to frames 1-3 and 17-20, which showed that the input particles were not suitable for sequential structural visualization with 3D-VA. Then, we employed the fully classified particles of GDP_{8s}-C1-4, and conducted 3D-VA. Successfully, all frame particles could be independently reconstructed at 2-4 Å resolution through NU-refinement, showing that each frame contained purified and homogeneous particles suitable for atomic model building.

Analysis of state transitions over incubation time

To visualize state transitions over time, two additional datasets, GDP_{60s} and GTP_{15s}, were processed by cryoSPARC v4.3 and RELION 5.0. The collected 14,214, 12,172, 12,073, and 17,834 movies were subjected to Patch motion correction and Patch CTF refinement in cryoSPARC. All particles were picked with a blob picker under unified conditions (Minimum particle diameter 80 (Å) and Maximum particle diameter 185 (Å)) and extracted from 9,000 micrographs, except for the GDP_{60s} and GTP_{15s} datasets. In these datasets, as the number of micrographs was less than 9,000, all of them (5,920 and 6,088 micrographs) were used. Through two rounds of 2D classification, the particles were mixed in the GDP and GTP datasets, respectively, and loaded into two rounds of heterogeneous refinement with five reference classes (decoy, NC state AHD-open, C state AHD-open, C state AHD-closed, and free G protein). In the first cycle, decoy-class particles were eliminated, and the others were subjected to the second cycle of classification. The resulting particles were used for particle-number evaluation and 3D map reconstruction with NU-refinement. Note that decoy-class particles were not used for particle-number evaluation, and the states that did not show obvious 3D protein maps were colored with hatching in the histogram.

Model building and refinement

For the NTSR1-Gq complex, the initial templates for NTSR1 and JMV449 were derived from the published NTSR1-Gi structure (PDB: 6OS9)¹⁶, the template for Gα_q was from the structure of the histamine H1 receptor in complex with Gq (PDB: 7DFL)⁵⁴, and the templates for Gβ, Gγ, and scFv16 were from the structure of the 5HT_{2A} serotonin receptor in complex with mini-Gsqi (PDB: 6WHA)⁵⁵. For the NTSR1-Go complex, the initial templates for NTSR1, JMV449, Gβ, Gγ, and scFv16 were derived from NTSR1-Gi (PDB: 6OS9), and the template for Gα_o was from the structure of the α_{2B} adrenergic receptor in complex with Go (PDB: 6K41)⁵⁶. These starting models were then subjected to iterative rounds of manual and automated refinement in COOT⁵⁷ (v0.9.8.92) and Refmac5⁵⁸ (v5.8.0419) in Servalcat pipeline⁵⁹ (v0.4.39), respectively. An external restraint from the published NTSR1-Gi C state structure (PDB: 6OS9) generated by ProSMART⁶⁰ was also used during refinements of the NTSR1-Go C state structure.

The models of NTSR1-Gi complexes from time-resolved cryo-EM analysis and NTSR1-delipidated Gi complexes from the conventional cryo-EM analysis were built from the published NTSR1-Gi structures (PDB: 6OS9 and 6OSA), except for GTP_{0-5s}-NC5. All starting models except for GTP_{0-5s}-NC5 were then subjected to iterative rounds of manual and automated refinement in COOT⁵⁷ and Refmac5⁵⁸ in Servalcat pipeline⁵⁹, respectively. The map of GTP_{0-5s}-NC5 showed some preferred orientation, thus we fit each chain of the NTSR1-Gi structure (PDB: 6OSA) as a rigid body,

and the rough-fit models were automatically accommodated into each map with the chain refine function in COOT under 4.3 Å self-restraints to avoid severe structural and geometric changes.

The final models were visually inspected for general fit to the map, and geometry was further evaluated using MolProbity⁶¹. The final refinement statistics are summarized in Supplementary Table 1. All molecular graphics figures were prepared with UCSF ChimeraX (v1.6.1)⁶² and CueMol2 (v2.3.0.454, <http://www.cuemol.org>).

NanoBiT-based G-protein dissociation assay

G-protein dissociation activity was measured using the NanoBiT-G-protein dissociation assay⁶³ with minor modifications. HEK293A cells were seeded in 6-well culture plates at a concentration of 2×10^5 cells/ml (2 ml per well in DMEM (Nissui) supplemented with 5% fetal bovine serum (Gibco), glutamine, penicillin, and streptomycin) one day prior to transfection. A NanoBiT-G-protein sensor comprising a Gα subunit fused with a large fragment (LgBiT) at the α-helical domain (Gai-LgBiT, Gao-LgBiT, or Gαq-LgBiT) and an N-terminally small fragment (SmBiT)-fused Gγ2 subunit containing a C68S mutation (SmBiT-Gγ2-CS) was expressed along with untagged Gβ1 subunit and the G-protein chaperone RIC8A (specifically for the Gq assay).

The transfection solution was prepared by mixing 5 μL (per well hereafter) of polyethylenimine (PEI) Max solution (1 mg/ml; Polysciences), 200 μL of Opti-MEM (Thermo Fisher Scientific), and a plasmid mixture consisting of 200 ng of N-terminally FLAG-tagged NTSR1 or untagged MOR or untagged BLT1, 100 ng of Gα-LgBiT, 500 ng of Gβ1 subunit, 500 ng of SmBiT-Gγ2-CS subunit, and 100 ng of RIC8A. After a 1-day incubation, the transfected cells were harvested with PBS containing 0.53 mM EDTA, centrifuged, and resuspended in 2 ml HBSS supplemented with 0.01% bovine serum albumin (BSA; protease- and fatty acid-free grade; SERVA) and 5 mM HEPES (pH 7.4), referred to as assay buffer. The cell suspension (80 μL per well) was dispensed into a white 96-well plate, followed by the addition of 20 μL of 50 μM coelenterazine (Angene) diluted in assay buffer. After incubation for 2 hours at room temperature, baseline luminescence was measured using a SpectraMax L (Molecular Devices) and analyzed by SoftMax Pro 7 (Molecular Devices). Subsequently, titrated concentrations of JMV449 (for NTSR1) or DAMGO (for MOR) or leukotriene B4 (for BLT1), prepared at 6-fold final concentrations, were manually added at 20 μL per well. The plate was immediately measured in kinetic mode, and luminescence counts recorded between 5 and 10 minutes after ligand addition were averaged and normalized to initial counts. The resulting fold-change values were further normalized against those of vehicle-treated samples and plotted to depict the G-protein dissociation response. Using GraphPad Prism 10 (v10.5) software (GraphPad), the G-protein dissociation signals were fitted to a four-parameter sigmoidal concentration-response curve with the HillSlope constrained to absolute values of less than 2.

Flow cytometry

Transfection was performed following the protocol described in the “NanoBiT-based G-protein dissociation assay” section. One day after transfection, cells were harvested by adding 200 μL of Dulbecco’s PBS (D-PBS) containing 0.53 mM EDTA, followed by the addition of 200 μL of Hank’s Balanced Salt Solution (HBSS) supplemented with 5 mM HEPES (pH 7.4). The cell suspension was then transferred in duplicate to a 96-well V-bottom plate. Cells were fluorescently labeled using an anti-FLAG epitope (DYKDDDDK) tag monoclonal antibody (Clone 1E6, FujiFilm Wako Pure

Chemicals; 10 µg/ml diluted in blocking buffer consisting of 2% goat serum and 2 mM EDTA in D-PBS) and a goat anti-mouse IgG secondary antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific, 10 µg/ml diluted in blocking buffer). After washing with D-PBS, cells were resuspended in 200 µL of D-PBS containing 2 mM EDTA and passed through a 40 µm filter.

Fluorescence intensity of single cells was quantified using an EC800 flow cytometer equipped with a 488 nm laser (Sony). Alexa Fluor 488 signals were recorded using the FL1 channel. Flow cytometry data were analyzed using FlowJo software (FlowJo). Live cells were gated with a forward scatter parameter (FS-Peak-Lin) cutoff set at 390 and a gain of 1.7. Mean fluorescence intensity (MFI) values from approximately 20,000 cells per sample were utilized for analysis.

Molecular Dynamics Simulations of the NTSR1–Gi protein complex

We performed twenty independent 1-µs all-atom molecular dynamics (MD) simulations of the NTSR1 bound to the Gi heterotrimer. Ten simulations were started from a modeled structure in which the shallow-bound GTP found in the C1 state was replaced by a deep-bound GTP conformation (Products 1-10). The other ten simulations were started from the original shallow-bound C1 state without GTP replacement (Products 11-20). The alpha-helical domain (AHD) of the Gα subunit was not resolved in the cryo-EM structure and was therefore modeled based on the Gi heterotrimer structure (PDB: 6CMO). To avoid steric interference between the AHD and Gβγ subunits, we performed a 5-ns MD simulation of the Gα subunit with positional restraints applied to the Ras-like domain and selected a frame at 5 ns in which the modeled AHD did not interact with Gβγ.

The complete NTSR1–Gi complex, including the modeled AHD, was used to construct the MD system, whereas the unresolved ICL3 was left unmodelled. Protonation states were assigned using PDB2PQR with the Adaptive Poisson-Boltzmann Solver (APBS) at pH 7.0. The complex was embedded in a pure 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) bilayer using CHARMM-GUI, forming a symmetric membrane with approximately 250 lipids per leaflet (503 total POPC lipids). The system was solvated with TIP3P water in a rectangular box of $\sim 13.6 \times 13.6 \times 19.9$ nm³, yielding $\sim 80,000$ water molecules. Na⁺ and Cl⁻ ions were added to neutralize the system and achieve a physiological salt concentration of 150 mM. These systems represent simplified models of mammalian membranes and cellular ionic environments.

All simulations were performed using AMBER with the CHARMM36m force field. Periodic boundary conditions were applied in all directions. The integration time step was 2 fs, and trajectory snapshots were saved every 10 ps. The temperature was maintained at 310.15 K using a Langevin thermostat, and pressure at 1 bar using a Monte Carlo (MC) barostat. The SHAKE algorithm was applied to constrain bonds involving hydrogen atoms. Short-range electrostatic and van der Waals interactions were calculated with a 1.2 nm cutoff.

Systems were first subjected to energy minimization under constant volume conditions. The system was then gradually heated to 310.15 K over 60 ps, applying harmonic positional restraints of 10.0 kcal/mol·Å² to the protein–ligand complex and 2.5 kcal/mol·Å² to the lipid bilayer. The equilibration protocol consisted of the following sequential steps:

1. 300 ps NVT equilibration with 10.0 kcal/mol·Å² (complex) and 2.5 kcal/mol·Å² (lipids) restraints
2. 300 ps NVT equilibration with 5.0 kcal/mol·Å² (complex) and 2.5 kcal/mol·Å² (lipids) restraints
3. 300 ps NPT equilibration with 5.0 kcal/mol·Å² (complex) and 2.5 kcal/mol·Å² (lipids) restraints
4. 1 ns NPT equilibration with 1.0 kcal/mol·Å² (complex) and 0.5 kcal/mol·Å² (lipids) restraints

5. 1 ns NPT equilibration with 0.5 kcal/mol·Å² (complex) and 0.1 kcal/mol·Å² (lipids) restraints

6. 1 ns NPT equilibration with 0.5 kcal/mol·Å² restraints on the complex only

Following equilibration, unrestrained production MD simulations were carried out for 1 μs per trajectory (20 μs in total).

Preparation of destabilized liposomes

We dissolved the phospholipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and cholesterol (Chol) (molar ratio of DOPC/Chol = 9/1) in chloroform in a glass tube and dried for at least 2 h in vacuo. The lipid film at the glass bottom was hydrated with buffer (10 mM HEPES [pH 7.5], 50 mM KCl) to form a liposome suspension (10 mg mL⁻¹). n-Dodecyl-β-d-maltopyranoside (DDM) was added to the liposome solution at 0.06% to solubilize the liposomes. This micellar solution of DDM and lipids was diluted with DDM-free buffer (20 mM HEPES-KOH (pH 7.5), 50 mM KCl, 0.8 μM JMV449) to a final DDM concentration of 0.006%, resulting in DDM-destabilized liposomes⁶⁴. All procedures were performed at room temperature.

Reconstitution of NTSR1 in lipid bilayers

NTSR1 with a His tag at the N-terminus was used for high-speed AFM observations. To suppress lateral diffusion of NTSR1 in the lipid bilayer, the surface was positively charged using aminosilane-modified mica. The aminosilane-modified mica was prepared by placing 0.01 vol% 3-aminopropyltriethoxysilane (APTES) solution on the mica substrate for 5 minutes and then washing with pure water. Next, the substrate was incubated with a 5 μg/mL NTSR1 solution (20 mM HEPES-KOH (pH 7.5), 50 mM KCl, 1 μM JMV449, 0.06 w% DDM, 10 mM imidazole (pH 7.6)) for 5 minutes, and then the floating NTSR1 was washed away by rinsing with NTSR1-free buffer. The solution was replaced with the destabilized liposome solution and incubated for 20 min, and then washed with DDM-free buffer (20 mM HEPES-KOH (pH 7.5), 50 mM KCl, 1 μM JMV449) to reconstitute NTSR1 in the lipid membrane⁶⁴. Finally, the substrate was placed in an imaging buffer (20 mM HEPES-KOH (pH 7.5), 50 mM KCl, 1 μM JMV449, 200 nM Gi1 or 500 nM Gq, 20 μM GDP) for at least 50 min before observation. Leaving it for a long time before observation allows the binding between NTSR1 and G proteins to reach equilibrium. All procedures were performed at room temperature.

High-speed AFM observation

To analyze the dynamics of G protein binding and dissociation from NTSR1, we observed the repeated binding of G proteins to NTSR1 in the lipid membrane under equilibrium conditions. The imaging buffer was 20 mM HEPES-KOH (pH 7.5), 50 mM KCl, 1 μM JMV449 containing G protein (200 nM Gi1 or 500 nM Gq) and 20 μM GDP. Since Gi and Gq have different binding affinities for NTSR1, their concentrations were adjusted to ensure sufficient binding and dissociation events could be observed. We used a laboratory-built HS-AFM^{65,66} and a cantilever AC10 (Olympus Co., Tokyo, Japan) with an electron beam deposition tip. The resonant frequency of AC10 in water is about 400 kHz. The scanning rate and range were 5 frames/s and 300 nm × 240 nm, respectively. Observations were carried out at room temperature.

Image analysis of HS-AFM data

Prior to analysis, we removed the background, lateral drift, and high-frequency noise of HS-AFM movies using background subtraction, the template matching plugin, and a Gaussian filter in ImageJ, respectively. To extract binding and dissociation time courses, we set ROIs (9.4 nm square) on the HS-AFM movies and corrected average heights in the ROIs. The time courses were then processed to remove the fluctuations in the baselines⁶⁷. The situation of repeated transition in particle height in the HS-AFM movies (Supplementary movies 1-3) was considered as repeated binding of G proteins to NTSR1, and ROIs were set up in such regions. In the absence of G proteins, height transitions are rarely observed (Supplementary movie 1). Due to the high concentration of G proteins in the imaging buffer, the AFM tip becomes blunt due to G-protein adsorption on the tip. This causes the recorded horizontal particle size to be larger. Therefore, the ROI is also set larger than the actual size of the G protein. Furthermore, while the horizontal size of the particle changes during imaging due to changes in the probe tip condition, the vertical size is basically not affected by the sharpness of the probe. Consequently, a height difference of approximately 2-3 nm, corresponding to the size of the G protein, can be stably recorded in both the G protein-bound and -dissociated states (Extended Data Fig. 2b, g, h).

Estimation of time constant using correlation function

The time courses were digitized assuming that NTSR1 is in the bound state when its height is greater than 1.2 nm and is in the dissociated state otherwise. The correlation function ($C(t)$) was calculated using the following equation⁶⁸.

$$C(t) = \langle \delta A(0) \delta A(t) \rangle / \langle \delta A(0)^2 \rangle$$

where the angular bracket represents the ensemble and time averaging. Here, $\delta A(t) = A(t) - \langle A \rangle$,

with $A(t)$ equal to 1 when NTSR1 is bound and 0 otherwise.

$C(t)$ was fitted using exponential functions: $\sum_{i=1}^3 a_i e^{-t/\tau_i}$ or $\sum_{i=1}^4 a_i e^{-t/\tau_i}$. The time constant from bound to dissociated state (τ_{10}) was calculated using the equation

$$\tau_{10} = \tau(n_0 + n_1)/n_0$$

where τ represents τ_1 , τ_2 , τ_3 , or τ_4 , and n_1 and n_0 are the numbers of frames in which NTSR1 is in bound and dissociated states, respectively.

Reporting Summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data Availability

The cryo-EM density maps and atomic coordinates have been deposited in the Electron Microscopy Data Bank (EMDB) and the PDB with accession codes EMD-64904 and 9VAT for NTSR1–Go, C (Nucleotide-free, AHD-open); EMD-64905 and 9VAU for NTSR1–Go, NC1 (Nucleotide-free, AHD-open); EMD-64906 and 9VAV for NTSR1–Go, NC2 (Nucleotide-free, AHD-open); EMD-64907 and 9VAW for NTSR1–Gq, C (Nucleotide-free, AHD-open); EMD-64908 and 9VAX for NTSR1–Gi GDP_{8s}-C1 (GDP-unbound, AHD-open); EMD-64909 and 9VAY for NTSR1–Gi GDP_{8s}-C2 (GDP-unbound, AHD-open); EMD-64910 and 9VAZ for NTSR1–Gi GDP_{8s}-NC1 (GDP-unbound, AHD-open); EMD-64911 and 9VB0 for NTSR1–Gi GDP_{8s}-NC2 (GDP-unbound, AHD-open); EMD-64912 and 9VB1 for NTSR1–Gi GDP_{8s}-NC3 (GDP-unbound, AHD-open); EMD-64913 and 9VB2 for NTSR1–Gi GDP_{8s}-C3 (GDP-bound, AHD-closed); EMD-64914 and 9VB3 for NTSR1–Gi GDP_{8s}-C4 (GDP-bound, AHD-closed); EMD-64915 and 9VB4 for NTSR1–Gi GDP_{15s}-C1 (GDP-bound, AHD-closed); EMD-64916 and 9VB5 for NTSR1–Gi GDP_{15s}-C2 (GDP-bound, AHD-closed); EMD-64917 and 9VB6 for NTSR1–Gi GTP_{8s}-C1 (GTP-bound, AHD-open); EMD-64918 and 9VB7 for NTSR1–Gi GTP_{8s}-C2 (GTP-bound, AHD-closed); EMD-64919 and 9VBA for NTSR1–Gi GTP_{8s}-C3 (GTP-bound, AHD-closed); EMD-67429 and 20ZG for NTSR1–Gi GTP_{0-5s}-C1 (GTP-unbound, AHD-open); EMD-67430 and 20ZH for NTSR1–Gi GTP_{0-5s}-C2 (GTP-unbound, AHD-open); EMD-67431 and 20ZI for NTSR1–Gi GTP_{0-5s}-NC1 (GTP-unbound, AHD-open); EMD-67432 and 20ZJ for NTSR1–Gi GTP_{0-5s}-NC2 (GTP-unbound, AHD-open); EMD-67433 and 20ZK for NTSR1–Gi GTP_{0-5s}-NC3 (GTP-unbound, AHD-open); EMD-67434 and 20ZL for NTSR1–Gi GTP_{0-5s}-NC4 (GTP-bound, AHD-open); EMD-67426 and 20ZC for NTSR1-delipidated Gi GTP_{0-5s}-C (GTP-unbound, AHD-open); EMD-67427 and 20ZD for NTSR1-delipidated Gi GTP_{0-5s}-NC (GTP-unbound, AHD-open), respectively. Maps and atomic coordinates for GTP_{0-5s}-NC5 and the 3DVA results of GDP_{8s} have been deposited in Zenodo (<https://zenodo.org/records/15755070> and <https://zenodo.org/records/17731630>). All other data are available upon request from the corresponding author.

Acknowledgements

We thank R. Danev and M. Kikkawa (UTokyo) for setting up the cryo-EM infrastructure; T. Kusakizako and Y. Sakamaki (UTokyo), and R. N. Burton-Smith and K. Murata (NIPS) for assistance with cryo-EM data collection; K. Hasegawa and A. Ohira (UTokyo) for administrative support; K. Sato, S. Nakano, and Ayumi Inoue (Tohoku University) for assistance with cell-based GPCR assays; D. Mukai (Kanazawa University) for technical assistance with HS-AFM; and J. Janetzko (University of Colorado) for helpful discussions. MD simulations were partially performed using computational resources at the Center for Computational Sciences, University of Tsukuba, and the Information Technology Center, The University of Tokyo. We also acknowledge ChatGPT, a multimodal large language model created by OpenAI, for providing guidance to improve the readability of this manuscript. Note that, after using this tool, we reviewed and edited the content as needed and took full

responsibility for the content of the publication. The electron microscopic observations were supported by Research Support Project for Life Science and Drug Discovery (Basis for Supporting Innovative Drug Discovery and Life Science Research [BINDS]) from the Japan Agency for Medical Research and Development (AMED) under grant number JP22ama121002, JP23ama121002, JP24ama121002, JP25ama121002, and JP25ama121005. Calculations were partly conducted on a supercomputer at the Research Center for Computational Science in Okazaki, Japan (project: 25-IMS-C097 to T.S.).

This work was supported by JSPS KAKENHI grants (JP24K18060/JP25H02243 to K. Kobayashi, JP23KJ0363/JP24K18286/JP25H01338 to K. Kawakami, JP22K21351/JP23KJ1997 to S.Y., JP24H02262/JP25K09525 to M.F., JP24K01308 to T.S., JP23K23187 to A.S., JP20H03230 to A.M., JP21H04791/JP24K21281/JP25H0101 to A.I., JP19H03163/JP22H00400/JP25H01338 to H.E.K.); AMED grant (JP223fa627001 to K. Kobayashi, JP21zf0127005 to S.Y., JP22ama121038/JP22zf0127007 to A.I., 24bm1123057h0001 to H.E.K.); JST PRESTO (JPMJPR24OF to M.F.), JST FOREST (JPMJFP224T to A.S., JPMJFR215T to A.I., JPMJFR204S to H.E.K.), JST Moonshot (JPMJMS2023 to A.I.) and JST CREST (JPMJCR21P3/JPMJCR23B1 to H.E.K.); Astellas Foundation for Research on Metabolic Disorders (K. Kobayashi); Kazato Research Foundation (K. Kobayashi); The Senri Life Science Foundation (K. Kawakami); The Naito Foundation (K. Kawakami); Hirose Foundation (K. Kawakami); The Uehara Memorial Foundation (K. Kawakami and A.I.); The Asahi Glass Foundation (H.E.K.); Takeda Science Foundation (H.E.K.).

Author Contributions

K. Kobayashi performed time-resolved cryo-EM experiments and analyzed the data. K. Kawakami and T.J.N. purified and prepared NTSR1–Gi complex under the supervision of K. Kobayashi. T.E.M. performed the cryo-EM analysis of NTSR1–Gq and NTSR1–Go complexes with the help of M.F. and J.K. S.Y. performed MD simulations and analyzed the data under the supervision of A.M.K. Kawakami, H.A, and M.T. performed the mutational assay and analysis under the supervision of A.I. M.T. and T.S. performed HS-AFM experiments under the supervision of A.S. K.Y. helped the model refinement. H.I. conducted cloning and mutagenesis. M.K. and B.K.K. provided input on structural considerations. K. Kobayashi, K. Kawakami, H.E.K. wrote the manuscript with input from all authors. H.E.K. supervised all aspects of the research.

Competing Interests

B.K.K. is a co-founder of, and consultant for, ConfometRx.

FIGURE LEGENDS

Figure 1 | Cryo-EM structures of NTSR1 complexes with Go or Gq in the nucleotide-free state. **a**, Cryo-EM density maps and structural models of the JMV449-bound NTSR1–Go complexes (C, NC1, and NC2 states) and the NTSR1–Gq complex (C state), resolved at 2.2–3.4 Å. **b**, Extracellular views of the superimposed structures, highlighting differences in the relative position of $G\alpha$ with respect to the receptor in the C versus NC states. Top: Comparison between C and NC states in the NTSR1–Gi1 complex (Kato et al., 2019). Middle: Comparison between C, NC1, and NC2 states in the NTSR1–Go complex (this study). Bottom: The NTSR1–Gq complex in the C state (this study). **c,d**, Close-up views of the interface between F174^{34.51} of NTSR1 and the hydrophobic pocket formed by the $\alpha N/\beta 1$ hinge, $\beta 2/\beta 3$ loop, and $\alpha 5$ helix ($\alpha 5H$) of the $G\alpha$ subunit in the C states (**c**) and NC states (**d**). **e,f**, Functional analysis of the F174^{34.51}A mutant using the NanoBiT-G-protein dissociation assay. Dose-response curves (**e**) and E_{max} values (**f**) for Gi, Go, and Gq dissociation upon JMV449 stimulation. Dashed lines represent wild-type (WT) NTSR1; solid lines represent the F174^{34.51}A mutant. Data represent mean $\pm$ SEM of three independent experiments performed in duplicate. ** represents $p < 0.01$ with two-way ANOVA followed by Dunnett's test for multiple comparison analysis with reference to WT. ns, not significantly different between the groups. See also the Source Data file for additional statistics and exact p -values.

Figure 2 | Time-resolved cryo-EM analysis of the NTSR1–Gi complex with GDP.

a,b, Cryo-EM density maps and structural models of the NTSR1–Gi complex captured 8 s after GDP addition. Four canonical-like (C) conformers (**a**, GDP_{8s}-C1 to C4) and three non-canonical-like (NC) conformers (**b**, GDP_{8s}-NC1 to NC3), resolved at 3.0–3.7 Å. GDP_{8s}-C1 and C2 are nucleotide-free with an open alpha-helical domain (AHD), whereas GDP_{8s}-C3 and C4 are GDP-bound with a closed AHD. **c**, Extracellular view of the superimposed structures of all seven conformers, highlighting the distinct positioning of $G\alpha$ relative to the receptor in the C versus NC states. **d**, Side view of the superposition of the four C conformers (GDP_{8s}-C1 to C4). **e–i**, Close-up views of key structural features: the αN helix (**e**), the AHD (**f**), the AHD–GDP–TCAT motif interface (**g**), the interaction between the TCAT motif and the C-terminal region of the $\alpha 5$ helix (**h**), and the interface between the $\alpha 5$ helix and F174^{34.51} of NTSR1 (**i**). **j**, Functional analysis of the three $G\alpha i$ mutants (D341^{G.H5.13}A, I344^{G.H5.16}A, K345^{G.H5.17}A) on signaling induced by NTSR1 (left) and MOR (right). Data represent mean $\pm$ SEM of three independent experiments performed in duplicate. ** represents $p < 0.01$ with two-way ANOVA followed by Dunnett's test for multiple comparison analysis with reference to WT. ns, not significantly different between the groups. See also the Source Data file for additional statistics and exact p -values. **k**, Structural comparison of the receptor– $G\alpha$ interface, highlighting the difference between F174^{34.51}(NTSR1) and V173^{34.51}(MOR) in accommodating the $\alpha 5$ helix.

Figure 3 | 3D-VA captures stepwise GDP-induced G-protein dissociation from the receptor.

a, Overall structural models representing six selected frames (frames 1, 6, 8, 9, 10, and 20) extracted from the 3D-VA of the GDP_{8s} dataset. **b–e**, Enlarged views highlighting key regions: GDP-binding pocket and AHD (**b**), $\alpha 1$ and $\alpha 5$ helices, GDP-binding region, and Q52^{G.H1.07} (**c**, **d**), and the interface between the $\alpha 5$ helix and the intracellular core of NTSR1 (**e**), as captured across the six frames. Black dashed lines represent hydrogen bonds, and the black dashed circle represents the unwound region of the $\alpha 5$ helix.

Figure 4 | Time-resolved cryo-EM analysis of the NTSR1–Gi complex with GTP.

a, Overall cryo-EM density maps and structural models captured 8 s after GTP addition, showing three canonical-like conformers (GTP_{8s}-C1 to C3), resolved at 3.0–3.4 Å. **b**, Superposition of GTP in the GTP_{8s}-C1 and GTP_{8s}-C2 states. The guanine ring binds more deeply into the pocket in the GTP_{8s}-C2 state. **c**, Representative traces (top) and snapshots (bottom) from simulations of the NTSR1–Gi complex initiated from the GTP_{8s}-C1 state with GTP bound at the shallow binding site. These traces are taken from “Product 10” in Extended Data Fig. 6b and are plotted with a magnified y-axis. **d**, **e**, Superposition of heterotrimeric G proteins, aligned based on Gα (**d**) or Gβ (**e**), from the indicated structures (GDP_{8s}-C3 to C4 and GTP_{8s}-C2 to C3). **f-h**, Surface representations highlighting the interface between the switch II region of Gα and Gβ in GDP_{8s}-C4 (**f**), GTP_{8s}-C2 (**g**), and GTP_{8s}-C3 (**h**).

Figure 5 | GTP-induced G-protein dissociation in the NC state.

a, Cryo-EM density maps and structural models of the NTSR1–Gi complex captured 5 s after GTP addition, showing two non-canonical-like (NC) conformers (GTP_{0-5s}-NC4 (top) and GTP_{0-5s}-NC5 (bottom)). Note that GTP could only be modeled for the GTP_{0-5s}-NC4 state because of the limited local resolution around the GTP-binding pocket. **b**, Extracellular (left) and side (right) views of the superimposed structures, showing that Gα undergoes a rotation in the NC states, with the αN helix swinging within the membrane plane (left) and tilting toward a more perpendicular orientation relative to the membrane (right). **c**, Comparison of GTP binding between the GTP_{8s}-C1 and GTP_{0-5s}-NC4 structures. While binding of the phosphate group is largely conserved, the guanine group in the GTP_{0-5s}-NC4 state points toward switch III loop. **d**, Superimposition of the GTP map and model in the GTP_{0-5s}-NC4 structure. **e**, Relative positioning of the complexes with respect to the membrane calculated using PPM 3.0⁶⁹ in the GTP_{8s}-NC1 and GTP_{0-5s}-NC4/NC5 structures.

Extended Data Fig. 1 | Cryo-EM analysis of nucleotide-free NTSR1-Go and NTSR1-Gq complexes.

a, Enlarged views of the $\alpha 5$ helices from three NTSR1-G protein complexes in C states. **b, c**, Enlarged views of the F174^{34,51}-G α interfaces from three NTSR1-G protein complexes in C states (**b**) and NC states (**c**). **d**, Cell surface expression levels for WT and F174^{34,51}A mutant NTSR1. Bars and error bars represent the mean and SEM, respectively, of three independent experiments, each performed in duplicate. **e**, Two orthogonal views of aligned structures from three complexes (NTSR1-Gi, Go, and Gq; all in C states). Dashed light green, orange, and red rectangles indicate regions highlighted in (**f**), (**g**), and (**h**), respectively. **f-h**, Enlarged views of ICL3 (**f**), TM2-ICL1 (**g**), and the receptor- $\alpha 5$ interface (**h**) from three complexes (NTSR1-Gi, Go, and Gq; all in C states). Black dashed lines represent hydrogen bonds.

Extended Data Fig. 2 | HS-AFM analysis of NTSR1-G protein interaction.

a,b, Representative HS-AFM images (**a**) and height time course (**b**) of the NTSR1-Gi complex in the presence of GDP. Scale bars, 20 nm (top) and 10 nm (bottom). In **b**, the red and blue boxes indicate the G-protein-bound and -unbound states, respectively. **c**, Histograms of molecular height for NTSR1-Gi derived from all HS-AFM measurements. **d**, Correlation functions (points) and fitted curves (lines) for the dwell times of NTSR1-Gi. The correlation function for NTSR1-Gi was best fitted with a quadruple-exponential decay ($F(x) = 0.205e^{-x/0.053} + 0.175e^{-x/1.16} + 0.203e^{-x/7.05} + 0.417e^{-x/88.7}$; time constants: $t_1 = 0.077$, $t_2 = 1.70$, $t_3 = 10.3$, $t_4 = 129.8$ sec; $R^2 = 1.000$). **e**, Histograms of molecular height for NTSR1-Gq derived from all HS-AFM measurements. **f**, Correlation functions (points) and fitted curves (lines) for the dwell times of NTSR1-Gq. NTSR1-Gq was fitted with a triple-exponential decay ($F(x) = 0.439e^{-x/0.053} + 0.175e^{-x/2.02} + 0.385e^{-x/50.1}$; time constants: $t_1 = 0.077$, $t_2 = 2.15$, $t_3 = 53.3$ sec; $R^2 = 1.000$). **g, h**, Individual height time courses for all analyzed particles of NTSR1-Gi ($n = 31$; **g**) and NTSR1-Gq ($n = 18$; **h**).

Extended Data Fig. 3 | Cryo-EM analysis of the NTSR1-Gi complex with GDP (GDP_{8s}).

a, Time-resolved cryo-EM workflow: 10 mM GDP (final concentration, 1 mM) was first applied to a grid, followed by application of purified nucleotide-free NTSR1-Gi complexes. The solution was mixed by pipetting (~1 s), incubated for 0–52 s, and then subjected to 7 s of blotting and immediate plunge-freezing (total time: 8–60 s). **b**, Overlay of the density map and the structural model of the four conformers (GDP_{8s}-C1 to C4) focused on the GDP-binding site. While there is no GDP density in the binding pocket of the GDP_{8s}-C1 and C2 states, clear GDP density is observed in the GDP_{8s}-C3 and C4 states. **c**, Side view of aligned structures from four conformers (GDP_{8s}-C1 to C4). The eye and arrow indicate the view shown in (**d**). Dashed green rectangle indicates the region highlighted in (**f**). **d-h**, Enlarged views highlighting key regions: TM5-6 of NTSR1 (**d**), ICL3 (**e**), ICL1 (**f**), ICL2 (**g**) and the ICL2- $\alpha 5$ interface (**h**). The dashed circle in (**g**) represents a potential steric clash between I344 and F174 if F174 does not change the orientation of its side chain. **i-l**, Functional analysis of the three G αi mutants (D341^{G.H5.13}A, I344^{G.H5.16}A, K345^{G.H5.17}A) on signaling induced by NTSR1 (**i**), BLT1 (**j, k**) and MOR (**l**). Data represent mean $\pm$ SEM of three independent experiments performed in duplicate. ** represents $p < 0.01$ with one-way ANOVA followed by Dunnett's test for multiple comparison analysis with reference to WT. ns, not significantly different between the groups. See also the Source Data file for additional statistics and exact p -values.

Extended Data Fig. 4 | Cryo-EM analysis of the NTSR1–Gi complex with GDP (GDP_{15s}) and population analysis across multiple incubation time points.

a, Cryo-EM density maps and structural models captured 15 s after GDP addition reveal two canonical-like conformers (GDP_{15s}-C1 and C2), resolved at 2.9 and 3.1 Å, respectively. **b**, Time-dependent population distribution of the conformational states. The percentage of particles assigned to each non-decoy class is plotted for the 8, 15 and 60 s datasets. Hatched boxes indicate that an interpretable cryo-EM map could not be reconstituted using the allocated particles. **c**, Overview of the image-processing workflow. Particles extracted from the 8, 15 and 60 s datasets were pooled and subjected to heterogeneous refinement using the indicated reference maps (“ref.”). Maps outlined in grey failed to yield interpretable structures, whereas maps outlined in red represent successfully reconstructed maps.

Extended Data Fig. 5 | 3D-VA of the GDP_{8s} NTSR1–Gi complex.

a, Data-processing workflow of 3D-VA (top) and local resolution maps (bottom) for the GDP_{8s} NTSR1–Gi complex.

Extended Data Fig. 6 | MD simulations comparing GTP binding stability starting from deep versus shallow binding positions.

a,b, Time courses of distances from 1-μs MD simulations (n = 10 independent runs) initiated from two distinct starting configurations: a modeled structure based on GTP_{8s}-C1 with GTP artificially placed in the deep binding pocket (**a**, Products 1–10), and the GTP_{8s}-C1 structure with GTP naturally located in the shallow binding pocket (**b**, Products 11–20). Blue traces represent the distance between the Cα atom of K46^{G.H1.01} and the O3A atom of the GTP phosphate group. Green traces represent the distance between the Cα atom of N269^{G.S5.07} and the C5 atom of the GTP guanine ring. **c, d**, Probability density histograms of the N269^{G.S5.07}–GTP (guanine) distance derived from the ten simulations in **a** (**c**, Products 1–10) and **b** (**d**, Products 11–20).

Extended Data Fig. 7 | Step-by-step comparison of switch I-III regions.

a–e, Structural comparisons of the switch I (Swi I) (top), switch II (Swi II) (middle), and switch III (Swi III) (bottom) regions between GDP-bound inactive Gαi (PDB: 1GP2) and GDP_{8s}-C1 (**a**), GDP_{8s}-C1 and GTP_{8s}-C1 (**b**), GTP_{8s}-C1 and GTP_{8s}-C2 (**c**), GTP_{8s}-C2 and GTP_{8s}-C3 (**d**), and GTP_{8s}-C3 and GppNHp-bound active Gαi (PDB: 1CIP) (**e**).

Extended Data Fig. 8 | Stepwise GTP-induced conformational changes in switch regions during G-protein dissociation.

a–e, Structural comparisons of switch I-III (Swi I-III) regions between GDP-bound inactive Gαi (PDB: 1GP2) and GDP_{8s}-C1 (**a**), GDP_{8s}-C1 and GTP_{8s}-C1 (**b**), GTP_{8s}-C1 and GTP_{8s}-C2 (**c**), GTP_{8s}-C2 and GTP_{8s}-C3 (**d**), and GTP_{8s}-C3 and GppNHp-bound active Gαi (PDB: 1CIP) (**e**). The black dashed circle represents the potential steric clash between the switch I loop in the GTP_{8s}-C2 state and the switch II-β3 loop in the GTP_{8s}-C1 state. **f–k**, Interactions between the switch II-β3 loop and the switch III loop. Both loops are ordered but do not strongly interact with each other in GDP-bound inactive Gαi (**f**). When the GDP is released, the switch III loop becomes disordered (**g**), and subsequently, when GTP binds, the switch III loop partially orders (**h**). The AHD closure brings these two loops close together

(i), and the interaction between them, including R205 and D237, pulls out the switch II helix (j). The rearranged structure of the switch II helix is further stabilized by multiple salt bridges (k). Black dashed lines represent salt bridges.

Extended Data Fig. 9 | Characterization of GTP_{0-5s} structures.

a, Structural models and local resolution maps of G-protein regions in the GTP_{0-5s}-NC4 (top) and GTP_{0-5s}-NC5 states (bottom). The local resolutions of G α , especially around the nucleotide-binding pocket, are markedly worse than in the receptor region. **b**, Superimposed images of the cryo-EM density map and model for the GTP_{0-5s}-NC4/5 and GTP_{8s}-C3 structures. AHD density is clearly visible next to G $\beta\gamma$ in both GTP_{0-5s}-NC4/5 states, indicating that AHD is not closed in either structure. **c**, Superimposed images of the cryo-EM density map and model for the free G-protein structures, observed under the GTP_{0-5s} conditions. While the left G α i structure, which shows a closed AHD and a withdrawn α 5 helix, could be reconstituted from other samples as well, the right G α i structure, characterized by an open AHD and an extended α 5 helix, could only be reconstituted from this sample. This suggests that G α i can be dissociated from the receptor without closing AHD and withdrawing the α 5 helix at an early reaction stage (0-5 s), supporting the idea that the Gi dissociation pathway from the NC state is significantly different from that from the C state.

Extended Data Fig. 10 | Working model of dynamic heterotrimeric Gi protein association and dissociation processes mediated by NTSR1.

Overall association and dissociation processes mediated by NTSR1. The processes are categorized into three primary steps: (1) association of GDP-bound G protein with the receptor (dashed orange box), (2) formation of a nucleotide-free state upon GDP release (dashed magenta box), and (3) dissociation of GTP-bound G protein from the receptor and heterotrimer separation (dashed green box). Note that dissociation process from the NC state proceeds through a pathway distinct from that of the C state. Both processes were analyzed starting from the most stable nucleotide-free state, with backward and forward reactions triggered by GDP and GTP, respectively.

Extended Data Fig. 11 | Structural comparison of NTSR1-Gi and β 2AR-Gs complexes obtained by time-resolved cryo-EM analysis.

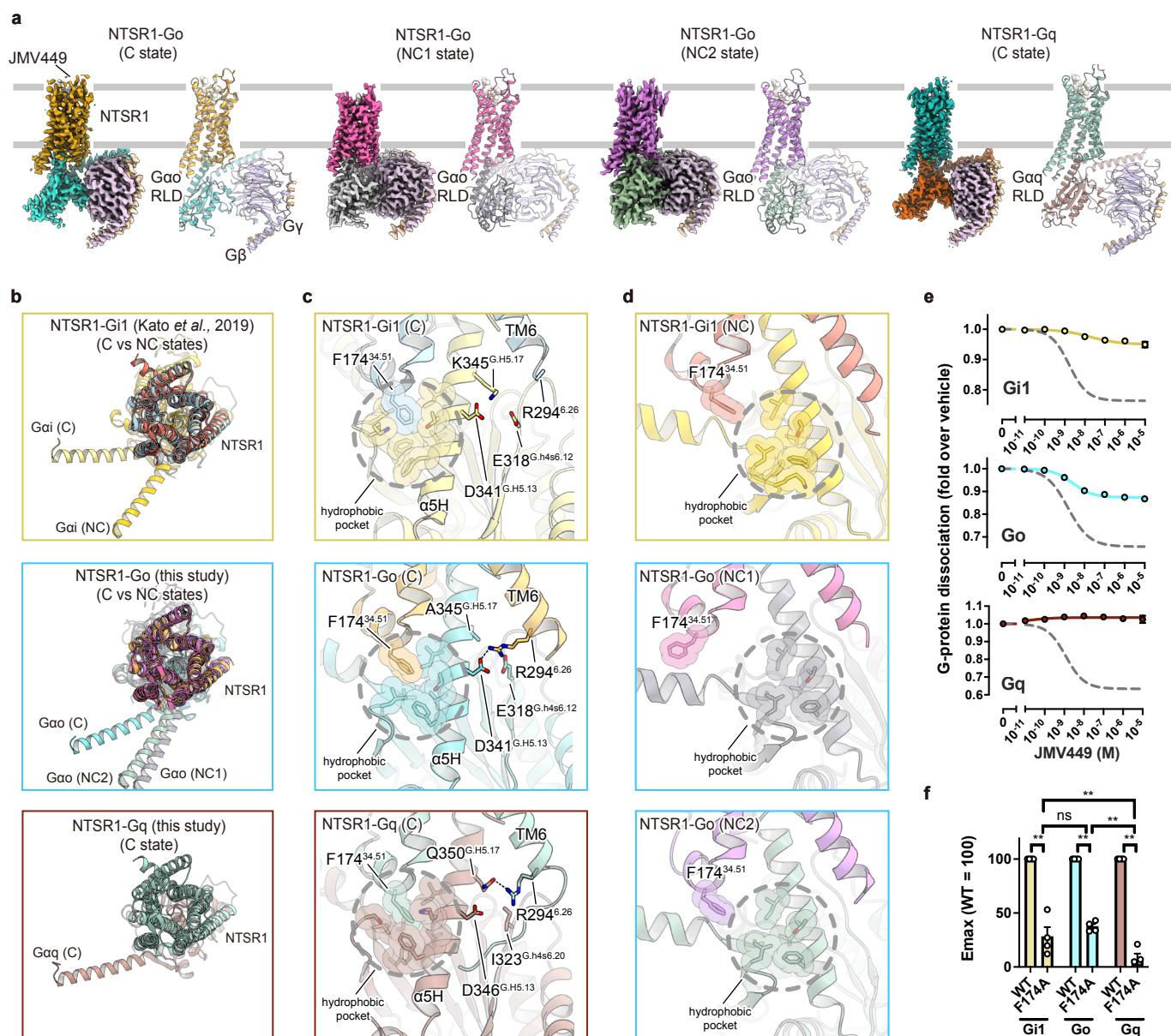
a,b, Comparison of frames 1 and 20 from the NTSR1-Gi structures extracted from 3D-VA of the 8-s GDP dataset (**a**) and the GDP-bound inactive Gi structure (PDB: 1GP2) (**b**), highlighting the α 1 and α 5 helices. **c,d**, Comparison of frames 1 and 20 from the β 2AR-Gs structures obtained from 3D-VA (PDB: 8UNL and 8UO4, respectively) (**c**) and the GDP-bound inactive Gs structure (PDB: 1BH2) (**d**), highlighting the α 1 and α 5 helices. **e,f**, Interaction between the α 5 helix and the β 2- β 3 loop from frame 1 of NTSR1-Gi (**e**) and frame 1 of β 2AR-Gs (**f**) complexes. The interaction is stronger in the β 2AR-Gs complex. **g**, Comparison of frame 1 of the NTSR1-Gi and β 2AR-Gs structures, highlighting the angle of the α 5 helix. **h**, Computational analysis of the potential steric clash between the α 1 and α 5 helices in the β 2AR-Gs complex. The α 5 helix from frame 1 is computationally superimposed onto the position of the α 5 helix in frame 20, while preserving its original orientation. This reveals a significant clash, suggesting that the α 5 helix must be unwound and pulled out via a curved trajectory

1320 to avoid the steric clash. The dashed circle indicates a steric clash between the $\alpha 5$ helix from frame 1
1321 and the αN helix. **i,j**, Comparison of $\alpha 5$ -helix conformations between Gi (**i**) and Gs (**j**) in GDP-bound
1322 inactive structures. While Gi maintains the helical structure except for the C-terminus, Gs has an
1323 unwound and kinked structure near C379^{G.H5.11}.
1324

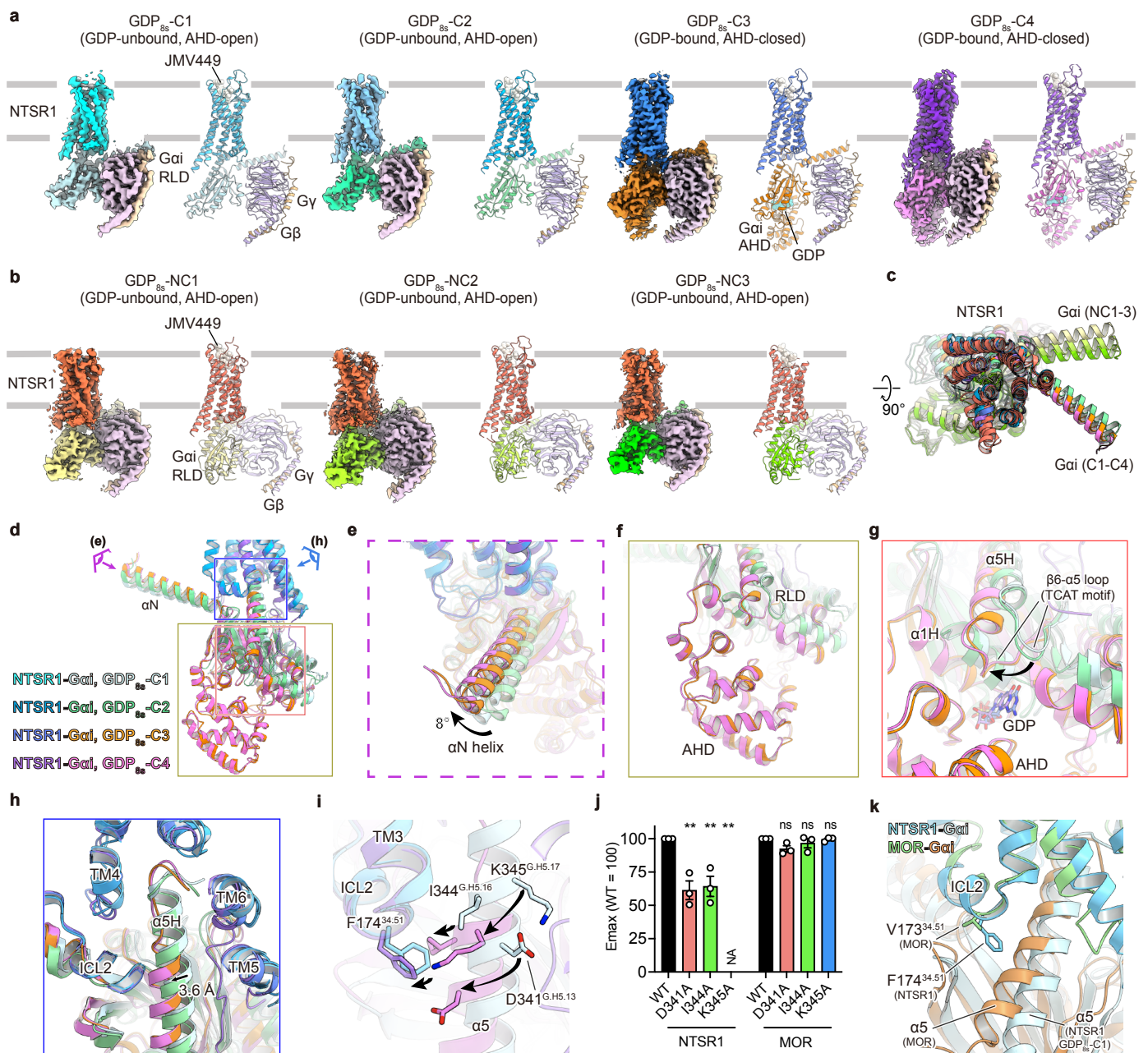
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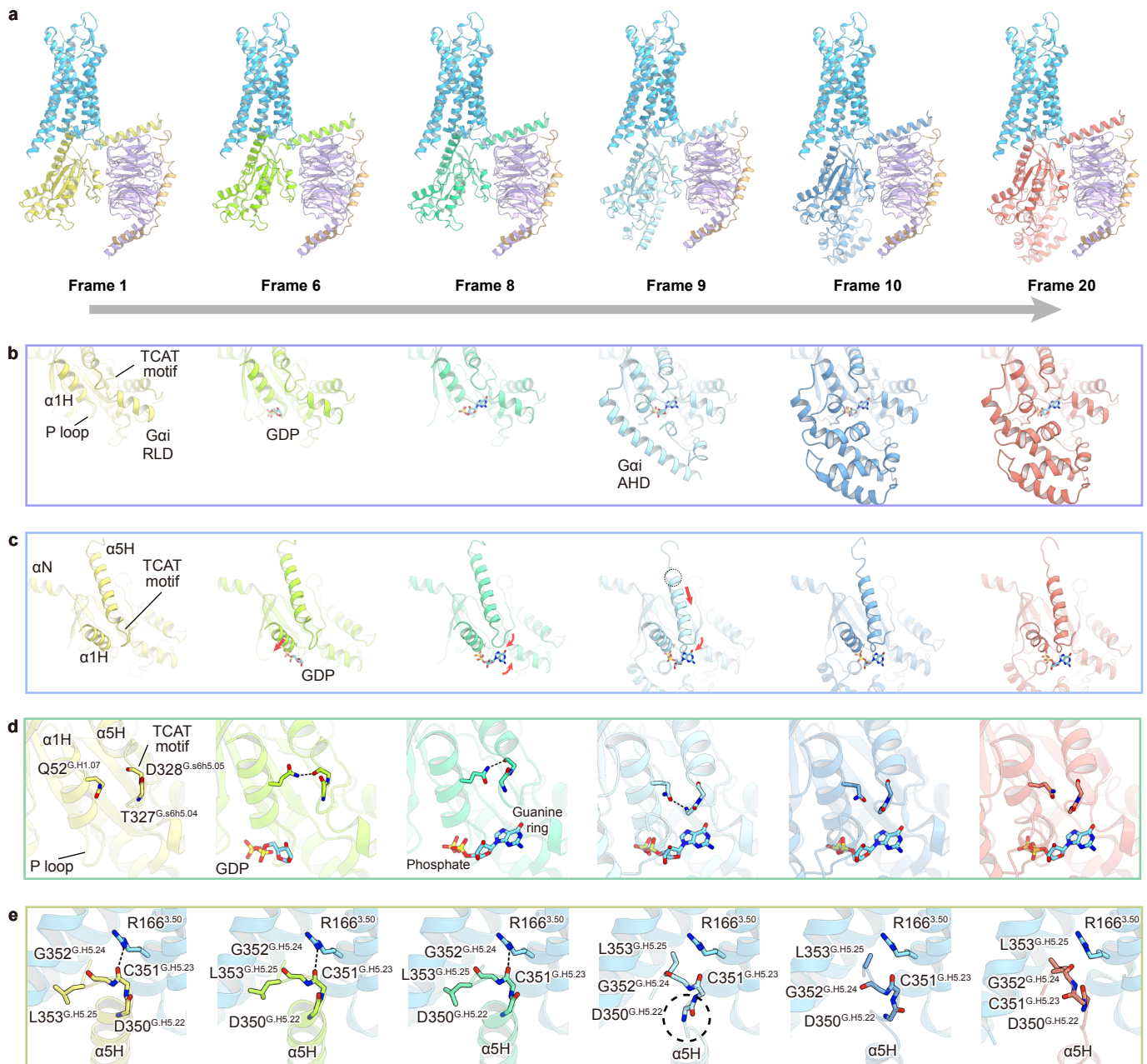
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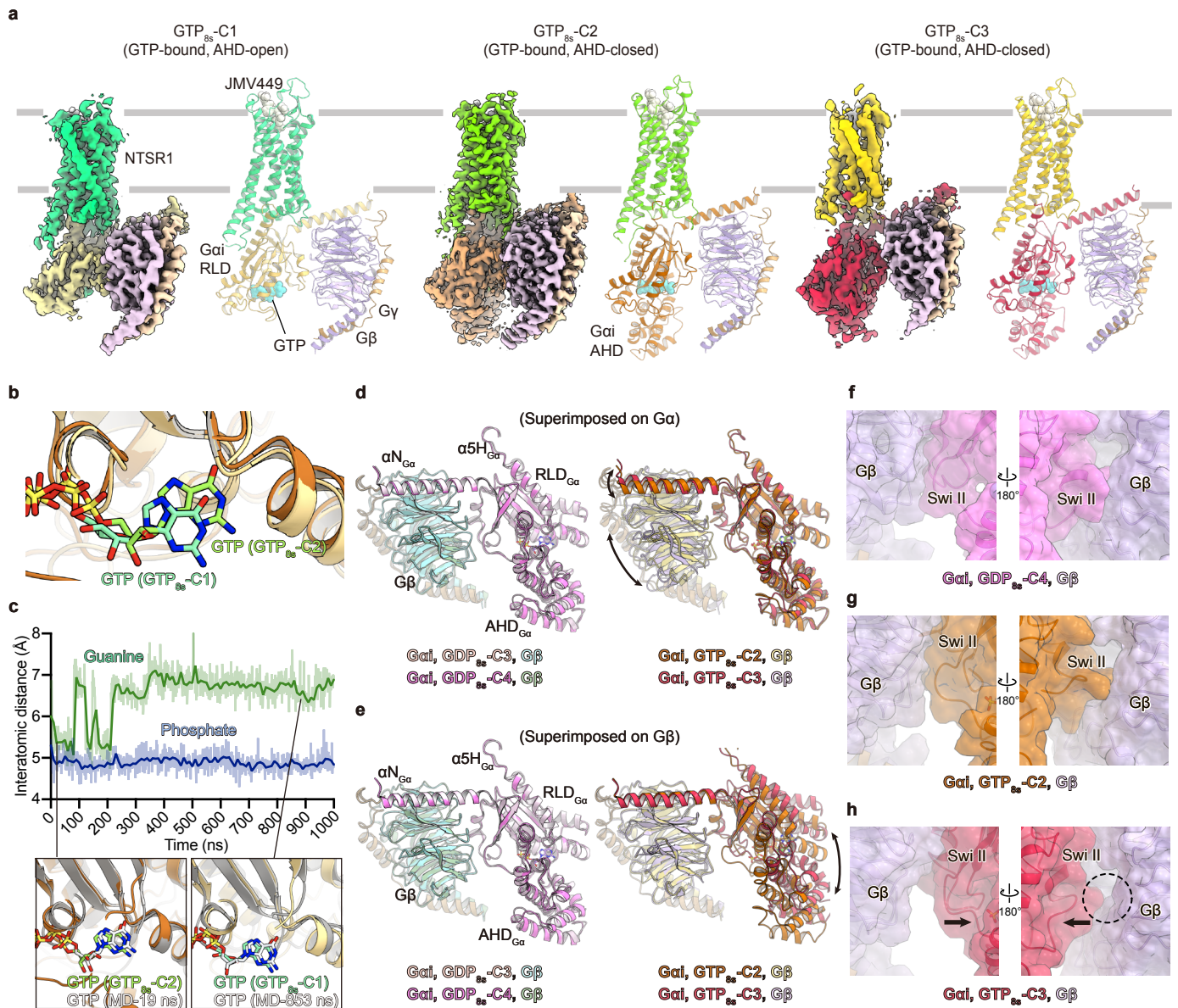
Kobayashi et al. Figure 1



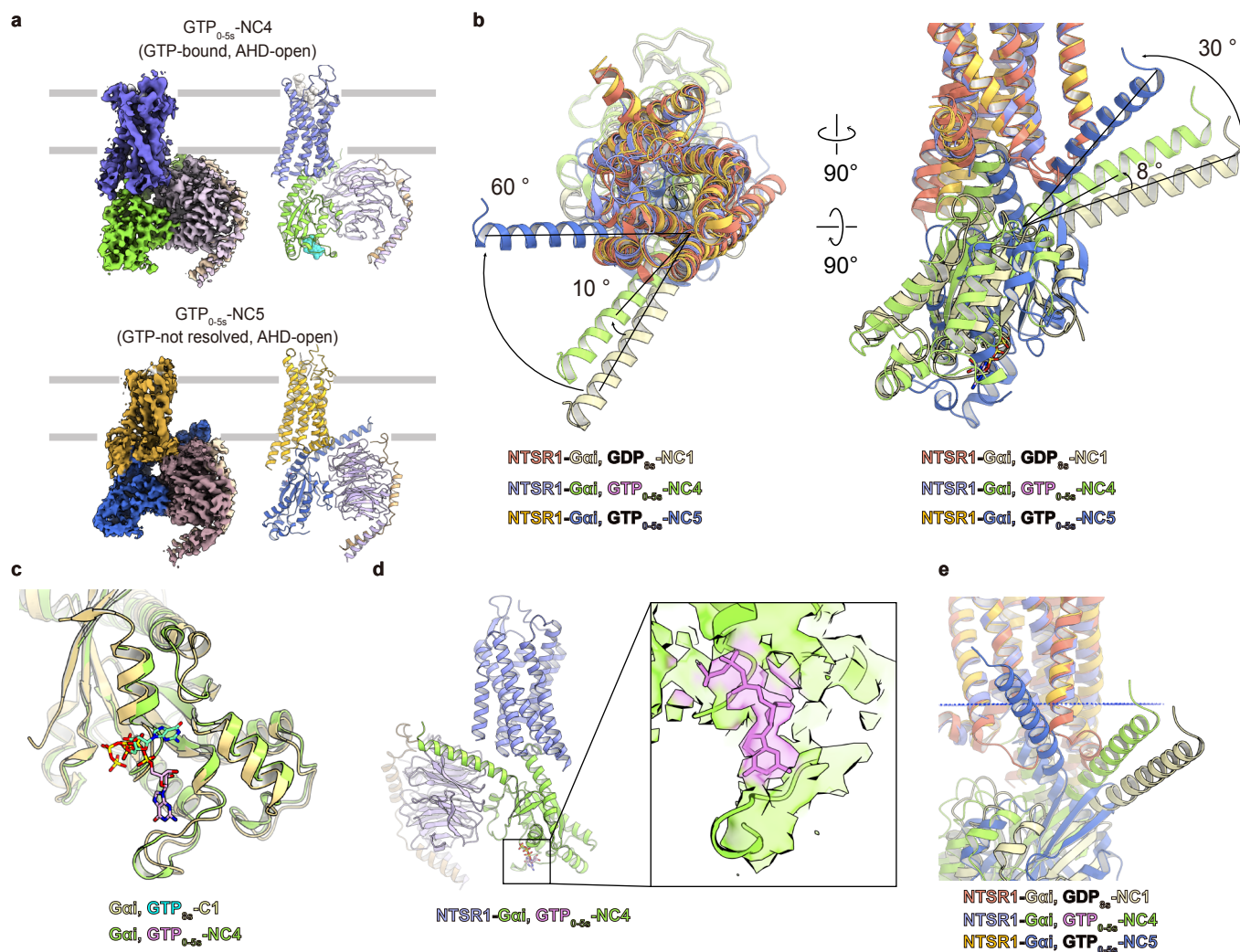
Kobayashi et al. Figure 2



Kobayashi et al. Figure 3



Kobayashi et al. Figure 4



Kobayashi et al. Figure 5