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Elucidation of a dynamic interplay between a beta-2 adrenergic receptor, its agonist and stimulatory G protein

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Abstract

G protein-coupled receptors (GPCRs) represent the largest group of membrane receptors for transmembrane signal transduction. Ligand-induced activation of GPCRs triggers G protein activation followed by various signaling cascades. Understanding structural and energetic determinants of ligand binding to GPCRs and GPCRs to G proteins is crucial to the design of pharmacological treatments targeting specific conformations of these proteins to precisely control their signaling properties. In this study we focused on interactions of a prototypical GPCR, beta-2 adrenergic receptor (β_2AR), with its endogenous agonist, norepinephrine (NE), and the stimulatory G protein (G_s). Using molecular dynamics (MD) simulations, we demonstrated stabilization of cationic NE, NE(+), binding to β_2AR by G_s protein recruitment, in line with experimental observations. We also captured the partial dissociation of the ligand from β_2AR and the conformational interconversions of G_s between closed and open conformations in the NE(+)-β_2AR-G_s ternary complex while it is still bound to the receptor. The variation of NE(+) binding poses was found to alter G_s α subunit (G_sα) conformational transitions. Our simulations showed that the interdomain movement and the stacking of G_sα α1 and α5 helices are significant for increasing the distance between the G_sα and β_2AR, which may indicate a partial dissociation of G_sα. The distance increase commences when G_sα is predominantly in an open state and can be triggered by the intracellular loop 3 (ICL3) of β_2AR interacting with G_sα, causing conformational changes of the α5 helix. Our results help explain molecular mechanisms of ligand and GPCR mediated modulation of G protein activation.

Significance Statement

G protein-coupled receptors (GPCRs) and G proteins work together to transmit signals from various hormone and neurotransmitter molecules across cell membranes, and their activation and subsequent dissociation initiate a cascade of downstream signaling events resulting in modulation of cellular behavior. Here we studied interactions of a prototypical GPCR, beta-2 adrenergic receptor in its active state, with neurotransmitter norepinephrine and stimulatory G protein using multi-microsecond-long atomistic computer simulations to understand how energetic and structural changes in this system could initiate cellular signaling. Our results provided us with intrinsic molecular mechanisms, which may control G protein dissociation from GPCRs, and highlighted the importance of protein domain and ligand dynamics in this crucial biological process.

Main Text

Introduction

GPCRs transduce intracellular signaling via coupling to G proteins. In the heart, sympathetic nervous system (SNS) activation increases cardiac output to supply the body with oxygenated blood by raising the heart rate, the force of contraction, and conduction rate (1). SNS activation in the cardiovascular system is triggered by binding of two catecholamine neurotransmitters, norepinephrine (NE) and epinephrine (Epi), to specific cell surface adrenergic receptors (βARs in human heart), which belong to the superfamily of GPCRs (2). There are three βAR subtypes in the nonfailing human heart (75% – 80% of β_1, 15% – 18% of β_2, and 2% – 3% of β_3), regulating cardiac rate and contractility by responding to NE and Epi (2, 3). Recently, β_2AR has been the focus of therapeutic interest, partly because of its relative preservation of expression in the failing human heart (4). After binding to agonists, β_2AR can activate the stimulatory G protein (G_s). G_s is a heterotrimer consisting of an α subunit ($G_s\alpha$) and a tightly associated βγ complex (5). The $G_s\alpha$ subunit harbors the guanine nucleotide-binding site and associates with the βγ complex in the inactive GDP-bound state (5). Binding of G_s to the agonist-bound β_2AR results in activation and dissociation of trimeric G proteins (5, 6). Both $G_s\alpha$ and βγ can transduce a cascade of downstream signaling events which eventually regulate cardiac rate and contractility (2, 4). However, the molecular determinants and the dynamics of the ternary complex during receptor signaling transduction remain incompletely understood.

The GDP release by G protein is a preparatory step of G protein activation which takes place between two stable end-point states: one is referred as “closed-out” with G protein closed and its βAR-interacting α5 helix outside the receptor, and the other is referred as “open-in” with G protein fully open and the α5 helix coupled to the receptor. In 2011, Rasmussen et al. crystallized the first high-resolution structure of β_2AR bound G_s (β_2AR-G_s) which is a ternary complex in the “open-in” state consisting of a high affinity agonist (BI-167107), an active-state receptor, and G_s (7). There $G_s\alpha$ subunit adopts an open state with a largely displaced α–helical domain ($G_s\alpha$AH) and Ras–like GTPase domain ($G_s\alpha$Ras) (7). More recently, a cryo-EM structure of the β_1AR-G_s complex bound to another high-affinity agonist (isoproterenol) was solved, in which $G_s\alpha$ subunit adopts a somewhat different but also open conformation (8). The agonist-bound structure is very distinct from the crystal structure of the receptor-free closed $G_s\alpha$-GTPγ complex (7, 9). In another work, an intermediate state of G_s between the GDP-bound G_s and GDP-free β_2AR-G_s complex was proposed by Liu et al. by crystalizing an active-state structure of the β_2AR stabilized by the last 14 residues of the $G_s\alpha$ terminal α5-helix (6). Su and Zhu et al. found that β_1AR induces a tilting of the α5 helix of $G_s\alpha$ which deforms the GDP/GTP-binding pocket and accelerates GDP release (8). Goricanec et al. performed NMR spectroscopic characterization of an inhibitory Gα subunit, $G_i\alpha$1, and showed that it adopts a more open conformation in the apo and GDP-bound forms, but a more compact and rigid state in the GTP-bound form with no interaction to GPCR (5). They proposed that the apo G_i protein eventually binds to GTP, leading to subunit dissociation and loss of affinity to the receptor (5).

Meanwhile, there have also been multiple atomistic modeling and simulation studies of βAR conformational dynamics and transitions (10-17), their interactions with G_s protein (18-24) and other regulatory proteins (25-27) as well as endogenous ligand and drug binding (28-36) (recently reviewed, e.g., in (37-39)). Dror et al. studied the structural basis for GDP/GTP exchange in G_s protein coupled with or uncoupled from β_2AR by combining long time scale MD simulation with experimental validations (23). Alhadeff et al. explored the free-energy landscape of β_2AR activation using coarse-grained (CG) modeling using multiple receptor and G_s protein conformational states (40). In a follow-up study, Bai et al. performed targeted MD simulations and free energy analysis based on the β_2AR-$G_s\alpha$ structure and found that the GDP could be released during the half-opening of the binding cavity in the transition to the G_s open state; the potential key residues on α5 were also validated by site-directed mutagenesis (41). Enhanced sampling

metadynamics simulations were used to predict energetics of small-molecule ligand binding to βARs and other GPCRs in good agreement with experimental affinities (42-45), but for the most part did not focus on the G protein dissociation and conformational transitions.

In the current study, we explore the relationship between the dissociation of G_s from the β_2AR and $G_s\alpha$ conformational change and characterize the molecular determinants of how and when G_s may dissociate from the receptor and how and why the G_s binding affects the endogenous agonist, cationic norepinephrine, NE(+), affinity to the receptor. We performed multiple microsecond-long all-atom MD simulations to study the molecular interactions within the ternary NE(+) β_2AR-G_s complex. We applied the open-in state based on PDB:3SN6 (7) as our simulation starting point (Figure 1) and focused on capturing the molecular conformational changes associated with dissociation of G_s from the receptor.

Results and Discussion

Two types of molecular systems were simulated: beta-2 adrenergic receptor (β_2AR) and its complex with the stimulatory G_s (β_2AR-G_s). The cationic norepinephrine, NE(+), bound at the orthosteric binding site, was present in each system. The snapshot of the β_2AR-G_s system is shown in Figure 1. Each system was embedded in a lipid bilayer hydrated by 0.15 M NaCl, corresponding to physiological conditions in the extracellular medium and equilibrated for 90 ns using restraints that were gradually reduced in the first 40 ns of these simulations. We then performed much longer production runs. For β_2AR, a 2.5 μs Anton 2 (Anton) unrestrained MD simulations and three Gaussian accelerated MD (GaMD) runs (600 ns each, 1800 ns in total) were performed. For β_2AR-G_s system, four different Anton runs (5.0 μs each for run 1, run 2, and run 4; 7.5 μs for run 3) and three GaMD runs (600 ns each, 1800 ns in total) were performed (see Table S1). As we observed NE(+) partial dissociation after 4.5 μs in Anton run 3, we extended it to 7.5 μs. Based on the simulation trajectories, we first checked the dominant and secondary NE(+) binding poses in the β_2AR and analyzed the role of G_s coupling in stabilizing the NE(+) binding. Then, we assessed the conformational changes in the α subunit of G_s ($G_s\alpha$) upon coupling with β_2AR. The intracellular loop 3 (ICL3) of β_2AR was found to be essential in interacting with $G_s\alpha$ and causing a conformational change in the α5 helix of $G_s\alpha$. The induced α5 helix conformational change controls the formation of an active-state receptor – G protein complex. To find the molecular determinants of $G_s\alpha$ conformational changes, structural parameters were analyzed, including opening/closing of $G_s\alpha$ and the distance between two $G_s\alpha$ domains. The geometric centers were used for all the distance and angle measurements. Finally, we analyzed distribution of those parameters converting them to two-dimensional free energy profiles to explore low-energy pathways for $G_s\alpha$ conformation changes and its dissociation from β_2AR. We also performed a posteriori implicit-solvent molecular mechanics Poisson-Boltzmann surface area (MM-PBSA) calculations to estimate β_2AR binding to NE and G_s.

1. Binding affinity of NE(+) to β_2AR and β_2AR-G_s

The starting point of our β_2AR-G_s simulations is the open-in $G_s\alpha$ state with $G_s\alpha$ in a fully open conformation and its α5 helix intruded into the intracellular part of the active-state β_2AR (see Figure 1) which is based on the agonist-bound X-ray structure of the complex (PDB ID: 3SN6) (7). In that study Rasmussen et al. discovered that, in the ternary complex, G_s binding increased the agonist binding affinity about 100-fold compared with β_2AR alone and that agonist binding promotes interactions of β_2AR with GDP-bound G_s heterotrimer, leading to the exchange of GDP for GTP followed by the functional dissociation of G_s into $G_s\alpha$-GTP and βγ subunits (7). Therefore, understanding the effect of G_s on the agonist binding is crucial. We performed multiple microsecond-long unbiased MD simulations (Anton runs) for the NE(+) bound β_2AR (referred to as β_2AR) and NE(+) bound β_2AR in complex with G_s (referred to as β_2AR-G_s) as shown in Table S1. To verify some of the observations, we also performed three GaMD runs for each of the above systems (Table S1).

We performed clustering for the NE(+) binding poses in the β_2AR and β_2AR-G_s based on their microsecond-long Anton run trajectories. Five clusters were found in each case as shown in Figure S1A – D. One representative pose with the lowest root-mean-square deviation (RMSD) compared with other frames was selected for each cluster (Figure S1C – D) and shown in the color matching histogram in Figure S1A – B. Figure 2 shows the NE(+) binding results based on Anton runs. Figure 2A shows the initial and three special representative poses found in β_2AR and in β_2AR-G_s systems. The time series of center-to-center distances between NE(+) and β_2AR for all runs are shown in Figure 2B with the three special representative poses matching the colors of the plots. All other representative poses can be found in Figure S1C – D. Figure 2C (the gray molecule) shows the initial pose, which is also the representative pose of the biggest cluster (cluster 2 in Figure S1A) in the β_2AR system. The amino acid residues in close contact with NE(+) forming the binding pocket were identified based on the frames collected in this cluster. The close contacts are defined as the amino acid residues within 3 Å of the NE(+) for more than half of the total MD simulation frames. The number of NE(+) poses in cluster 2 accounts for the largest proportion (28%) of the overall binding poses for β_2AR, and it is the initial and dominant binding pose in this system (referred as NE(+)-d). The amino acid residues forming the binding pockets of NE(+)-d are D113$^{3.32}$, V114$^{3.33}$, and V117$^{3.36}$ on transmembrane helix 3 (TM3), F193$^{45.52}$ on ECL2, S203$^{5.42}$ and S207$^{5.46}$ on TM5, F289$^{6.51}$ and F290$^{6.52}$ on TM6, N312$^{7.39}$ and Y316$^{7.43}$ on TM7, among which D113$^{3.32}$, S203$^{5.42}$ and N312$^{7.39}$ form hydrogen bonds with NE(+). The residue superscripts denote the Ballesteros-Weinstein (BW) numbering of GPCRs (46). The residues forming the binding site of NE(+) on the active β_2AR are mainly from helices TM3, TM5, TM6, and TM7, which matches the findings of Dror et al. (12), where they observed that helices TM5, TM6, and TM7 contribute to the shift of β_2AR conformation between inactive and active states, while the helix TM3, TM5, and TM6 interactions also play an important role in this process.

Figure 2D shows the representative binding pose of NE(+) (magenta molecule) in the second biggest cluster (cluster 4) of β_2AR (referred to as NE(+)-s1). This binding pose is considered special because it shows a different orientation from all other poses in β_2AR and has the biggest deviation from the initial binding pose of NE(+) in β_2AR as shown in Figure S1C. It is also the 2nd most abundant pose, existing in 24.7% of the simulation frames (Figure S1A). A similar NE(+) binding pose (red in Figs. 2 and S1) is also identified in the β_2AR-G_s system as cluster 5, which is also the 2nd most abundant with 21.3% (see Figs. S1B and S1D). The residues in close contact with NE(+)-s1 are identified in the same way as stated previously. Compared with the binding pocket of NE(+)-d, four new ligand-binding residues appear in the case of NE(+)-s1, which are T110$^{3.29}$ on TM3, Y174$^{45.33}$ and R175$^{45.34}$ on ECL2, and Y199$^{5.38}$ on TM5. D113$^{3.32}$, V114$^{3.33}$, F193$^{45.52}$, N312$^{7.39}$, and Y316$^{7.43}$ are preserved in the NE(+)-s1 pocket, where D113$^{3.32}$ and N312$^{7.39}$ form H-bonds with NE(+)-s1, while V117$^{3.36}$, S203$^{5.42}$, S207$^{5.46}$, F289$^{6.51}$ and F290$^{6.52}$ are not interacting with NE(+) in this pose.

Figure 2E shows a special representative binding pose of NE(+) (light-blue molecule), which is captured in cluster 4 of β_2AR-G_s system (Figure S1D) and is referred to as NE(+)-s2 hereafter. It shows an almost opposite orientation compared to NE(+)-s1 (Figure 2D) and has an 8.85% population for the β_2AR-G_s and is not represented in the β_2AR alone (see Figure S1). This binding pose mostly corresponds to a low-value plateau in the NE(+) to β_2AR distance for β_2AR-G_s run 1 from ~2.8-5 μs, as shown by a blue curve in Figure 2B. Compared with NE(+)-d (Figure 2C), three new interacting residues (W286$^{6.48}$ on TM6, L311$^{7.38}$ and G315$^{7.42}$ on TM7) are found, while six residues (V114$^{3.33}$, F193$^{45.52}$, S203$^{5.42}$, S207$^{5.46}$, F290$^{6.52}$, and Y316$^{7.43}$) are missing in the binding pocket of NE(+)-s2. As noted above, the red NE(+) molecule shown in Figures 2A and 2B is another binding pose of NE(+) similar to NE(+)-s1 of β_2AR but was found in β_2AR-G_s cluster 5. It corresponds to NE(+) position plateaus in β_2AR-G_s run 3 at ~3.5 μs and 4.5 – 7.5 μs (red curve in Figure 2B) as well as at 2.6 – 3.9 μs of run 4 (purple curve in Figure 2B).

The above results indicate that NE(+) can have different degrees of dissociation from its dominant binding pose and pocket regardless of the G_s binding. However, those special binding

poses appear later during simulations in the β_2AR-G_s cases compared to simulations with β_2AR alone, as shown in Figure 2B. The partial dissociation of NE(+) can be attributed to the β_2AR residue movements, evidenced by the significant variations of its RMSD values, as shown in Figure S2B. We found three special representative binding poses out of ten clusters, and only one special pose (shown in light-blue in Figure 2) moves deeper inside the β_2AR (based on the center-to-center distance) closer to the intracellular side. In two other special poses (shown as red and magenta in Figure 2) we observed outward movement of NE(+) towards the extracellular side, which may indicate its partial dissociation from the receptor. Most other poses, which are dominant in both β_2AR and β_2AR-G_s simulations (Anton runs), are slight variations of the original pose with different degrees of shifting or rotation. Similar results were found in the GaMD runs as shown in Figure S3, where the representative binding poses were captured for both β_2AR and β_2AR-G_s, except that the NE(+) in one of the β_2AR GaMD runs almost completely dissociates from β_2AR as shown in Figures S4A and S4B (the gray molecule), and the full ligand dissociation may be possible to sample in longer runs and/or using e.g., ligand GaMD (LiGaMD) approach (47) to be explored in the follow-up studies.

In short, in all our MD simulations we observed partial NE(+) dissociation, which adopted alternative binding positions in the receptor interior, in most cases closer to an extracellular side. G_s association in β_2AR-G_s complexes seems to stabilize NE(+) binding to the orthosteric site in the β_2AR, as was evidenced by its delayed partial dissociation (Figure 2B), although a random fluctuation could potentially cause this delay. Ligand (antagonist) dissociation was also observed in an adenosine A_{2A} receptor where a multistep ligand dissociation pathway featured by different ligand poses during dissociation was suggested based on temperature-accelerated MD simulation (48). Similarly, using GaMD, different binding poses were also revealed for a partial agonist in the orthosteric pocket of a muscarinic receptor in the absence or presence of G protein mimic (nanobody) (49). These studies suggest that multiple ligand binding poses may be common in GPCR systems with or without bound G protein.

We also computed MM-PBSA binding energies between β_2AR and NE(+) and RMSDs for β_2AR based on Anton runs, as shown in Table 1. In most runs of β_2AR-G_s, free energies of binding between β_2AR and NE(+) are more favorable than that for β_2AR, in agreement with the experiment (7). The reason for the stabilized NE(+) binding in the β_2AR-G_s complex can be attributed to the stabilization of β_2AR active state by the open G_s, suggested experimentally (7) and by previous coarse-grained simulations (40). We checked the RMSDs for the β_2AR (not including the intracellular loops) alone and in the presence of G_s. Using the averaged β_2AR structure as the reference, we computed the mean RMSD value and its standard deviation (SD) for each run (Table 1) using VMD (50). RMSD time series for the receptor, G_s protein, NE(+) and the entire β_2AR-G_s complex can be found in Figure S2. Half of the β_2AR-G_s runs show lower mean RMSD values compared with the β_2AR alone. Moreover, all the SDs (a measure of the amount of variation from the mean) for the β_2AR-G_s cases are lower than that of β_2AR alone, indicating more stable conformations of β_2AR in complex with G_s. These analyses confirm that NE(+) binding to β_2AR-G_s is more favorable than to β_2AR alone due to the stabilized β_2AR structure in the complex with G_s. In a recent GaMD study it was also found that removal of the G protein mimic leads to a conformational transition of a muscarinic receptor M_2 to an inactive state along with multiple orthosteric ligand dissociation and binding events consistent with extensive experimental and computational studies of other GPCRs (49).

The MM-PBSA binding energies between β_2AR/ β_2AR-G_s and NE(+) based on GaMD runs can be found in Table S2. Due to the nature of GaMD simulations, where different boost potentials were added to the β_2AR and β_2AR-G_s systems to accelerate dynamics of both the protein and NE(+), it is impossible to compare the binding energies between β_2AR and β_2AR-G_s systems directly, unless the energy values are reweighted properly. Despite this, it is still true that the most displaced NE(+) binds weaker to the β_2AR or β_2AR-G_s, as demonstrated using non-reweighted MM-PBSA ΔG values for β_2AR-GaMD run 1 as well as β_2AR-G_s-GaMD runs 2 and 3 (Table S2

and Figure S4). Since the reweighting of entropy turned out to be exceedingly noisy, we only reweighted the MM-PBSA enthalpy, ΔH, term by using the distribution of interaction energies based on a cumulant expansion (details can be found in the Materials and Methods section) as shown in the last column of Table S2. The reweighed ΔH show somewhat different trends from the non-reweighted ones, but still reflect the weaker NE(+) binding affinity in β_2AR-GaMD run 1 and β_2AR-G_s-GaMD runs 2 and 3.

2. G_s conformational changes after binding with β_2AR

After checking the effect of G_s on NE(+) binding to β_2AR, we analyzed the conformational changes of G_s when it couples with β_2AR. In the published β_2AR-G_s complex structure (PDB: 3SN6), used as a starting point of our simulations, the $G_s\alpha$ preserves an open state with the α–helical domain ($G_s\alpha$AH) largely displaced from the Ras–like GTPase domain ($G_s\alpha$Ras) as shown in Figure 1. The $G_s\alpha$AH rotated as a rigid body with an angle of approximately 127° from the domain junction compared to the crystal structure of the closed $G_s\alpha$-GTPγ (PDB: 1AZT) (7, 9). However, a different $G_s\alpha$ conformation was discovered in the complex of isoproterenol-bound β_1AR-G_s, which is partly based on cryo-EM, due to the dynamic nature of $G_s\alpha$AH (8). The $G_s\alpha$ in β_1AR-G_s is less open compared with that in the crystalized β_2AR-G_s complex (7) but still can be considered as a fully open state in comparison with $G_s\alpha$ alone (PDB: 1AZT) (9). $G_s\alpha$ conformational transitions were thoroughly tested via long-scale MD simulations by Dror et al., who found that the separation of $G_s\alpha$Ras and $G_s\alpha$AH domains occurs only in the absence of β_2AR, whereas GDP release can only be observed after restraining $G_s\alpha$ α5 in the distal conformation like that in the β_2AR-G_s complex, indicating the need of an internal structural rearrangement of the $G_s\alpha$Ras to weaken its nucleotide binding affinity (23).

As shown in Figure 3 (based on Anton runs), we used the geometric center-to-center distance (referred to as "distance" hereafter for all the distances) between the $G_s\alpha$AH residue $A161^{H.HD.5}$ and $G_s\alpha$Ras residue $E299^{G.HG.6}$ as an indicator for the opening and closing of $G_s\alpha$ (the same one as used in the work of Dror et al. (23)), e.g., a larger distance between $A161^{H.HD.5}$ and $E299^{G.HG.6}$ indicates a more open $G_s\alpha$ conformation. The residues are labeled by residue number and common Gα numbering (CGN) system (51) in their superscripts. The systems corresponding to different Anton simulations are referred to as runs (with GaMD runs labeled differently). If the distance is greater than or equal to 55 Å, we define $G_s\alpha$ conformation as fully open; if the distance is in the range of 45 Å – 55 Å, we define it as semi-open; if the distance is in the range of 35 Å – 45 Å then it is a semi-closed structure, and if the distance is less than or equal to 35 Å then it is a closed structure.

Transition of $G_s\alpha$ from open to closed conformation was observed e.g., in a 5.0 μs long MD run 1 of β_2AR-G_s complex: the distance between $A161^{H.HD.5}$ and $E299^{G.HG.6}$ changes from 62 to 34 Å (Figure 3A). Interestingly, such transition was not captured by the previous multi-microsecond long MD simulations by Dror et al., instead, an opposite conformational change of GDP bound $G_s\alpha$, from closed to fully open conformation was observed but only in the receptor-free systems (23). They proposed that this conformational transition favors the closed state in the absence of the receptor (23). When it comes to the receptor bound case, they only sampled fully open and nucleotide free $G_s\alpha$ during their multi-microsecond long MD simulations. They also proposed that the loss of GDP after G_s binding to β_2AR shifts the equilibrium toward a widely open $G_s\alpha$ state (23).

In run 3, we observed a very dynamic conformational transition of $G_s\alpha$ between open and semi-closed states in terms of A161–E299 distance as shown in Figure S7A. This conformational transition to a semi-closed state also correlates with the increase in NE(+) to β_2AR distance in Figure 2B. Specifically, the decrease in $G_s\alpha$ A161-E299 distance during ~4.0-5.5 μs in Figure S7A seems to correlate with an increase in NE(+) to β_2AR distance in Figure 2B, i.e. partial agonist dissociation, especially evident after ~4.5 μs. A similar, but less evident correlation can be seen for β_2AR-G_s run 4, where transient rearrangements of $G_s\alpha$ to a semi-closed state may be

related to NE(+) partial dissociation from ~2.6 to 3.9 μs (cf. Figs. S7A and 2B). Interestingly, $G_s\alpha$ transition to a fully closed state in β_2AR-G_s run 1 discussed above may eventually lead to a decreased NE(+) to β_2AR distance at ~2.8 μs, i.e., agonist movement deeper towards the intracellular side (Figure 2B). These trends indicate the potential correlation between NE(+) binding poses and G_s conformational changes.

In another β_2AR-G_s simulation run (run 2), we observed similar open $G_s\alpha$ conformation as was observed in Dror et al.'s work (23) throughout the entire 5 μs-long MD simulation (Figures 3B and S7A). Interestingly, in that run we observed partial unwinding of the $G_s\alpha$ α5 helix (referred to as α5), a key interaction site with the receptor (see Figure 3B and bottom inset). We correlate this α5 conformational transition with the interaction between $G_s\alpha$ and flexible ICL3 of the β_2AR as will be discussed below. Snapshots for other β_2AR-G_s runs can be found in Figures S5 and S6, where different levels of $G_s\alpha$ closing and opening, different $G_s\alpha$ conformations and interaction details between α5 and ICL3 are shown.

Due to its unstructured nature, ICL3 region is either unresolved or completely removed and replaced by T4-lysozyme (T4L) in experimental structures (15). Thus, very limited experimental (52) and simulation (15) studies have discussed the possible effect of ICL3 on the intrinsic dynamics of the receptor. Ozcan et al. found through MD simulation that ICL3 contributes to a transition of β_2AR to a "very inactive" conformation (15). DeGraff et al. explored the function of ICL3 of α_2-adrenergic receptors in determining subtype specificity of arrestin interaction (52). Yet, it is well accepted that direct interaction of ICL3 with G-proteins probably has a significant role in the receptor's dynamics and the activation/inactivation pathways (12, 15). However, due to the absence of ICL3 in receptor structures, its function is not well understood. We examined specific interactions between ICL3 and $G_s\alpha$ α5 as shown in the insets of Figure 3 and Figure S5 with the key amino acid residues in close contact between ICL3 and α5 labeled. K232, D234 and K235 are the common amino acid residues from ICL3 involved in the interactions with α5 in both run 1 and run 2. Table S3 shows the number of amino acid residues in close contact between different parts of the proteins. The amino acid residues in ICL3 run 2 interact more extensively with α5 with 72.5% average percentage interaction time compared to those in run 1 with 65.7% average percentage interaction time. With the partial unwinding of α5 in run 2, the number of amino acid residues in the entire β_2AR in close contact with α5 is reduced to 22 with 85.0% average percentage interaction time compared to 26 amino acid residues with 86.7% average percentage interaction time in run 1, indicating partial dissociation of α5 from the β_2AR interior in run 2. These analyses suggest that ICL3 involvement may trigger the conformational change of $G_s\alpha$ α5, which favors the dissociation of α5 from the β_2AR interior. Moreover, the conformational change of α5 is not correlated with the opening and closing of $G_s\alpha$, because we observed no significant changes in α5 conformation with closed $G_s\alpha$ in run 1 (Figure 3A), with partially open $G_s\alpha$ in runs 3 and 4 shown in Figure S5, and with open $G_s\alpha$ in the GaMD simulations (Figure S6). An important question arises here: is there any correlation between different protein domains and what is the relationship between the G_s conformational changes and its dissociation?

To answer this question, we performed analysis of time series for multiple distances and angles between different protein residues and domains based on Anton runs as shown in Figure S7. The average values of those distances and angles based on the last 2 μs simulation for each run are shown as scatter plots in Figures 4A and 4B. Figure S7A shows the time series of A161 – E299 distance. A special attention should be given to run 3, where the distance between A161 – E299 (51 Å at the end of the run) indicates a partially open structure, but it represents a closed $G_s\alpha$ as shown in Figure S5A, because the $G_s\alpha$AH domain flipped upwards with A161 pointing up. We then analyzed an angle between two vectors representing GsαAH and GsαRas domains indicating their relative orientation (Figure S7B). As shown in Figure 4C, vector 1 goes through the centers of the $G_s\alpha$AH domain and residue A161 and vector 2 goes through the centers of the

G_sαRas domain and residue E299. Time series of G_sαAH – G_sαRas center-to-center distance, NPxxY – α5 distance, β_2AR – α5 distance, and α1 – α5 distance are shown in Figures S7C-S7F.

As demonstrated using different distance and angle measurements in Figures 4 and S7, we captured different conformations of $G_s\alpha$ in our multiple microsecond-long Anton simulations for β_2AR-G_s. The closing/opening conformational transition of $G_s\alpha$ is due to the movement of G_sαAH relative to G_sαRas. G_sαAH moves more like a rigid body as shown in RMSD plots when this domain is aligned with β_2AR or itself (Figure S8), which is in line with experimental findings (7, 53). The initial distance between A161 and E299 is about 62 Å based on the crystal structure PDB: 3SN6. In run 1 (Figure S7A), we mostly captured the closed $G_s\alpha$, resembling the closed inactive $G_s\alpha$ (PDB: 1AZT) (9), with the final distance of ~ 34 Å, as shown in Figure 3A. In run 2, $G_s\alpha$ goes through a short period of partial closing with a minimum distance of ~ 47 Å at the very beginning of the run, but the dominant conformation is fully open with a distance of ~64 Å (Figure S7A and Figure 3B). In both run 3 and run 4, G_sα shows dynamical nature, switching between fully open and semi-open states (Figure S7A). The GαAH flexibility is a reason for its low electron density in the recent cryo-EM structure of the β_1AR-G_s complex (8, 53). As mentioned in the previous section, run 3 shows the flip up G_sαAH orientation, but it cannot be identified by A161 to E299 distance. Thus, we analyzed the angle between G_sαAH and G_sαRas domains and the distance between the G_sαAH and G_sαRas centers (Figures S7B and S7C). The angle is defined by two vectors shown in Figure 4C. This angle weakly correlates with the opening and closing of G_sα (Figure 4A), specifically, the big separation of A161 and E299 in run 2 does not guarantee a large interdomain angle, indicating seemingly random drifting of the domains in 3D space during conformational change of G_sα. The Pearson correlation coefficients (Table S4), *r*, were calculated among the data points in Figures 4A and 4B collected from the average values of the last 2 μs of each Anton runs. The value of *r* for the interdomain angle and A161 – E299 distance is 0.61 validating a relatively weak correlation.

To track a possible partial dissociation of G_s from β_2AR, we analyzed the distance between G_sα helix α5 and the conserved motif NPxxY in β_2AR's transmembrane domain 7 (TM7) (Figure S7D) as done by Miao et al. in their GaMD simulations for different GPCR systems, adenosine receptors (54). Our β_2AR-G_s Anton runs 1 and 2 show almost identical displacement of α5 with the largest dissociation distance among all the runs, but this does not match with our previous analysis of dissociation in terms of the number of amino acid residue contacts (Table S3), where run 2 shows a more dissociated β_2AR-G_s complex than run 1. Thus, we think the NPxxY to α5 distance may be not suitable to accurately predict displacement of α5 from β_2AR in our systems, because NPxxY motif can be easily affected by the relative movement of TM7 to other TMs in our systems, which adds random noise into the measured distances. As α5 is a major element of the G protein - GPCR interacting interface (8, 23, 41, 54), researchers in a recent study used it as a cognate peptide to probe the kinetics of its binding to and activation of β_2AR, which is at least on the order of seconds (55), much longer than a time scale of our MD simulations. Despite this, we think that the center-to-center distance between β_2AR and α5 may be suitable to check the displacement of α5 from β_2AR which can be used as a sign for a commencement of G_s dissociation, and the corresponding plot is shown in Figure S7E. However, there is still no obvious correlation between the $G_s\alpha$ conformational change and β_2AR-G_s partial dissociation as the values of *r* between β_2AR – α5 distance and A161 – E299 distance is 0.53, $G_s\alpha$ interdomain orientation angle is 0.07, $G_s\alpha$AH – $G_s\alpha$Ras distance is 0.46 (Table S4, row 4). These results indicate that closing or opening of $G_s\alpha$ by itself cannot control the suggested partial dissociation of G_s from β_2AR. Instead, the internal arrangement of protein secondary structure elements may matter. To validate our assumption, we further analyzed the center-to-center distance between $G_s\alpha$ helices α1 and α5 as shown in Figure S7F (the illustration of these two helices in $G_s\alpha$ is shown in Figure 4D). We found a strong negative correlation between α1 – α5 distance and β_2AR – α5 distance with the *r* of –0.80. The temporal variation of value of *r* between α1 – α5 distance and β_2AR – α5 distance in each Anton run was also calculated in terms of lag time (Figure S9A). The negative correlation was found in runs 2, 3 and 4 when the lag time is less than 1 μs and

where conformational transition is clearly seen in the latter two runs. Thus, we think the stacking of α1 and α5 mostly causes the dislocation of α5 from β_2AR. Importantly, we also found that the opening of $G_s\alpha$ (indicated by $G_s\alpha$AH – $G_s\alpha$Ras interdomain distance and A161 to E299 distance) is negatively correlated with the α1 – α5 distance with relatively large *r* values of –0.65 (Table S4, row 5). This indicates that the opening of $G_s\alpha$ in the nucleotide free state is related to the stacking of α1 and α5 following the dislocation of α5 from β_2AR. However, the direct correlation between $G_s\alpha$AH – $G_s\alpha$Ras interdomain distance and β_2AR – α5 distance with an *r* of 0.46 is not as strong as expected, indicating the importance of the internal domain rearrangement in the suggested partial dissociation of G_s. The role of α1 and α5 movements has been highlighted in the structural analysis of β_2AR – G_s coupling/association and GDP release processes (56). Specifically, it was found that α5 interacts with α1, β2 and β3 through highly conserved hydrophobic contacts in the GDP-bound closed $G_s\alpha$, and structural perturbation of α1 accelerates GDP release and opening of inactive $G_s\alpha$ (56). Here, in our study of G_s partial dissociation, α1 and α5 were found to be important in regulating the conformational change of $G_s\alpha$. The stacking of α1 and α5 may cause the opening of $G_s\alpha$ (or vice versa), pulling the α5 away from the interior part of β_2AR, which facilitates the G_s dissociation. In the GaMD runs, the $G_s\alpha$ is almost always in a fully open state (Figures S6 and S10), except at the end of β_2AR-G_s-GaMD-run2 where a semi-open state appears. We did not see large $G_s\alpha$ conformational changes in the enhanced sampling GaMD runs as observed in the unbiased Anton runs 1 and 4 which could be due to random fluctuations. We do not anticipate any correlations for the interdomain distances when there is no obvious $G_s\alpha$ conformational change. In our study we used general GaMD methodology, which boosts the overall potential of the system (57) and may not have been sufficient to trigger a $G_s\alpha$ conformational transition. Using a more directed approach such as protein–protein interaction-GaMD (PPI-GaMD) (58) may solve this issue in the follow-up studies.

We then calculated the free energy or potential of mean force (PMF, in kcal/mol) 2D profiles (see Figures 5 and S11-S12) based on $G_s\alpha$ conformation and its β_2AR partial dissociation to further validate the correlation analyzed in the previous section. As shown in Figure 5A, the 2D PMF for the A161 – E299 distance on the *x* axis versus the β_2AR – α5 distance on the *y* axis exhibits two free energy minima, the closed $G_s\alpha$ (at *x* = ~ 32 Å) and the open $G_s\alpha$ (at *x* = ~ 58 Å). There is a small free energy barrier of about 2 – 3 kcal/mol between the two minima, but the open state is more energetically favorable, which is in line with the proposition in the earlier work of Dror et al. (23). Interestingly, only one minimum was found in the GaMD run (Figure S11A) at an even more open $G_s\alpha$ state (*x* = ~ 67 Å). It can also be seen that the open $G_s\alpha$ (Figure 5A) favors a larger distance between α5 and β_2AR compared with the closed $G_s\alpha$. Notably, there are also more chances for the dislocation of α5 from its β_2AR binding site when $G_s\alpha$ is open because of the bigger area within the 0.5 kcal/mol low-energy contour line associated with the open state. Similarly, Figure S12E shows the 2D PMF for the $G_s\alpha$AH – $G_s\alpha$Ras interdomain distance versus the β_2AR – α5 distance, also indicating a larger chance of α5 dislocation in the open state. However, the open $G_s\alpha$ conformation by itself cannot guarantee the dissociation, as the structures in runs 3 and 4 at around 3 μs (Figure S7A) correspond to the open $G_s\alpha$, but they are not in a suggested partially dissociated state (Figure S7E). We previously proposed that some internal structural rearrangements may occur during the opening and closing of $G_s\alpha$, triggering the dissociation. We again found that the relative movement between $G_s\alpha$ helices α5 and α1 is well correlated with the dislocation of α5 from β_2AR. As shown in Figure 5B, decreasing the distance between $G_s\alpha$ α5 and α1, as marked with the yellow arrow, can lead to the dislocation of α5 with minimal energy barriers (~ 0.1 kcal/mol). Also, Figure S12B shows the 2D PMF for the $G_s\alpha$AH – $G_s\alpha$Ras interdomain distance versus $G_s\alpha$ α1 – α5 interhelical distance, which exhibits a negative correlation in line with the Pearson correlation coefficient calculations in the previous section. These analyses indicate that the stacking of α1 and α5 helices can be the molecular determinant for the partial dissociation of G_s from β_2AR in the absence of guanine nucleotide binding. The interaction between α1 and α5 was previously found to be important in the allosteric

activation of $G_s\alpha$ using structural and phylogenetic analyses (51). The interruption of the contacts between α1 and α5 was found to be the key step for GDP release during the association of $G_s\alpha$ to its receptor (51). And, in our study we observed that the interaction between α1 and α5 favors suggested partial dissociation of $G_s\alpha$ from its receptor, thus sharing similar structural rearrangements to their association process. This indicates that interaction between α1 and α5 could be a molecular control for the association and dissociation kinetics of $G_s\alpha$ and β_2AR.

To estimate the relative binding affinities between the G_s and β_2AR, we calculated corresponding MM-PBSA interaction energies2 as shown in Table 2. These results can be compared with different conformations of $G_s\alpha$ (Figures 3 and S5) to give insights into the correlation between G_s conformation and its possible partial dissociation from β_2AR. As discussed previously, during the last 2 μs, run 1 corresponds to the fully closed $G_s\alpha$; run 2 has a fully open $G_s\alpha$; in run 3 and run 4, $G_s\alpha$ is very dynamic, transitioning between open and intermediate states, which makes predicting the trends in MM-PBSA interaction energy challenging. Run 1 with the final closed G_s conformation shows the lowest (most favorable) free energies of binding, while run 2 with a fully open structure shows relatively higher (less favorable) binding free energy, indicating more chances of G_s dissociation with the open state. This result is in line with the 2D PMF analysis (discussed above) where the minimum for $G_s\alpha$ open states span a larger range of distances between $G_s\alpha$ α5 and β_2AR indicating a larger chance for dissociation. Moreover, we found fewer interacting amino acid residues between α5 and β_2AR and a bent α5 conformation in run 2 with an open state compared with run 1 where $G_s\alpha$ is mostly in a closed state. Also, the number of interacting amino acid residues at the G_s – β_2AR binding interface shows a clear trend of decrease in the longer run, run 3, also possibly suggesting a partial G_s dissociation (Figure S13). Altogether, we found that the opening of $G_s\alpha$ favors its partial dissociation from β_2AR but is not sufficient. The interdomain rearrangement, namely, the stacking of $G_s\alpha$ helices α1 and α5 is necessary for the partial G_s dissociation process. We have to mention that we only considered nucleotide-free and receptor-bound open-in G_s initial state in this work. The effect of GTP/GDP binding to the G_s conformational transitions and dissociation will be evaluated in a follow-up study.

Conclusions

Combining all-atom multi-microsecond-long MD simulations with a posteriori implicit-solvent MM-PBSA calculations, we found that G_s binding to β_2AR can stabilize the NE(+) binding to β_2AR through stabilizing the structure of the active β_2AR conformation. Different binding poses and partial dissociation of NE(+) were captured in both free and G_s bound β_2AR systems. The partial dissociation of NE(+) can be attributed to the altered β_2AR structure due to its interactions with G_s, evidenced by the variances of β_2AR RMSD values. The waggling of NE(+) binding to β_2AR, i.e., presence of alternative binding poses closer to extra- or intracellular sides than the orthosteric binding site, was found to be related to the $G_s\alpha$ conformational transition to a semi-closed or closed state. Using all-atom MD simulations, we also observed interaction between β_2AR's ICL3 and G_s which caused the partial unwinding of the $G_s\alpha$ α5 helix in the open-in state of this subunit, suggesting the important role of ICL3 in the G_s dissociation. ICL3 was included in our models but usually missing in the available PDB structures (7, 8, 53), thus very limited information can be found about its function in related works (6, 12, 41). We also captured multiple closed and semi-closed conformations of the $G_s\alpha$ subunit in the β_2AR-G_s system. These conformations are absent in previous simulation works (6, 23, 40, 41) and hard to obtain from experiments due to the highly dynamic nature of $G_s\alpha$AH (8, 56). Our simulation data indicate the possibility of G_s closing before its partial dissociation from β_2AR, which was not observed in previous simulation studies to the best of our knowledge. However, the closed $G_s\alpha$ conformation is less favorable compared with the open one in promoting the dislocation of $G_s\alpha$ α5 from its β_2AR binding site. Instead, the internal $G_s\alpha$Ras domain stacking between helices α1 and α5 was found to be

necessary. We found that the open $G_s\alpha$ favors a more stacked $\alpha1$ and $\alpha5$ arrangement, which can drive the dissociation of $G_s\alpha$ $\alpha5$ from the receptor. Yet, the binding of guanine nucleotides may have a different effect on the G protein conformational changes and dislocation of $G_s\alpha$ $\alpha5$ from its receptor binding site, which will be evaluated in our subsequent studies. The results of this study may help explain molecular determinants and underlying mechanisms on why bound G_s protein can stabilize NE(+) binding to β_2AR and how G protein dissociation from the receptor may commence in the nucleotide-free state. These questions are important for understanding the activation of GPCRs and their modulation by G protein interactions in normal physiological and pathophysiological conditions. Our results can also be used to inform the next generation of multiscale functional kinetic models of sympathetic nervous stimulation in cardiac myocytes and other excitable cells, which is a powerful tool to complement experimental and clinical research.

Materials and Methods

Protein structures

The 3D coordinates of adrenaline-bound β_2AR were obtained from the published X-ray crystallographic structure (PDB: 4LDO) (59) to serve as a template for the activated receptor. The G_s heterotrimer template was obtained from the 3D coordinates of the crystal structure of β_2AR-G_s complex (PDB: 3SN6) bound to agonist BI-167107 (P0G) (7). 3D coordinates were oriented via the Orientations of Proteins in Membranes (OPM) database (60). The adrenaline-bound receptor from PDB 4LDO was aligned to protein complex structure from PDB 3SN6 via UCSF Chimera (61) Matchmaker to replace the P0G-bound receptor of PDB 3SN6, then all ligands and non-physiological proteins were removed. The resulting template, which combined the receptor of 4LDO with the G_s heterotrimer of 3SN6, was then assessed for clashing van der Waals radii before proceeding.

As the β_2AR structure was published without 3D coordinates for the intracellular loop 3 (ICL3), this region as well as omitted regions of the published G_s model in PDB 3SN6 were remodeled using the ROSETTA implementation of fragment-based cyclic coordinate descent (CCD) (62, 63). Target sequences for *de novo* modeling of both the human β_2AR and the G_s heterotrimer were obtained via UniProt (64). Rosetta comparative modeling (RosettaCM) was used with the Rosetta Membrane Energy Function to generate 10,000 decoy models of sequence-complete β_2AR-G_s complex (65-67). Rosetta clustering analysis was used to assess convergence of decoys into different microstates using their RMSDs with a cluster radius of 2.5 Å. The lowest-energy decoy of the most populated cluster was selected as a model for further refinement.1,000 energy-minimized decoys were then generated from the sequence-complete model using the Rosetta Fast Relax application in conjunction with the membrane energy function (68). Relaxation was permitted only to residues that were modeled *de novo*. The lowest energy structure was then selected for ligand docking and MD simulations.

Ligand docking

RosettaLigand (69) was used for all docking simulations of NE(+) to β_2AR and β_2AR-G_s. Ligand rotamers and parameters were generated by OpenEye Omega (70) and ROSETTA scripts. A box size of 5 Å was used for ligand transformations along with 7 Å ligand distance cutoff for side chain and backbone reorientations (with <0.3 Å C_α restraint). 50,000 structures were generated in each run with top 10% selected by total score, out of which 50 lowest-interfacial score structures were validated for their convergence with the crystalized adrenaline of the original template structure 4LDO. Subsequent simulations were conducted using the lowest-interfacial score structures.

Molecular dynamics simulations

MD simulation systems of ~222,000 or ~302,000 atoms were generated using CHARMM-GUI (71-73) and consisted of β_2AR protein or β_2AR-G_s protein complex in lipid bilayers soaked by a 0.15 M NaCl aqueous solution. The outer bilayer leaflet contained pure 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) whereas the inner leaflet had ~70% POPC and ~30% 1-Palmitoyl-2-oleoylphosphatidylserine (POPS) as in a previous MD simulation study (23). The same ionizable protein residue protonation states, post-translational modifications (lipidations and disulfide bonds based on UniProt data) and C- and N- protein termini as in that study (23) were used as well. All-atom biomolecular CHARMM36m protein (74), C36 lipid (75) and general CHARMM (CGENFF) (76) force field and TIP3P water (77) were used. CGENFF program (78, 79) was used to generate cationic norepinephrine, NE(+), force field parameters by analogy, which were validated and had to be optimized for one dihedral angle using an established quantum-mechanics (QM) based protocol (76).

MD simulations were run in the *NPT* ensemble at 310 K and 1 atm pressure using tetragonal periodic boundary condition. The systems were equilibrated for 90 ns with gradually reducing protein restraints in the first 40 ns using NAMD (80). MD equilibration runs were then followed by multi-microsecond long production runs on the Anton 2 (81) supercomputer or using enhanced sampling Gaussian accelerated MD (GaMD) (57) runs, respectively. The GaMD module implemented in the NAMD (82) was applied to perform GaMD simulations, which included a 10-ns short conventional MD (cMD) simulation (after the previous 90 ns MD equilibration), used to collect potential statistics for calculating the GaMD acceleration parameters, 50-ns GaMD equilibration after adding the boost potential, and finally three independent GaMD production runs with randomized initial atomic velocities for each system. All GaMD simulations were run at the "dual-boost" level by setting the reference energy to the lower bound. The upper limit of the boost potential standard deviation (SD), σ_0 was set to 6.0 kcal/mol for both the dihedral and the total potential energy terms. Simulation analyses were performed using VMD (50) and lab generated codes. The PyReweighting toolkit (83) was used to reweight the PMF profiles based on the distances and angles for GaMD trajectories to account for the effect of the boost potential on GaMD simulated distributions. A bin size of 0.5 Å was used for the interatomic distances and 5° for angles. The cutoff was set to 10 configurations in one bin for 2D PMF calculations. For the Anton simulations, PMF profiles did not need to be reweighted.

MM-PBSA binding energies

Free energy calculations for β_2AR-NE(+) binding and β_2AR-G_s binding were performed using the Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) approach with all-atom MD simulation trajectories by MMPBSA.py program in Amber Tools (84). The Chamber module of ParmEd program was used to convert CHARMM-style forcefields to Amber-style forcefields (85). Aqueous solution (ionic strength 150 mM) and lipid membrane were treated implicitly using dielectric constants (water ε_w=80, lipid bilayer ε_l=2 and protein ε_p= 4). Solvent probe radius is set to 1.4 Å and the atomic radii were set according to the converted force field parameters. To obtain the enthalpy (ΔH) contributions of solvation and gas-phase free energies, the particle-particle particle-mesh (P3M) procedure was used (86). These calculations were performed with implicit membrane, where the electrostatic energy includes both reaction filed and Coulombic electrostatic energies. Entropy was calculated separately by the interaction entropy method (87). This method was shown to increase the entropy calculation efficiency and possibly improve the accuracy of MM-PBSA in estimating protein-protein interactions (88). To use the interaction entropy method, gas-phase interaction energies including Coulombic electrostatic and van der Waals components were computed. In order to get the gas-phase Coulombic energy separated from the reaction filed energy contribution, each system energy was recalculated by using dielectric boundary surface charges method in the implicit ionic solution. In this study we focused on trends in relative binding free energies for the same or similar (β_2AR and β_2AR-G_s) protein systems, which may justify usage of a standard MM-PBSA approach (84) along with interaction entropy calculations (87). However, to obtain more accurate absolute and relative protein-protein

binding free energy estimates we may need to use recently developed MM-PBSA method with a screened electrostatic energy (88) in subsequent studies.

To reweight the MM-PBSA energies computed from GaMD simulations, we used the PyReweighting toolkit (83) to generate a corresponding PMF (W) value for each bin of the energy histogram generated from the simulation trajectories as described above for distance and angle PMFs. The probability for each bin can then be computed as $P_{bin} = e^{-\beta W}$, where $\beta = 1/(k_B T)$, k_B is Boltzmann constant and T is temperature. The average MM-PBSA energy in the GaMD boost-potential biased ensemble (notated with an asterisk, $\langle E^* \rangle$) is then converted to the canonical ensemble value $\langle E \rangle$ using probabilities, P_{bin}, and energies, E^*_{bin}, for each bin as $\langle E \rangle = \frac{\sum_{bin=1}^{N} P_{bin} E^*_{bin}}{\sum_{bin=1}^{N} P_{bin}}$. The bin width was kept as 0.5 kcal/mol. Similar reweighting approach can be in principle applied to interaction entropies using e.g., a cumulant expansion approach outlined in (89), but results for our systems were found to be noisy and unreliable (divergent) due to domination of higher order terms.

Binding pose clustering

The clustering for the NE(+) binding poses was performed by TTClust program (90). The trajectories were first aligned to the first frame of β_2AR (without intracellular loop 3). The RMSDs of NE(+) between all pairs of frames was calculated and stored into a matrix. This matrix was then used to calculate a linkage matrix by the hierarchical cluster linkage function of the SciPy package (91). Ward's method within the SciPy module was used to minimize the variance within clusters and allows more demarcated clusters to be obtained (90). K-means clustering with the Elbow algorithm was used to find the optimal number of clusters (90).

Pearson correlation coefficients

The Pearson correlation coefficients (values of *r*) shown in Table S4 were calculated among the data points in Figures 4A and 4B collected from the average values of the last 2 μs of each Anton run.

The time-lag correlation analysis was performed using MATLAB version 2022b. Calculations of the Pearson correlation coefficients (values of *r*) were performed using the built-in *corrcoef* function. The lag time defines a delay between two different MD simulation measurements, e.g., the distance between two protein residues as compared to the angle between two protein domains. A lag time of zero indicates that the distance and angle observations are compared from the same simulation time points, whereas a lag time of 50 ns, for example, indicates that distance observations for time *t* will be compared with angle observations from time (*t*+50) for the duration of the simulation. The lag time was varied from zero to half of the MD simulation length (e.g., 2.5 μs for a 5 μs long simulation).

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Figure captions

Figure 1. NE(+) bound β_2AR coupled with G_s protein. Different subunits and loops are illustrated by different colors (Green – β_2AR, Gray –intracellular loop 3 or ICL3, Pink – $G_s\alpha$AH domain, Red – $G_s\alpha$Ras domain, Blue – Gβ, Yellow – Gγ).

Figure 2. NE(+) binding poses and time series of center-to-center distances between NE(+) and β_2AR. (A) The initial (gray) and three special representative binding poses of NE(+) found in β_2AR (cluster 4 – in magenta) and β_2AR-G_s (cluster 4 – in light-blue and cluster 5 – in red) systems. See Figure S1 for binding pose clustering information (B) Time series for center-to-center distances between NE(+) and β_2AR (without intracellular loops) with the three special poses in panel A matching the plot colors. (C) The initial and dominant NE(+) binding pose and interacting β_2AR residues. C atoms are shown in gray for NE(+) and in cyan for residues of β_2AR, O atoms are in red, N atoms are in blue, H atoms are omitted. H-bonds between NE(+) and β_2AR residues S203$^{5.42}$, N312$^{7.39}$ and D113$^{3.32}$ are shown as dashed lines. (D) The special representative binding pose of NE(+) found in β_2AR system cluster 4 (magenta) and interacting β_2AR residues. H-bonds between the NE(+) and N312$^{7.39}$, D113$^{3.32}$ are shown as dashed lines. The preserved residues from the initial binding pocket in panel C are shown with cyan C atoms, whereas new residues in the binding pocket are shown with gray C atoms. (E) The special representative NE(+) binding pose from β_2AR-G_s cluster 4 (light blue) and interacting β_2AR residues in the binding pocket, which follow the same rendering style as in panel D. The geometric centers were used for the distance measurements. The Ballesteros-Weinstein (BW) numbering for the residues can be found in the text and is omitted in the figure for clarity.

Figure 3. All-atom MD simulations of the active-state human β_2AR-G_s with NE(+) bound based on Anton runs. (A) run 1 with the top inset. (B) run 2 with the bottom inset. Final structures are captured from the 5 μs long unbiased MD simulation runs. Individual protein chains / subunits are labeled and shown in the ribbon representation using different colors. $G_s\alpha$ α5 helix and β_2AR intracellular loop 3 (ICL3) are colored in yellow and gray. C_α atoms of residues A161 on $G_s\alpha$AH domain and E299 on $G_s\alpha$Ras domain are shown as blue and green balls, and distances between them are shown by light-blue dashed arrows. The quantification of the interactions between ICL3 and α5 helix can be found in Table S3. The geometric centers were used for the distance measurements. The common Gα numbering (CGN) numbers (D381$^{G.H5.13}$, D378$^{G.H5.10}$, N377$^{G.H5.9}$, R374$^{G.H5.6}$, R385$^{G.H5.17}$) for residues in $G_s\alpha$ α5 as well as A161$^{H.HD.5}$ and E299$^{G.HG.6}$ are omitted in the figure for clarity.

Figure 4. Analysis of $G_s\alpha$ conformation and its possible partial dissociation from β_2AR based on all-atom MD Anton runs. The distances and angle shown in each run are based on their average values during the last 2 μs of MD simulations. The distances and angles were measured between geometric centers of protein residues or domains. (A) A161–E299 distances indicating G_s protein conformational change (opening or closing), $G_s\alpha$AH–$G_s\alpha$Ras distances indicating relative movement between the two domains, the angle between the two vectors of $G_s\alpha$AH and $G_s\alpha$Ras domains indicating their relative orientation (B) α1 – α5 distances indicating relative movement between α1 and α5 helices in $G_s\alpha$, β_2AR – α5 distances indicating possible partial dissociation of $G_s\alpha$ α5 helix from the receptor, and β_2AR NPxxY motif – α5 helix distances also indicating $G_s\alpha$ α5 partial dissociation. (C) Illustration of the angle between $G_s\alpha$AH and $G_s\alpha$Ras domains; vector 1 goes through $G_s\alpha$AH and A161 centers; vector 2 goes through $G_s\alpha$Ras and E299 centers. (D) Illustrations of $G_s\alpha$ α5 helix (yellow), α1 helix (cyan), and β_2AR NPxxY motif (blue helix on transmembrane domain 7).

Figure 5. 2D potential of mean force (PMF) or free energy profiles (in kcal/mol) based on $G_s\alpha$ conformation and its possible partial dissociation from β_2AR based on all-atom Anton MD

simulations of the active state of the human β_2AR-G_s complexes with NE(+). The 0.5 kcal/mol contour lines are shown as bold black curves. Relative free energy values from 0 to 8 kcal/mol are indicated by different colors from blue to red. All distances were measured between geometric centers of protein residues or domains. (A) A161 – E299 distance indicating $G_s\alpha$ opening or closing is shown as *X* axis; distance between $G_s\alpha$ α5 and β_2AR indicating possible partial G_s dissociation is shown as *Y* axis. (B) $G_s\alpha$ α1 – α5 distance is shown as *X* axis; distance between $G_s\alpha$ α5 and β_2AR is shown as *Y* axis. The contour lines are smoothed for better visualization.

References

1. M. N. Alshak, J. M. Das, *Neuroanatomy, Sympathetic Nervous System* (StatPearls Publishing, Treasure Island (FL), 2021).
2. A. Lymperopoulos, G. Rengo, W. J. Koch, Adrenergic nervous system in heart failure: pathophysiology and therapy. *Circ. Res.* **113**, 739-753 (2013).
3. D. B. Bylund *et al.*, International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol. Rev.* **46**, 121-136 (1994).
4. S. B. Wachter, E. M. Gilbert, Beta-adrenergic receptors, from their discovery and characterization through their manipulation to beneficial clinical application. *Cardiology* **122**, 104-112 (2012).
5. D. Goricanec *et al.*, Conformational dynamics of a G-protein alpha subunit is tightly regulated by nucleotide binding. *Proc. Natl. Acad. Sci. U.S.A.* **113**, E3629-3638 (2016).
6. X. Liu *et al.*, Structural Insights into the Process of GPCR-G Protein Complex Formation. *Cell* **177**, 1243-1251 e1212 (2019).
7. S. G. Rasmussen *et al.*, Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature* **477**, 549-555 (2011).
8. M. Su *et al.*, Structural Basis of the Activation of Heterotrimeric Gs-Protein by Isoproterenol-Bound beta1-Adrenergic Receptor. *Mol. Cell* **80**, 59-71 e54 (2020).
9. R. K. Sunahara, J. J. G. Tesmer, A. G. Gilman, S. R. Sprang, Crystal Structure of the Adenylyl Cyclase Activator Gsα. *Science* **278**, 1943-1947 (1997).
10. R. O. Dror *et al.*, Identification of two distinct inactive conformations of the β2-adrenergic receptor reconciles structural and biochemical observations. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 4689-4694 (2009).
11. S. Vanni, M. Neri, I. Tavernelli, U. Rothlisberger, Observation of "ionic lock" formation in molecular dynamics simulations of wild-type β1 and β2 adrenergic receptors. *Biochemistry* **48**, 4789-4797 (2009).
12. R. O. Dror *et al.*, Activation mechanism of the β2-adrenergic receptor. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 18684-18689 (2011).
13. R. Nygaard *et al.*, The dynamic process of β2-adrenergic receptor activation. *Cell* **152**, 532-542 (2013).
14. M. I. Mahmood, X. Liu, S. Neya, T. Hoshino, Influence of lipid composition on the structural stability of g-protein coupled receptor. *Chem. Pharm. Bull.* **61**, 426-437 (2013).
15. O. Ozcan, A. Uyar, P. Doruker, E. D. Akten, Effect of intracellular loop 3 on intrinsic dynamics of human β2-adrenergic receptor. *BMC Struct. Biol.* **13**, 1-17 (2013).
16. C. Neale, H. D. Herce, R. Pomès, A. E. García, Can specific protein-lipid interactions stabilize an active state of the beta 2 adrenergic receptor? *Biophys. J.* **109**, 1652-1662 (2015).
17. O. Fleetwood, P. Matricon, J. Carlsson, L. Delemotte, Energy landscapes reveal agonist control of G protein-coupled receptor activation via microswitches. *Biochemistry* **59**, 880-891 (2020).
18. A. Goetz, H. Lanig, P. Gmeiner, T. Clark, Molecular dynamics simulations of the effect of the G-protein and diffusible ligands on the β2-adrenergic receptor. *J. Mol. Biol.* **414**, 611-623 (2011).
19. Z. Feng, T. Hou, Y. Li, Studies on the interactions between β2 adrenergic receptor and Gs protein by molecular dynamics simulations. *J. Chem. Inf. Model* **52**, 1005-1014 (2012).

20. Q. Bai, Y. Zhang, Y. Ban, H. Liu, X. Yao, Computational study on the different ligands induced conformation change of β2 adrenergic receptor-Gs protein complex. *PLoS one* **8**, e68138 (2013).
21. R. C. Kling, H. Lanig, T. Clark, P. Gmeiner, Active-state models of ternary GPCR complexes: determinants of selective receptor-G-protein coupling. *PloS one* **8**, e67244 (2013).
22. X. Sun, H. Ågren, Y. Tu, Microsecond molecular dynamics simulations provide insight into the allosteric mechanism of the Gs protein uncoupling from the β2 adrenergic receptor. *J. Phys. Chem. B* **118**, 14737-14744 (2014).
23. R. O. Dror *et al.*, Structural basis for nucleotide exchange in heterotrimeric G proteins. *Science* **348**, 1361-1365 (2015).
24. V. Kumar *et al.*, GDP Release from the Open Conformation of Gα Requires Allosteric Signaling from the Agonist-Bound Human β2 Adrenergic Receptor. *J. Chem. Inf. Model.* **60**, 4064-4075 (2020).
25. K. E. Komolov *et al.*, Structural and functional analysis of a β2-adrenergic receptor complex with GRK5. *Cell* **169**, 407-421. e416 (2017).
26. Y. Chen, O. Fleetwood, S. Pérez-Conesa, L. Delemotte, Allosteric Effect of Nanobody Binding on Ligand-Specific Active States of the β2 Adrenergic Receptor. *J. Chem. Inf. Model.* **61**, 6024-6037 (2021).
27. L. Zhao, X. He, H. Jiang, X. Cheng, Computational characterization of transducer recognition of β2 adrenergic receptor. *Biochem. Biophys. Res. Commun.* **592**, 67-73 (2022).
28. T. Wang, Y. Duan, Ligand entry and exit pathways in the β2-adrenergic receptor. *J. Mol. Biol.* **392**, 1102-1115 (2009).
29. R. O. Dror *et al.*, Pathway and mechanism of drug binding to G-protein-coupled receptors. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 13118-13123 (2011).
30. A. González, T. Perez-Acle, L. Pardo, X. Deupi, Molecular basis of ligand dissociation in β-adrenergic receptors. *PLoS one* **6**, e23815 (2011).
31. S. Vanni, M. Neri, I. Tavernelli, U. Rothlisberger, Predicting novel binding modes of agonists to β adrenergic receptors using all-atom molecular dynamics simulations. *PLoS Comput. Biol.* **7**, e1001053 (2011).
32. I. G. Tikhonova, B. Selvam, A. Ivetac, J. Wereszczynski, J. A. McCammon, Simulations of biased agonists in the β2 adrenergic receptor with accelerated molecular dynamics. *Biochemistry* **52**, 5593-5603 (2013).
33. A. Plazinska, M. Kolinski, I. W. Wainer, K. Jozwiak, Molecular interactions between fenoterol stereoisomers and derivatives and the β2-adrenergic receptor binding site studied by docking and molecular dynamics simulations. *J. Mol. Model.* **19**, 4919-4930 (2013).
34. A. Ranganathan, R. O. Dror, J. Carlsson, Insights into the role of Asp792. 50 in β2 adrenergic receptor activation from molecular dynamics simulations. *Biochemistry* **53**, 7283-7296 (2014).
35. A. Tandale, M. Joshi, D. Sengupta, Structural insights and functional implications of inter-individual variability in β2-adrenergic receptor. *Sci. Rep.* **6**, 1-11 (2016).
36. A. Plazinska, W. Plazinski, Stereoselective binding of agonists to the β2-adrenergic receptor: insights into molecular details and thermodynamics from molecular dynamics simulations. *Mol. Biosyst.* **13**, 910-920 (2017).

37. J. Wang, Y. Miao, Recent advances in computational studies of GPCR-G protein interactions. *Adv. Protein Chem. Struct. Biol.* **116**, 397-419 (2019).
38. D. Hilger, The role of structural dynamics in GPCR‐mediated signaling. *FEBS J.* **288**, 2461-2489 (2021).
39. J. M. L. Ribeiro, M. Filizola, Allostery in G protein-coupled receptors investigated by molecular dynamics simulations. *Curr. Opin. Struct. Biol.* **55**, 121-128 (2019).
40. R. Alhadeff, I. Vorobyov, H. W. Yoon, A. Warshel, Exploring the free-energy landscape of GPCR activation. *Proc. Natl. Acad. Sci. U.S.A.* **115**, 10327-10332 (2018).
41. C. Bai *et al.*, Exploring the Activation Process of the beta2AR-Gs Complex. *J. Am. Chem. Soc.* **143**, 11044-11051 (2021).
42. D. Provasi, A. Bortolato, M. Filizola, Exploring molecular mechanisms of ligand recognition by opioid receptors with metadynamics. *Biochemistry* **48**, 10020-10029 (2009).
43. S. Schneider, D. Provasi, M. Filizola, "The dynamic process of drug–GPCR binding at either orthosteric or allosteric sites evaluated by metadynamics" in G Protein-Coupled Receptors in Drug Discovery. (Springer, 2015), pp. 277-294.
44. N. Saleh, P. Ibrahim, G. Saladino, F. L. Gervasio, T. Clark, An efficient metadynamics-based protocol to model the binding affinity and the transition state ensemble of G-protein-coupled receptor ligands. *J. Chem. Inf. Model.* **57**, 1210-1217 (2017).
45. P. Ibrahim, T. Clark, Metadynamics simulations of ligand binding to GPCRs. *Curr. Opin. Struct. Biol.* **55**, 129-137 (2019).
46. J. A. Ballesteros, H. Weinstein, *Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors* S. C. Sealfon, Ed., Methods in neurosciences (Academic Press, 1995), vol. 25, pp. 366–428.
47. Y. Miao, A. Bhattarai, J. Wang, Ligand Gaussian Accelerated Molecular Dynamics (LiGaMD): Characterization of Ligand Binding Thermodynamics and Kinetics. *J. Chem. Theory Comput.* **16**, 5526-5547 (2020).
48. D. Guo *et al.*, Molecular Basis of Ligand Dissociation from the Adenosine A2A Receptor. *Mol. Pharmacol.* **89**, 485-491 (2016).
49. Y. Miao, J. A. McCammon, Graded activation and free energy landscapes of a muscarinic G-protein-coupled receptor. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 12162-12167 (2016).
50. W. Humphrey, A. Dalke, K. Schulten, VMD - Visual Molecular Dynamics. *J. Mol. Graph.* **14**, 33-38 (1996).
51. T. Flock *et al.*, Universal allosteric mechanism for Galpha activation by GPCRs. *Nature* **524**, 173-179 (2015).
52. J. L. DeGraff, V. V. Gurevich, J. L. Benovic, The third intracellular loop of alpha 2-adrenergic receptors determines subtype specificity of arrestin interaction. *J. Biol. Chem.* **277**, 43247-43252 (2002).
53. K. O. Alegre *et al.*, Structural basis and mechanism of activation of two different families of G proteins by the same GPCR. *Nat. Struct. Mol. Biol.* **28**, 936-944 (2021).
54. J. Wang, Y. Miao, Mechanistic Insights into Specific G Protein Interactions with Adenosine Receptors. *J. Phys. Chem. B* **123**, 6462-6473 (2019).
55. K. J. Culhane, T. M. Gupte, I. Madhugiri, C. J. Gadgil, S. Sivaramakrishnan, Kinetic model of GPCR-G protein interactions reveals allokairic modulation of signaling. *Nat. Commun.* **13**, 1202 (2022).

56. D. Hilger, M. Masureel, B. K. Kobilka, Structure and dynamics of GPCR signaling complexes. *Nat. Struct. Mol. Biol.* **25**, 4-12 (2018).
57. Y. Miao, V. A. Feher, J. A. McCammon, Gaussian Accelerated Molecular Dynamics: Unconstrained Enhanced Sampling and Free Energy Calculation. *J. Chem. Theory Comput.* **11**, 3584-3595 (2015).
58. J. Wang, Y. Miao, Protein–Protein Interaction-Gaussian Accelerated Molecular Dynamics (PPI-GaMD): Characterization of Protein Binding Thermodynamics and Kinetics. *J. Chem. Theory Comput.* **18**, 1275-1285 (2022).
59. A. M. Ring *et al.*, Adrenaline-activated structure of β2-adrenoceptor stabilized by an engineered nanobody. *Nature* **502**, 575-579 (2013).
60. M. A. Lomize, I. D. Pogozheva, H. Joo, H. I. Mosberg, A. L. Lomize, OPM database and PPM web server: resources for positioning of proteins in membranes. *Nucleic Acids Res* **40**, D370-D376 (2012).
61. E. F. Pettersen *et al.*, UCSF Chimera--a visualization system for exploratory research and analysis. *J. Comput. Chem.* **25**, 1605-1612 (2004).
62. C. Wang, P. Bradley, D. Baker, Protein-protein docking with backbone flexibility. *J. Mol. Biol.* **373**, 503-519 (2007).
63. A. A. Canutescu, R. L. Dunbrack, Jr., Cyclic coordinate descent: A robotics algorithm for protein loop closure. *Protein Sci.* **12**, 963-972 (2003).
64. T. U. Consortium, UniProt: the universal protein knowledgebase in 2021. *Nucleic Acids Res* **49**, D480-D489 (2020).
65. Y. Song *et al.*, High-resolution comparative modeling with RosettaCM. *Structure* **21**, 1735-1742 (2013).
66. V. Yarov-Yarovoy, J. Schonbrun, D. Baker, Multipass membrane protein structure prediction using Rosetta. *Proteins* **62**, 1010-1025 (2006).
67. R. F. Alford *et al.*, The Rosetta All-Atom Energy Function for Macromolecular Modeling and Design. *J. Chem. Theory Comput.* **13**, 3031-3048 (2017).
68. P. Conway, M. D. Tyka, F. DiMaio, D. E. Konerding, D. Baker, Relaxation of backbone bond geometry improves protein energy landscape modeling. *Protein Sci.* **23**, 47-55 (2014).
69. J. Meiler, D. Baker, ROSETTALIGAND: protein-small molecule docking with full side-chain flexibility. *Proteins* **65**, 538-548 (2006).
70. P. C. D. Hawkins, A. G. Skillman, G. L. Warren, B. A. Ellingson, M. T. Stahl, Conformer Generation with OMEGA: Algorithm and Validation Using High Quality Structures from the Protein Databank and Cambridge Structural Database. *J. Chem. Inf. Model.* **50**, 572-584 (2010).
71. J. Lee *et al.*, CHARMM-GUI Input Generator for NAMD, GROMACS, AMBER, OpenMM, and CHARMM/OpenMM Simulations Using the CHARMM36 Additive Force Field. *J. Chem. Theory Comput.* **12**, 405-413 (2016).
72. B. R. Brooks *et al.*, CHARMM: the biomolecular simulation program. *J. Comput. Chem.* **30**, 1545-1614 (2009).
73. S. Jo, T. Kim, V. G. Iyer, W. Im, CHARMM-GUI: a web-based graphical user interface for CHARMM. *J. Comput. Chem.* **29**, 1859-1865 (2008).
74. J. Huang *et al.*, CHARMM36m: an improved force field for folded and intrinsically disordered proteins. *Nat. Methods* **14**, 71 (2017).

75. J. B. Klauda *et al.*, Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. *J. Phys. Chem. B* **114**, 7830-7843 (2010).
76. K. Vanommeslaeghe *et al.*, CHARMM General Force Field: A Force field for Drug-Like Molecules Compatible with the CHARMM All-Atom Additive Biological Force Field. *J. Comput. Chem.* **31**, 671-690 (2010).
77. W. L. Jorgensen, J. Chandrasekhar, J. D. Madura, R. W. Impey, M. L. Klein, Comparison of Simple Potential Functions for Simulating Liquid Water. *J. Chem. Phys.* **79**, 926-935 (1983).
78. K. Vanommeslaeghe, A. D. MacKerell Jr, Automation of the CHARMM General Force Field (CGenFF) I: bond perception and atom typing. *J. Chem. Inf. Model.* **52**, 3144-3154 (2012).
79. K. Vanommeslaeghe, E. P. Raman, A. D. MacKerell, Automation of the CHARMM General Force Field (CGenFF) II: Assignment of Bonded Parameters and Partial Atomic Charges. *J. Chem. Inf. Model.* **52**, 3155-3168 (2012).
80. J. C. Phillips *et al.*, Scalable molecular dynamics with NAMD. *J. Comput. Chem.* **26**, 1781-1802 (2005).
81. D. E. Shaw *et al.* (2014) Anton 2: Raising the Bar for Performance and Programmability in a Special-Purpose Molecular Dynamics Supercomputer. in *SC '14: Proceedings of the International Conference for High Performance Computing, Networking, Storage and Analysis*, pp 41-53.
82. Y. T. Pang, Y. Miao, Y. Wang, J. A. McCammon, Gaussian Accelerated Molecular Dynamics in NAMD. *J. Chem. Theory Comput.* **13**, 9-19 (2017).
83. Y. Miao *et al.*, Improved Reweighting of Accelerated Molecular Dynamics Simulations for Free Energy Calculation. *J. Chem. Theory Comput.* **10**, 2677-2689 (2014).
84. B. R. Miller, 3rd *et al.*, MMPBSA.py: An Efficient Program for End-State Free Energy Calculations. *J. Chem. Theory Comput.* **8**, 3314-3321 (2012).
85. M. R. Shirts *et al.*, Lessons learned from comparing molecular dynamics engines on the SAMPL5 dataset. *bioRxiv* 10.1101/077248, 077248 (2016).
86. Q. Lu, R. Luo, A Poisson–Boltzmann dynamics method with nonperiodic boundary condition. *J. Chem. Phys.* **119**, 11035-11047 (2003).
87. L. Duan, X. Liu, J. Z. Zhang, Interaction Entropy: A New Paradigm for Highly Efficient and Reliable Computation of Protein-Ligand Binding Free Energy. *J. Am. Chem. Soc.* **138**, 5722-5728 (2016).
88. Y. J. Sheng, Y. W. Yin, Y. Q. Ma, H. M. Ding, Improving the Performance of MM/PBSA in Protein-Protein Interactions via the Screening Electrostatic Energy. *J. Chem. Inf. Model.* **61**, 2454-2462 (2021).
89. W. M. Menzer, C. Li, W. Sun, B. Xie, D. D. Minh, Simple entropy terms for end-point binding free energy calculations. *J. Chem. Theory Comput.* **14**, 6035-6049 (2018).
90. T. Tubiana, J. C. Carvaillo, Y. Boulard, S. Bressanelli, TTClust: A Versatile Molecular Simulation Trajectory Clustering Program with Graphical Summaries. *J. Chem. Inf. Model.* **58**, 2178-2182 (2018).
91. P. Virtanen *et al.*, SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat. Methods* **17**, 261-272 (2020).

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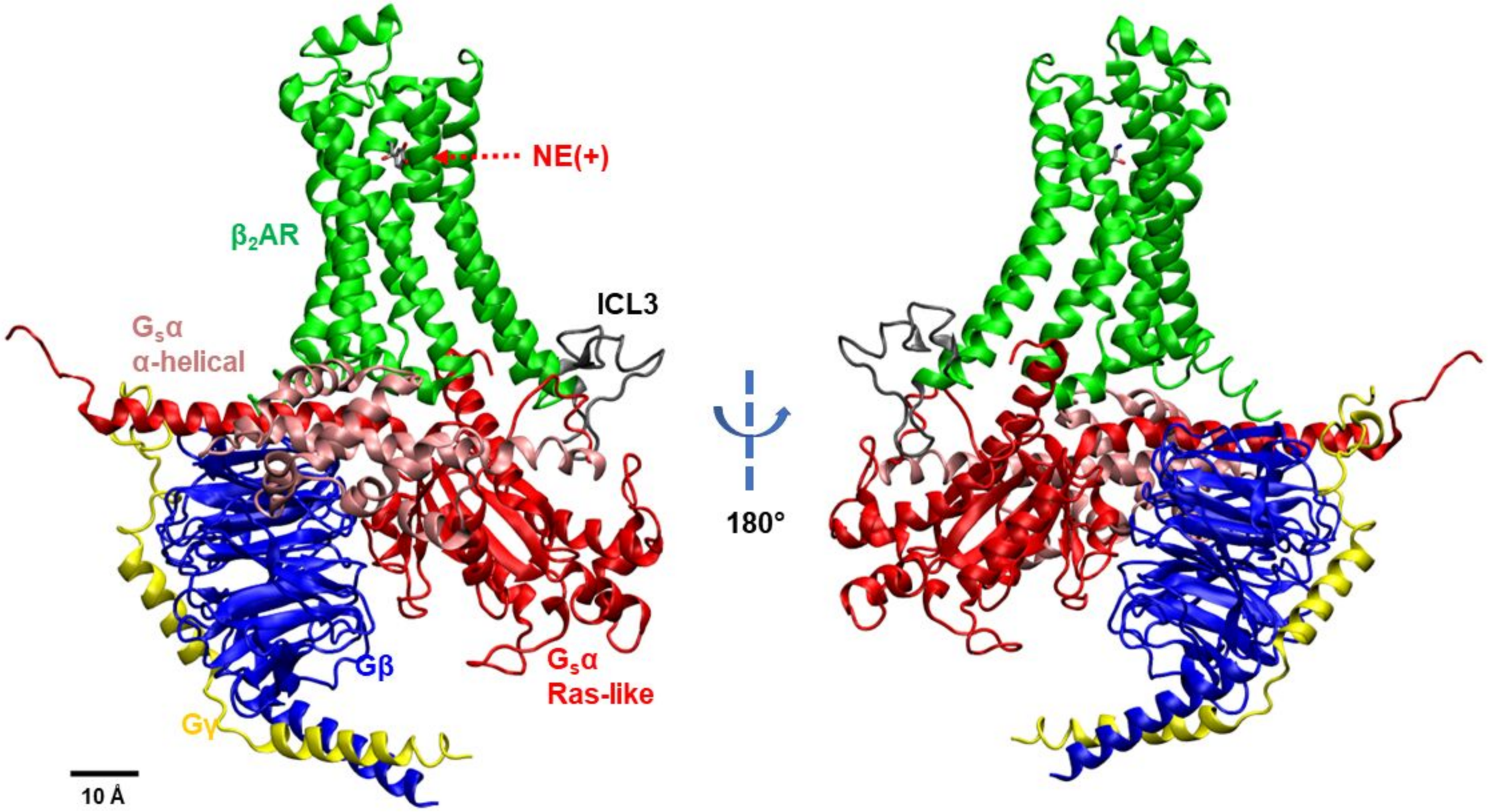
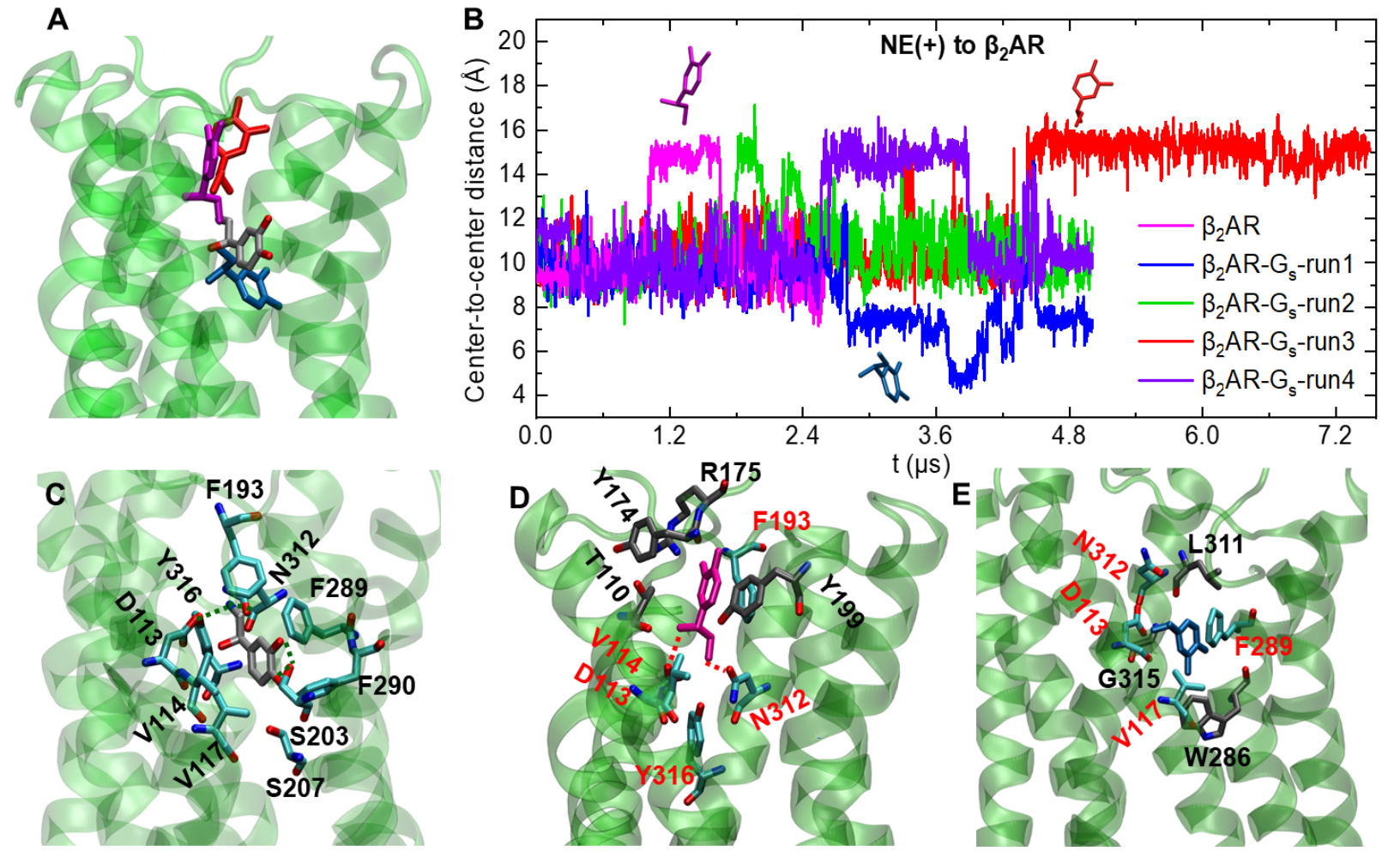
Table 1. MM-PBSA interaction free energies (ΔG) between NE(+) and β_2AR (in kcal/mol) along with their standard errors of mean (SEM) computed using block averages, enthalpic (ΔH) and entropic ($-T\Delta S$) components as well as mean RMSD values (in Å) along with their standard deviations (SD) for β_2AR without loops (the average structure was taken as reference; analysis was performed for the last 2 μs of Anton trajectories). See also Figure S14 for analysis of correlations between MM-PBSA interaction energies, β_2AR – NE(+) distances and RMSD values.

System	Time	ΔH	$-T\Delta S$	$\Delta G \pm$ SEM	RMSD (SD)
β_2AR	0.5 – 2.5 μs	-21.61	6.88	-14.73± 0.92	1.65 (0.26)
β_2AR-G_s - run1	3.0 – 5.0 μs	-27.54	11.92	-15.62± 2.00	1.79 (0.23)
β_2AR-G_s – run2	3.0 – 5.0 μs	-25.09	6.10	-18.99±0.44	1.56 (0.21)
β_2AR-G_s – run3	5.5 – 7.5 μs	-23.70	7.91	-15.79 ±0.45	1.52 (0.15)
β_2AR-G_s – run4	3.0 – 5.0 μs	-22.42	10.81	-11.61 ±1.11	1.67 (0.16)

Table 2. MM-PBSA interaction free energies between β_2AR and G_s (in kcal/mol) along with their standard errors of mean (SEM) computed using block averages, enthalpic (ΔH) and entropic ($-T\Delta S$) components (based on the last 2 μs of Anton trajectories).

System	Time	ΔH	$-T\Delta S$	$\Delta G \pm$ SEM
β_2AR-G_s - run1	3.0 – 5.0 μs	-145.4	105.1	-40.3±8.2
β_2AR-G_s - run2	3.0 – 5.0 μs	-111.8	82.9	-28.9±8.6
β_2AR-G_s – run3	5.5 – 7.5 μs	-154.6	105.4	-49.2±17.2
β_2AR-G_s – run4	3.0 – 5.0 μs	-109.6	83. 6	-26.0±4.9


NE(+)
β2AR
ICL3
Gsα
α-helical
Gβ
Gγ
Gsα
Ras-like
180°
10 Å


A
B
NE(+) to β2AR
Center-to-center distance (Å)
t (μs)
β2AR
β2AR-Gs-run1
β2AR-Gs-run2
β2AR-Gs-run3
β2AR-Gs-run4
C
F193
Y316
N312
F289
D113
F290
V114
S203
V117
S207
D
R175
Y174
F193
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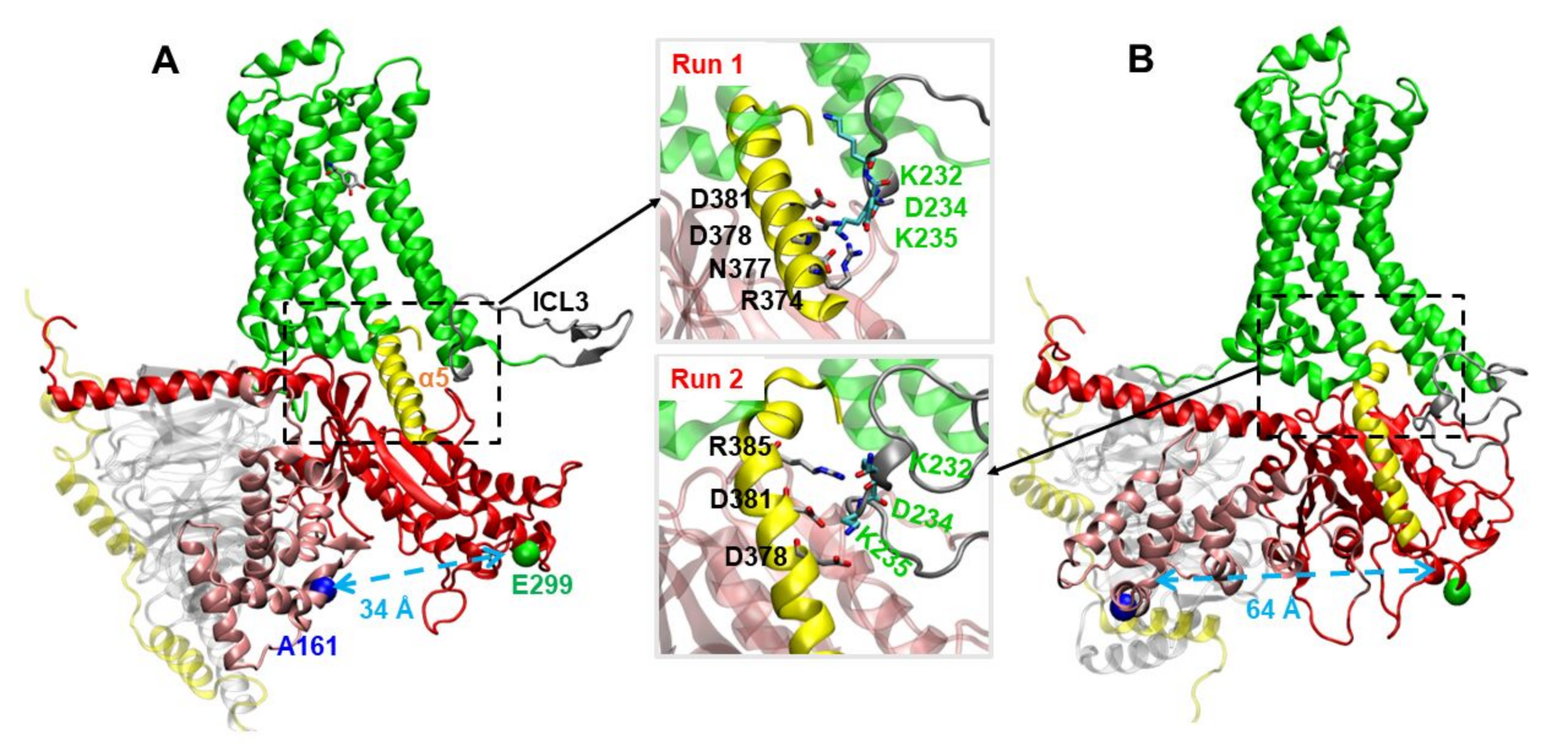
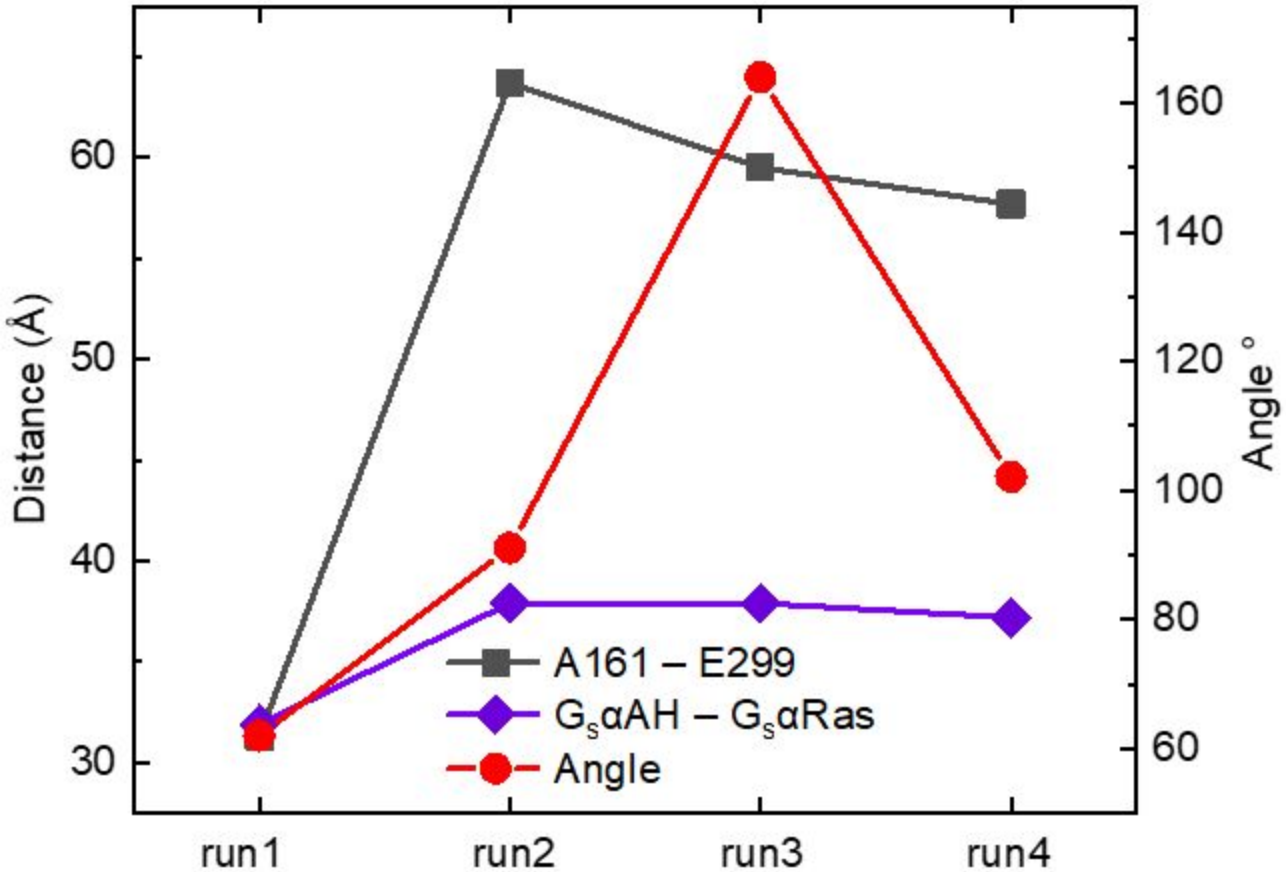
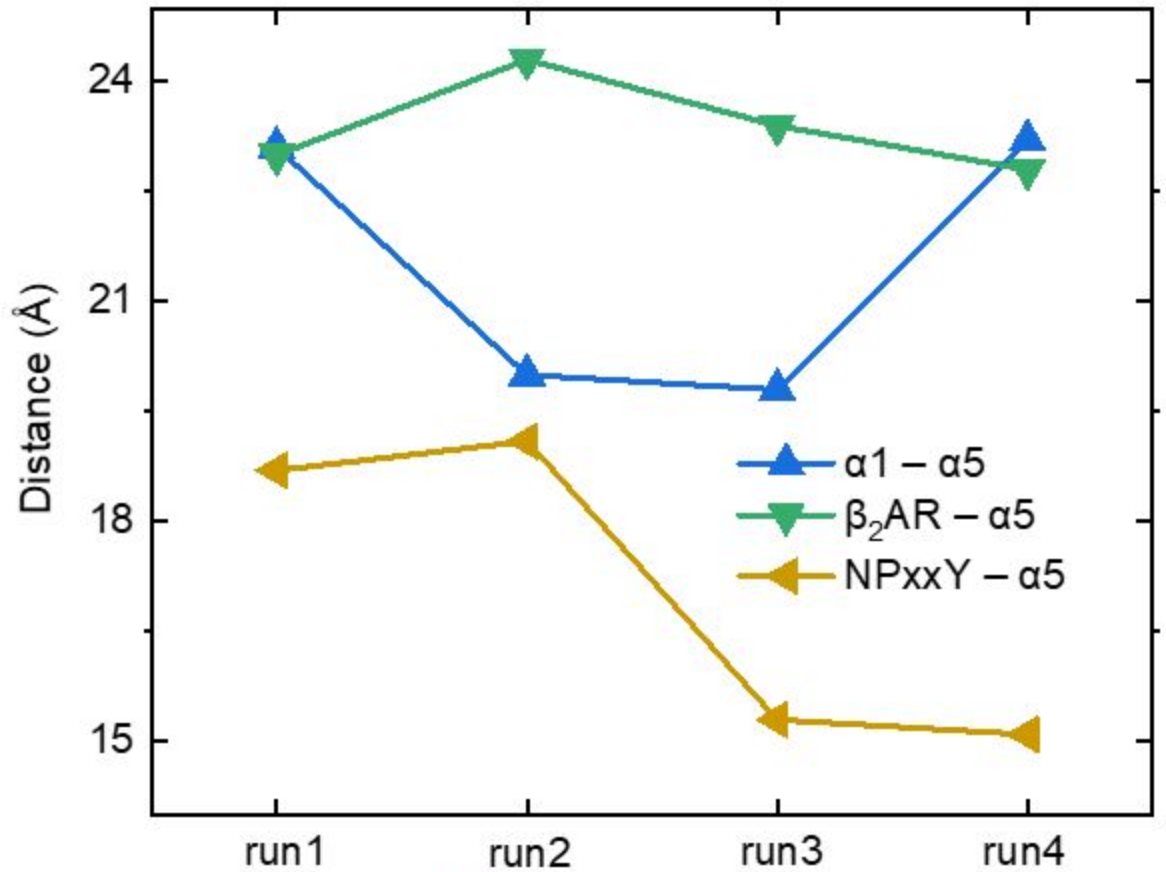
L311
N312
D113
F289
G315
V117
W286


A
ICL3
α5
E299
34 Å
A161
Run 1
D381
D378
N377
R374
K232
D234
K235
Run 2
R385
D381
D378
K232
D234
K235
B
64 Å

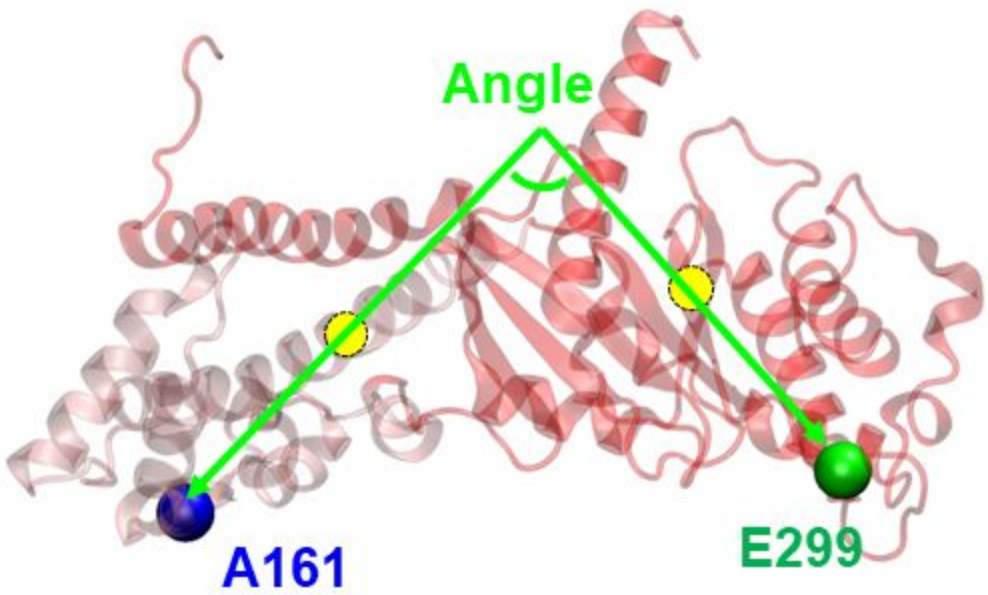
A


Distance (Å)
60
50
40
30
Angle °
160
140
120
100
80
60
run1
run2
run3
run4
A161 – E299
G_sαAH – G_sαRas
Angle

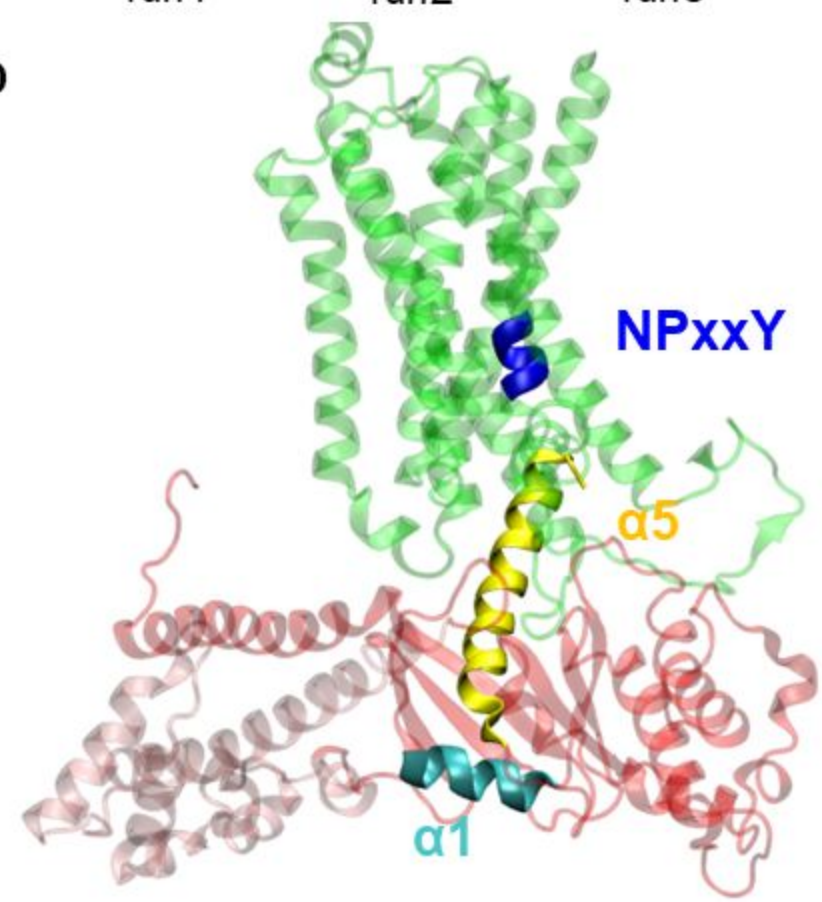
B

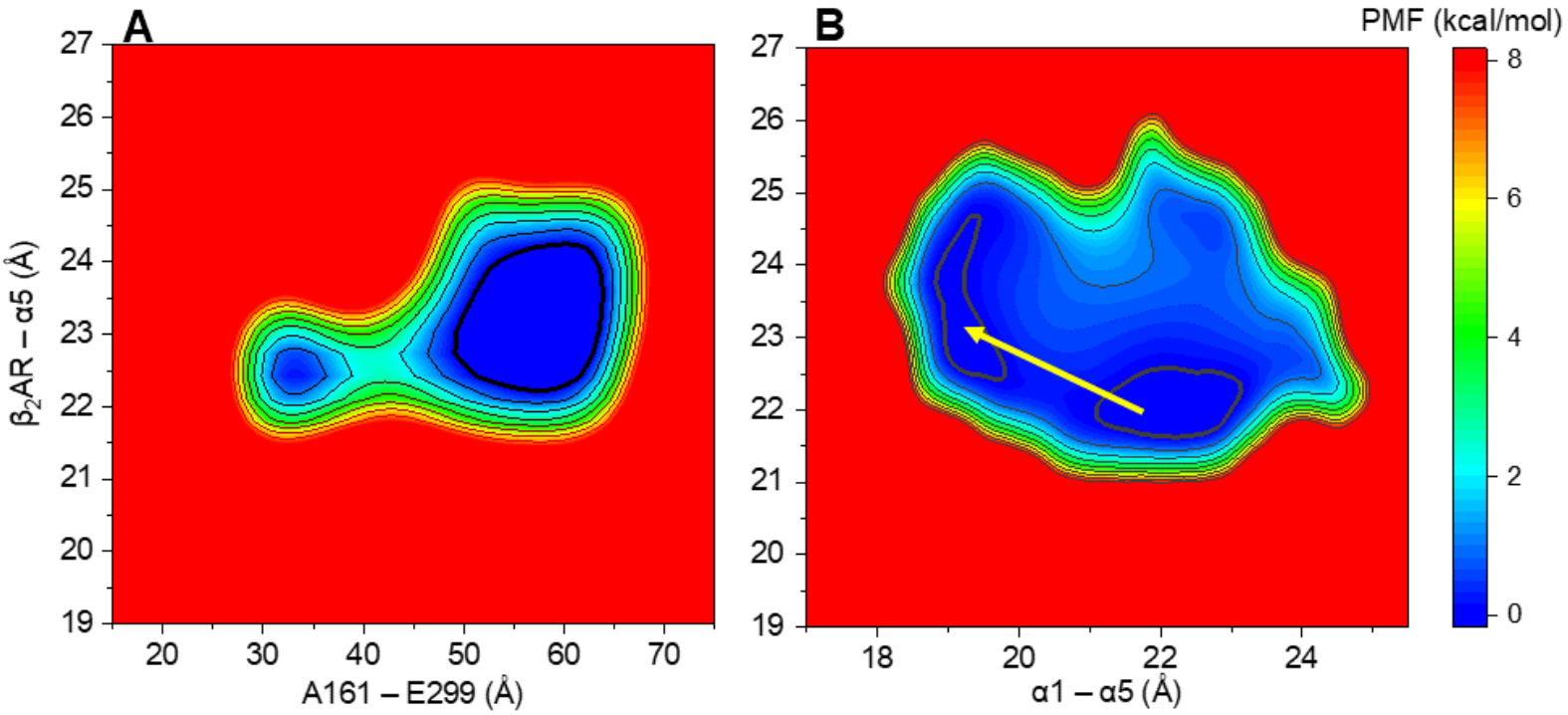

Distance (Å)
24
21
18
15
run1
run2
run3
run4
α1 – α5
β_2AR – α5
NPxxY – α5

C


Angle
A161
E299

D


NPxxY
α5
α1


A
B
PMF (kcal/mol)
8
6
4
2
0
27
26
25
24
23
22
21
20
19
β_2AR – α5 (Å)
20
30
40
50
60
70
A161 – E299 (Å)
18
20
22
24
α1 – α5 (Å)

PNAS

Supporting Information for

Elucidation of a dynamic interplay between a beta-2 adrenergic receptor, its agonist and stimulatory G protein

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This PDF file includes:

Tables S1 to S5

Figures S1 to S14

Table S1. Molecular systems simulated, their simulation times (in ns or μs), average boost potentials (Δ*V*) and standard deviations (std) in kcal/mol for GaMD runs. All simulation systems were first subject to 90 ns long equilibration (eq) MD runs after which microsecond-long unbiased Anton 2 MD or enhanced sampling GaMD simulations commenced. See main text "*Materials and Methods*" section for more details.

System name	Eq MD	Anton 2 MD		GaMD
β_2AR – NE(+)	90 ns	2.5 μs		Run 1, 600 ns, Δ*V* = 14.56, std = 4.29 Run 2, 600 ns, Δ*V* = 15.14, std = 4.35 Run 3, 600 ns, Δ*V* = 14.65, std = 4.29
β_2AR – G_s – NE(+)	90 ns	Run 1	5.0 μs	Run 1, 600 ns, Δ*V* = 18.60, std = 4.78 Run 2, 600 ns, Δ*V* = 18.95, std = 4.86 Run 3, 600 ns, Δ*V* = 16.73, std = 4.64
		Run 2	5.0 μs	
		Run 3	7.5 μs	
		Run 4	5.0 μs	

Table S2. MM-PBSA interaction free energies (Δ*G*) between NE(+) and β_2AR or β_2AR – G_s (in kcal/mol) along with their standard errors of mean (SEM) computed using block averages, enthalpic (Δ*H*) and entropic (-*T*Δ*S*) components. Calculations were based on GaMD trajectories (600 ns each). See "*Materials and Methods*" section of the main text for a description of the reweighting procedure.

System	Δ*H*	-*T*Δ*S*	Δ*G* ± SEM	Reweighted Δ*H*
β_2AR-GaMD-run1	-26.00	8.93	-17.07±1.38	-25.96
β_2AR-GaMD-run2	-25.61	5.30	-20.31±0.44	-26.74
β_2AR-GaMD-run3	-26.25	7.71	-18.54±1.35	-27.69
β_2AR-G_s-GaMD-run1	-25.87	6.58	-19.29±1.46	-27.72
β_2AR-G_s-GaMD-run2	-24.22	7.15	-17.07±0.66	-22.21
β_2AR-G_s-GaMD-run3	-22.22	5.19	-17.03±0.54	-21.29

Table S3. Amino acid residue (AA) contact information between different components of G_s and β_2AR proteins from Anton 2 MD runs of β_2AR – G_s – NE(+) system. Close contacts are defined as AAs within 3 Å of each other. The stable contacts are defined as AA interacting more than 50% of the simulation time. The average percentage interaction time was calculated by averaging the interaction times of the stable AA contacts in the third column.

Contacts		**Number of stable contacts**	**Average percentage interaction time**
AA in β_2AR interact with $G_s\alpha$ α5	Run 1	26	86.7%
	Run 2	**22**	**85.0%**
	Run 3	25	89.5%
	Run 4	25	88.5%
AA in $G_s\alpha$ α5 interact with β_2AR ICL3	Run 1	4	53.0%
	Run 2	3	69.8%
	Run 3	4	67.2%
	Run 4	4	62.9%
AA in β_2AR ICL3 interact with $G_s\alpha$ α5	Run 1	3	65.7%
	Run 2	3	72.5%
	Run 3	3	66.6%
	Run 4	2	75.3%
AA in β_2AR ICL3 interact with G_s	Run 1	5	70.6%
	Run 2	9	88.1%
	Run 3	10	78.8%
	Run 4	3	72.7%

Table S4. Pearson correlation coefficients (*r)* calculated for any two MD simulation averaged geometric criteria characterized in main-text Figure 4 based on Anton 2 MD runs of β_2AR – G_s – NE(+) system: **A** – $G_s\alpha$ A161 to E299 distance, **B** – angle between two vectors of $G_s\alpha$AH and $G_s\alpha$Ras domains, **C** – $G_s\alpha$AH and $G_s\alpha$Ras interdomain distance, **D** – β_2AR NpxxY to $G_s\alpha$ α5 distance, **E** – β_2AR to $G_s\alpha$ α5 distance, **F** – $G_s\alpha$ α1 to α5 distance.

Row #					
1	A and B				
	0.61				
	A and C	B and C			
2	0.99	0.69			
	A and D	B and D	C and D		
3	-0.36	-0.71	-0.46		
	A and E	B and E	C and E	D and E	
4	0.53	0.07	0.46	0.55	
	A and F	B and F	C and F	D and F	E and F
5	-0.65	-0.63	-0.65	-0.06	-0.80

Table S5. MM-PBSA interaction free energies (Δ*G*) between β_2AR and G_s (in kcal/mol), along with their standard errors of mean (SEM) computed using block averages, enthalpic (Δ*H*) and entropic (-*T*Δ*S*) components based on GaMD trajectories (600 ns each). See the "*Materials and Methods*" section of the main text for a description of the reweighting procedure.

System	Δ*H*	-*T*Δ*S*	Δ*G*±SEM	Reweighted Δ*H*
β_2AR-G_s–GaMD-run1	-142.3	97.9	-44.4±11.9	-144.4
β_2AR-G_s–GaMD-run2	-154.2	98.8	-55.3±13.8	-135.0
β_2AR-G_s–GaMD-run3	-119.5	94.4	-25.1±18.3	-132.2

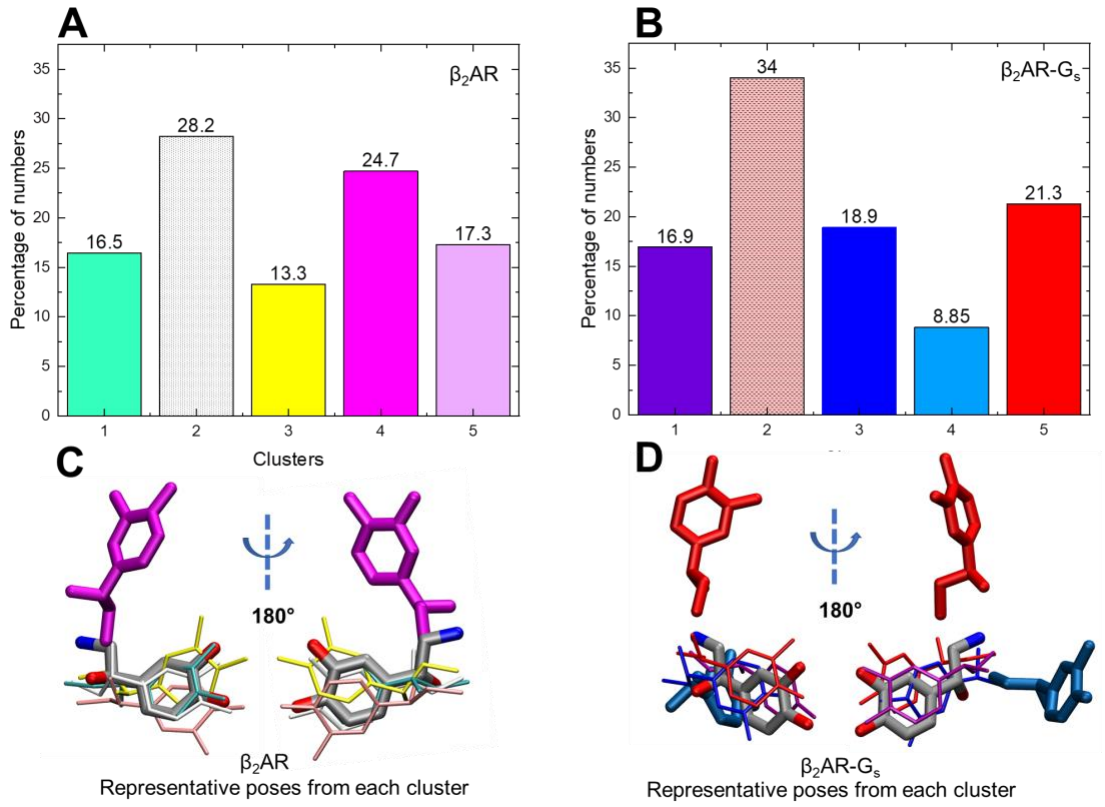

Fig. S1. (A) Clustering for binding poses of NE(+) in β_2AR Anton 2 run, percentage of pose numbers out of all poses in each cluster is shown on top of each bar. **(B**) Clustering for binding poses of NE(+) in β_2AR-G_s (Four Anton 2 runs combined), percentage of pose numbers out of all poses in each cluster is shown on top of each bar. **(C)** Representative binding poses found for β_2AR, the coloring of molecules matches the histogram in (A), the white molecule corresponding to cluster 2 in (A). **(D**) Representative binding poses for β_2AR-G_s, the coloring of molecules matches the histogram in (B), the red molecule with thin bonds corresponds to cluster 2.

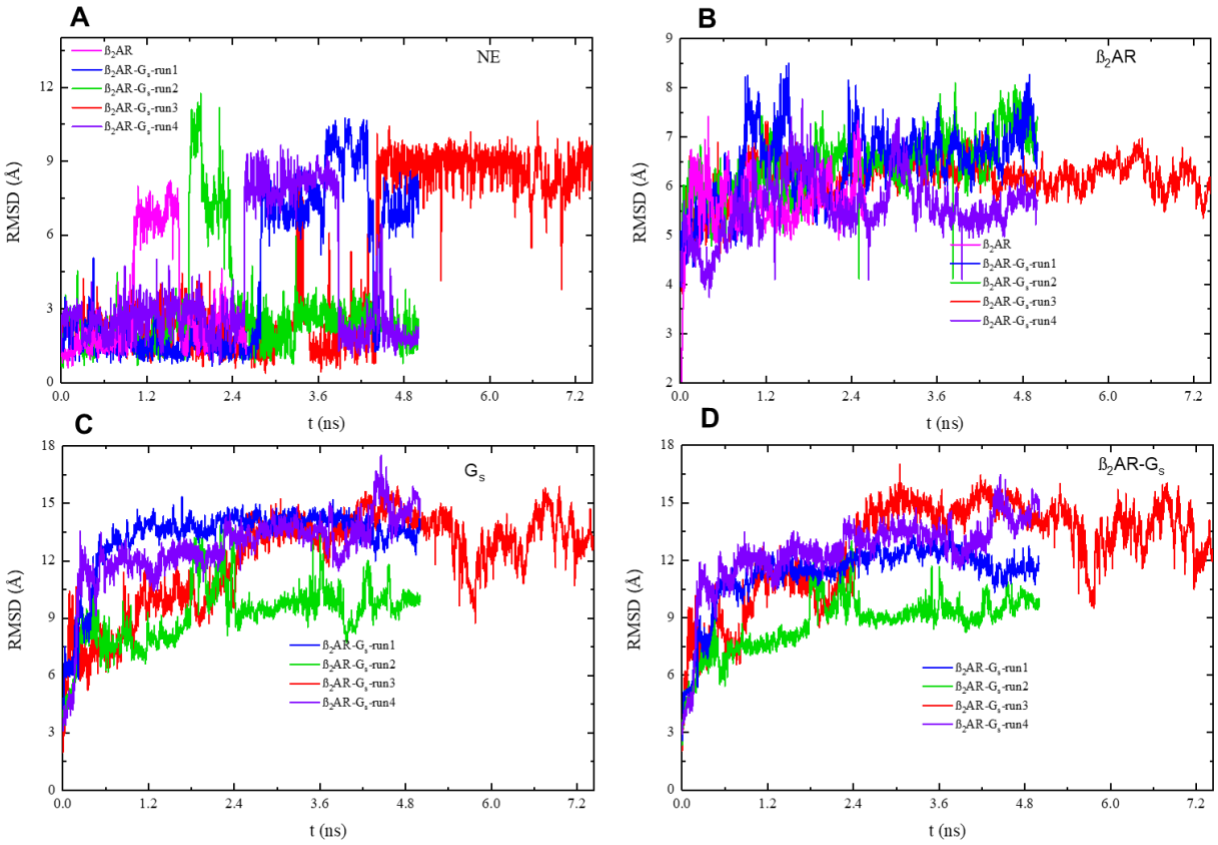

Fig. S2. RMSD time series of **(A**) NE(+) in different Anton 2 runs, trajectories were aligned to the β_2AR without loops with the first frame as reference; **(B**) β_2AR in different Anton 2 runs, trajectories were aligned to β_2AR with the first frame as reference; **(C)** G_s in different Anton 2 runs, trajectories were aligned to G_s with the first frame as reference; **(D)** β_2AR-G_s complex in different Anton 2 runs, trajectories were aligned to β_2AR-G_s with the first frame as reference.

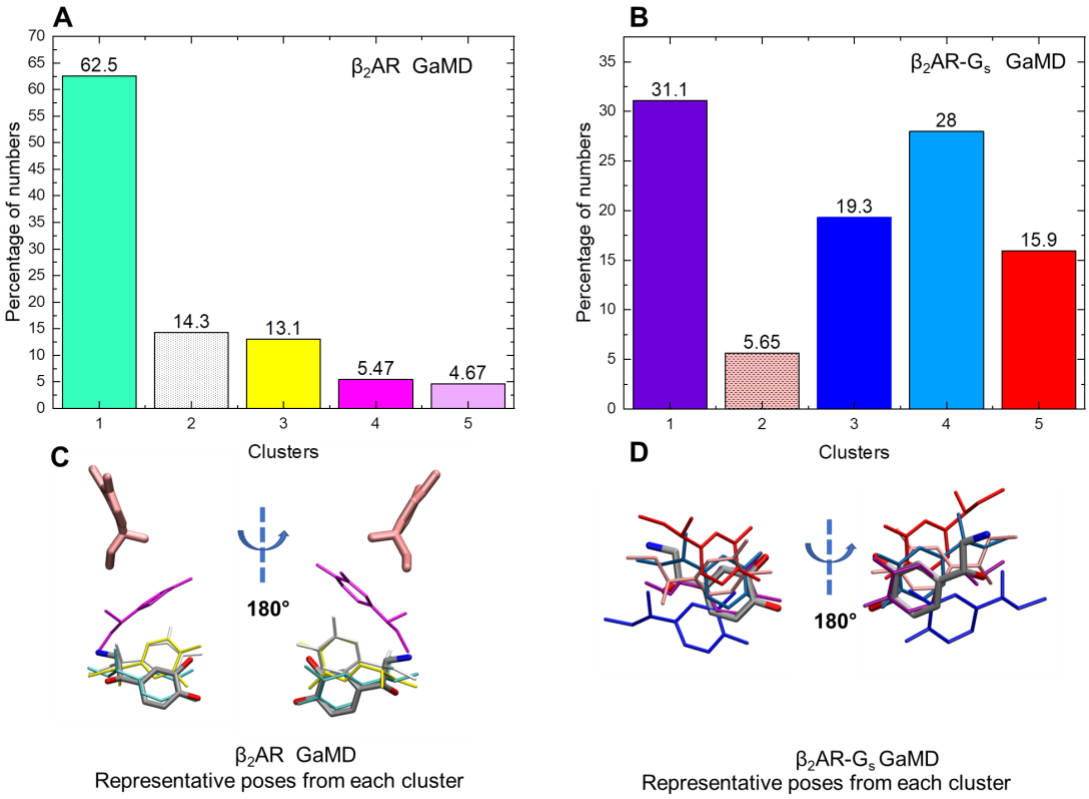

Fig. S3. (A) Clustering for binding poses of NE(+) in β_2AR GaMD runs, percentage of pose numbers out of all poses in each cluster is shown on top of each bar. **(B**) Clustering for binding poses of NE(+) in β_2AR-G_s GaMD runs, percentage of pose numbers out of all poses in each cluster is shown on top of each bar. **(C)** Representative binding poses found for β_2AR, the coloring of molecules matches the histogram in (A), the white molecule corresponds to cluster 2 in (A). **(D)** Representative binding poses for β_2AR-G_s, the coloring of molecules matches the histogram in (B), the pink molecule corresponds to cluster 2.

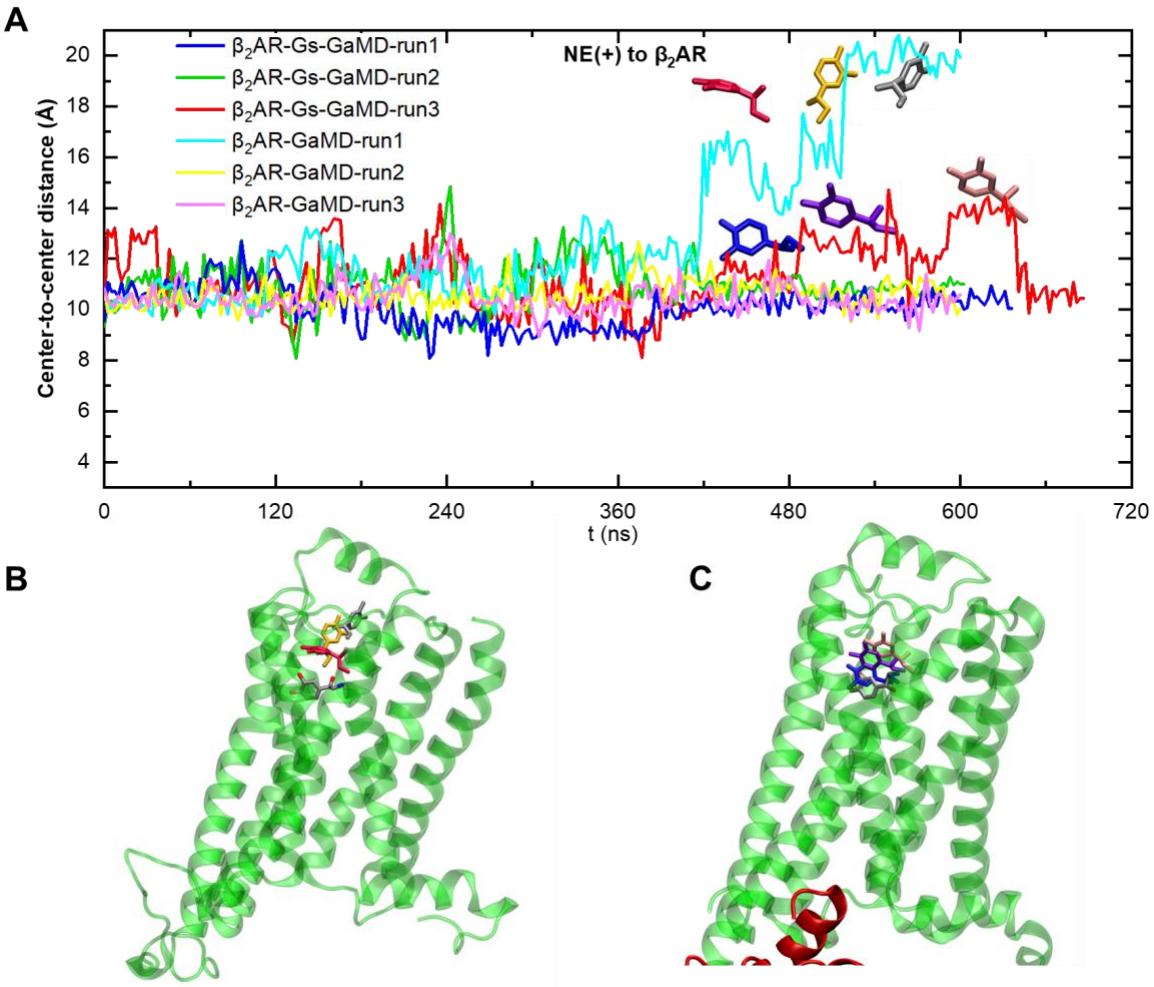

Fig. S4. **(A)** Time series of center-to-center distance between NE(+) and β_2AR geometric centers based on GaMD simulations; **(B)** Representative binding poses of NE(+) from β_2AR-GaMD-run1 (NE(+) colors correspond to those in panel A); **(C)** Representative binding poses of NE(+) from β_2AR-G_s-GaMD-run3 (NE(+) colors correspond to those in panel A).

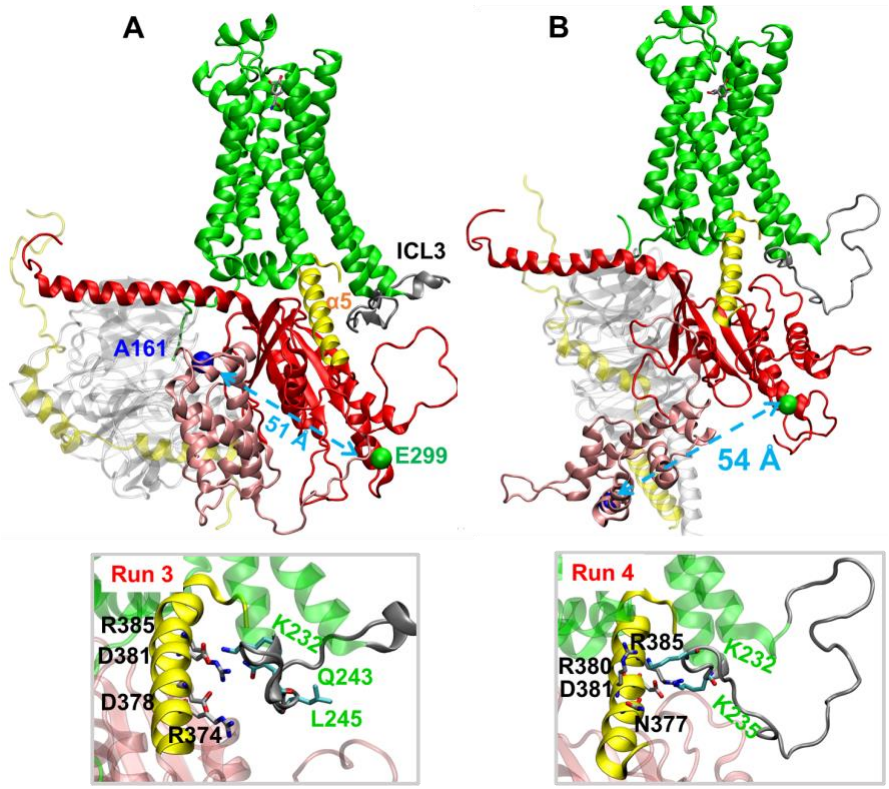

Fig. S5. All-atom Anton 2 MD simulations of the active state of the human β_2AR-G_s complex with NE(+) bound. **(A)** run 3 with the inset at the bottom. **(B)** run 4 with the inset at the bottom. Final structures from 5 μs long unbiased MD simulation runs on Anton 2. Individual protein chains / subunits are shown in the ribbon representation using different colors and labeled. $G_s\alpha$ α5 helix and β_2AR intracellular loop 3 (ICL3) are shown as yellow and dark gray. $G_s\alpha$ α-helical domain residue A161 and Ras-like domain residue E299 are shown as blue and green balls, and distances between them are shown by light-blue dashed arrows.

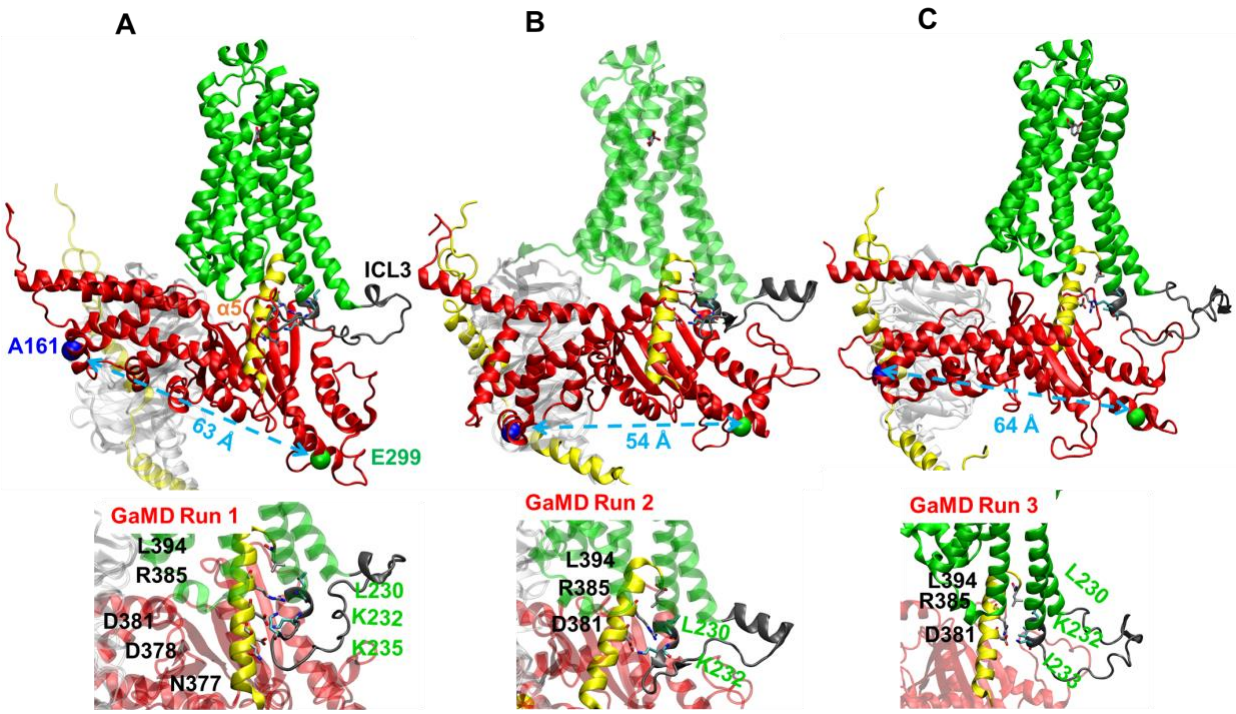

Fig. S6. All-atom GaMD simulations of the active state of the human β_2AR-G_s complex with NE(+) bound. **(A)** GaMD run 1 with the inset at the bottom. **(B)** GaMD run 2 with the inset at the bottom. **(C)** GaMD run 3 with the inset at the bottom. Final protein structures from 600-ns long GaMD simulation runs are shown. Individual protein chains / subunits are shown in the ribbon representation using different colors and labeled. $G_s\alpha$ α5 helix and β_2AR intracellular loop 3 (ICL3) are shown as yellow and dark gray. $G_s\alpha$ α-helical domain residue A161 and Ras-like domain residue E299 are shown in blue and green balls, and distances between them are shown by light-blue dashed arrows.

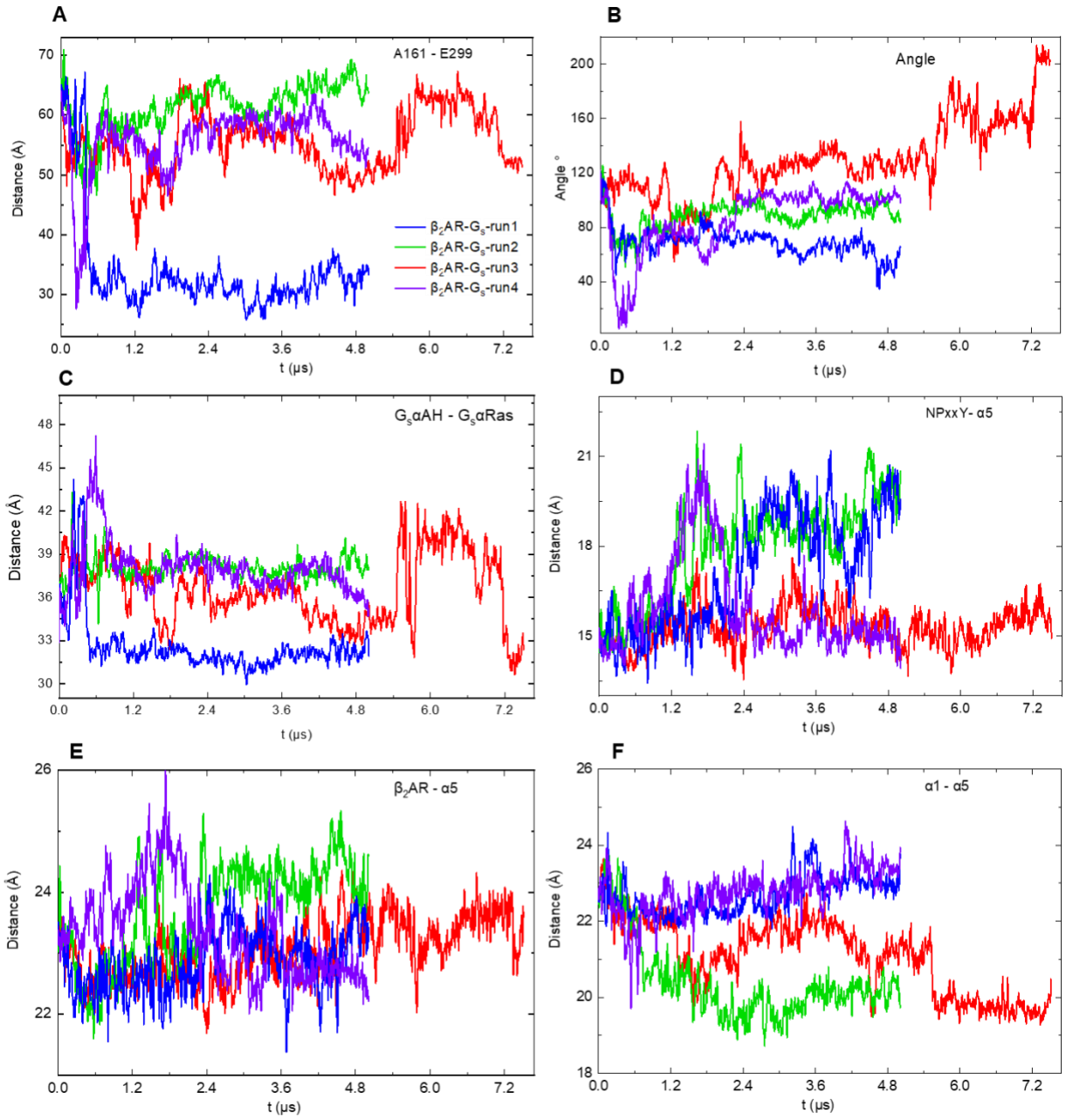

Fig. S7. Time series of geometric criteria from all-atom Anton 2 MD simulations of β_2AR-G_s-NE(+) system: **(A)** $G_s\alpha$ A161 to E299 distance indicating protein conformational changes (opening or closing); **(B)** angle between two vectors found in $G_s\alpha$AH and $G_s\alpha$Ras domains indicating the relative orientation of two domains. Vector 1 goes through $G_s\alpha$AH and A161 centers, vector 2 goes through $G_s\alpha$Ras and E299 centers (see main-text Figure 4C); **(C)** distance between $G_s\alpha$AH and $G_s\alpha$Ras domains; **(D)** distance between NPxxY (on the TM7 of β_2AR) and $G_s\alpha$ α5 helix indicating possible partial β_2AR-G_s dissociation; **(E)** distance between β_2AR and $G_s\alpha$ α5 indicating possible partial β_2AR-G_s dissociation; **(F)** $G_s\alpha$ α1 to α5 distance indicating relative movement of α1 and α5 helices. (The geometric centers were used for the distance and angle measurements.)

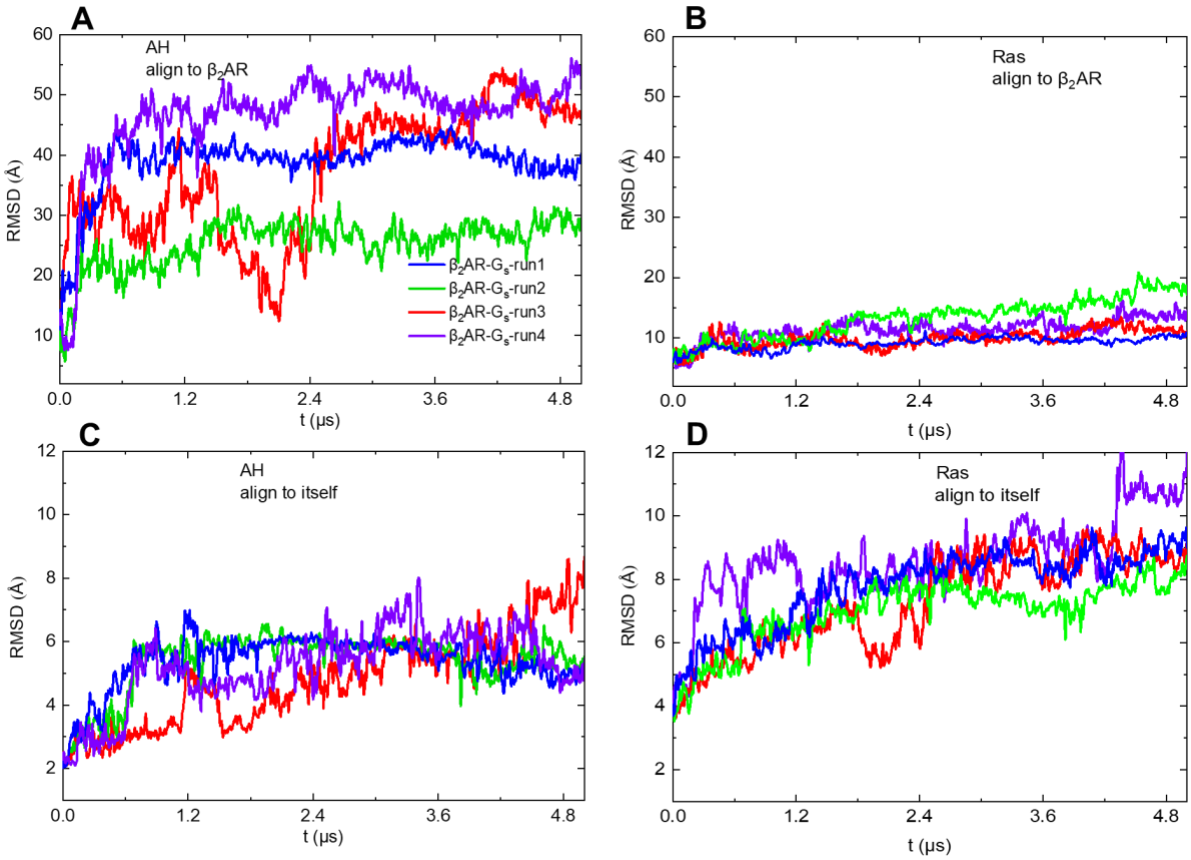

Fig. S8. RMSD time series from all-atom Anton 2 MD simulations of β_2AR-G_s-NE(+) system: **(A)** $G_s\alpha$AH domain C_α atoms aligned with respect to β_2AR; **(B)** $G_s\alpha$Ras domain C_α atoms aligned with respect to β_2AR; **(C)** $G_s\alpha$AH domain C_α atoms aligned with respect to its initial structure; **(D)** $G_s\alpha$Ras domain C_α atoms aligned with respect to its initial structure.

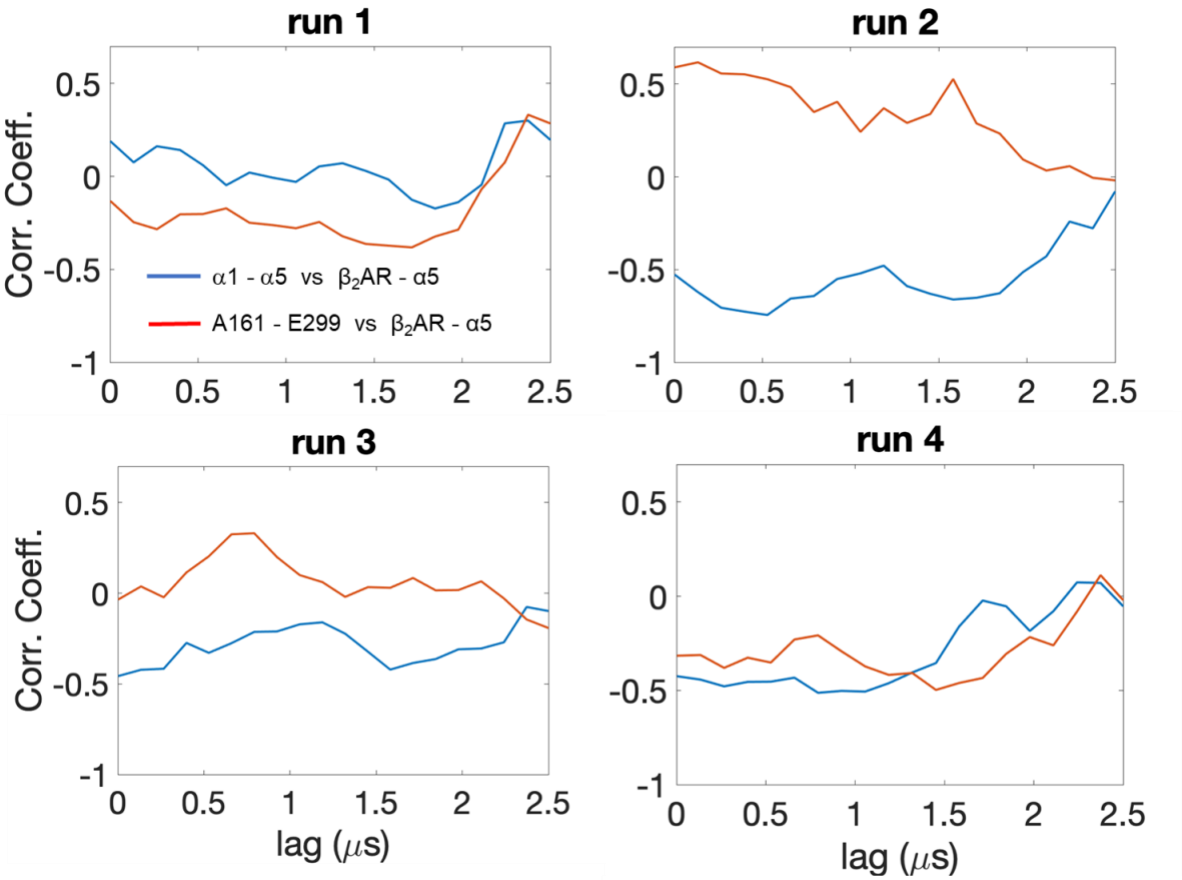

Fig. S9. Pearson correlation coefficients (Corr. Coeff) *r* as a function of lag time calculated for $G_s\alpha$ α1 - α5 distance vs. β_2AR - $G_s\alpha$ α5 distance (blue) and $G_s\alpha$ A161 - E299 distance vs. β_2AR - $G_s\alpha$ α5 distance (red). These data are based on all-atom Anton 2 MD simulations of β_2AR-G_s-NE(+) system.

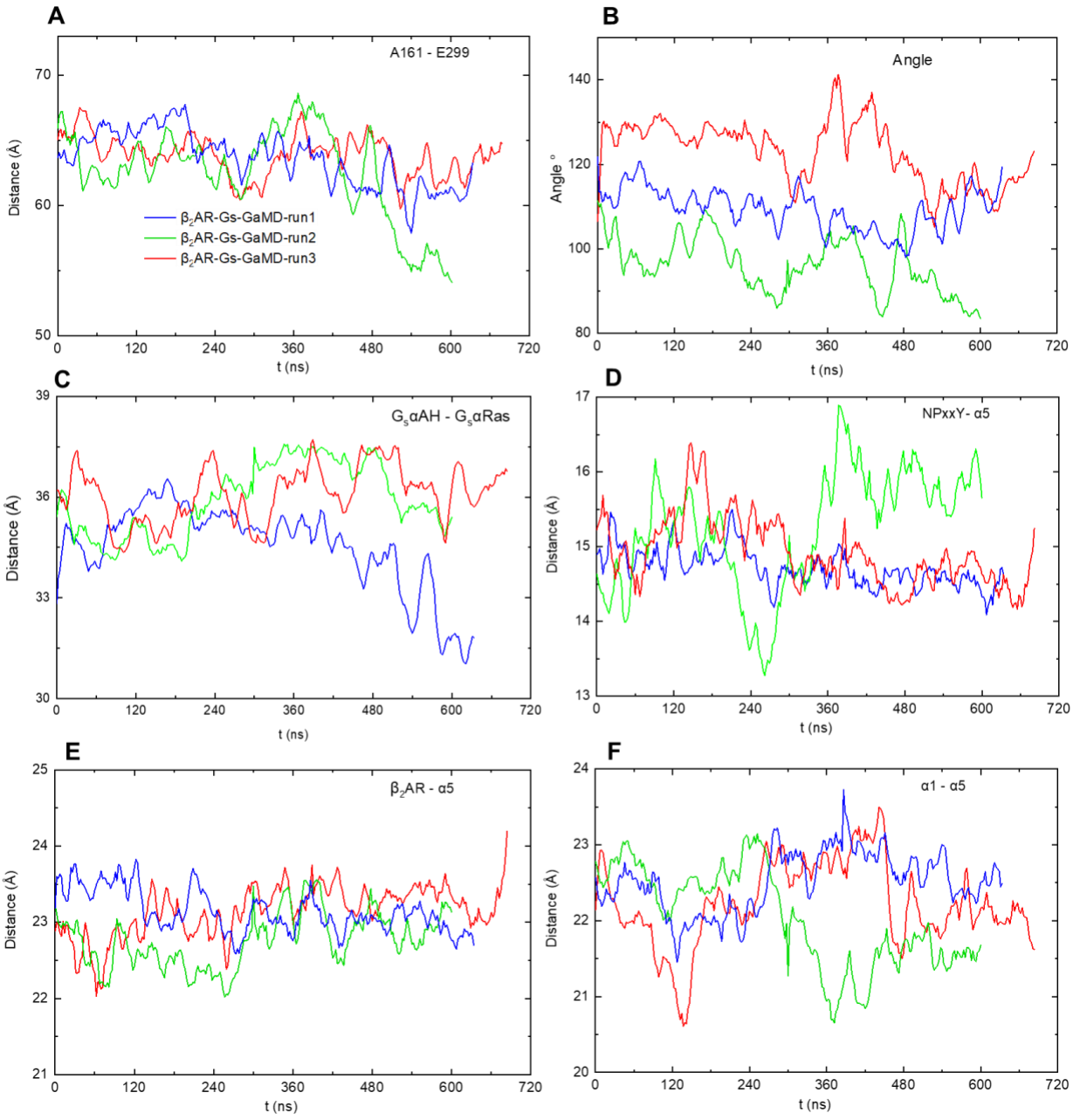

Fig. S10. Time series of geometric criteria from all-atom GaMD simulations of β_2AR-G_s-NE(+) system: **(A)** $G_s\alpha$ A161 to E299 distance indicating protein conformational changes (opening or closing); **(B)** Angle between two vectors found in $G_s\alpha$AH and $G_s\alpha$Ras domains indicating the relative orientation between the two domains. Vector 1 goes through $G_s\alpha$AH and A161 centers, vector 2 goes through $G_s\alpha$Ras and E299 centers (see main text Fig. 4C); **(C)** Distance between $G_s\alpha$AH and $G_s\alpha$Ras domains; **(D)** Distance between NPxxY (on the TM7 of β_2AR) and $G_s\alpha$ α5 indicating possible partial β_2AR-G_s dissociation; **(E)** Distance between β_2AR and $G_s\alpha$ α5 indicating possible partial β_2AR-G_s dissociation; **(F)** Distance between $G_s\alpha$ α1 and α5 indicating relative movement of helices α1 and α5. (The geometric centers were used for the distance and angle measurements.)

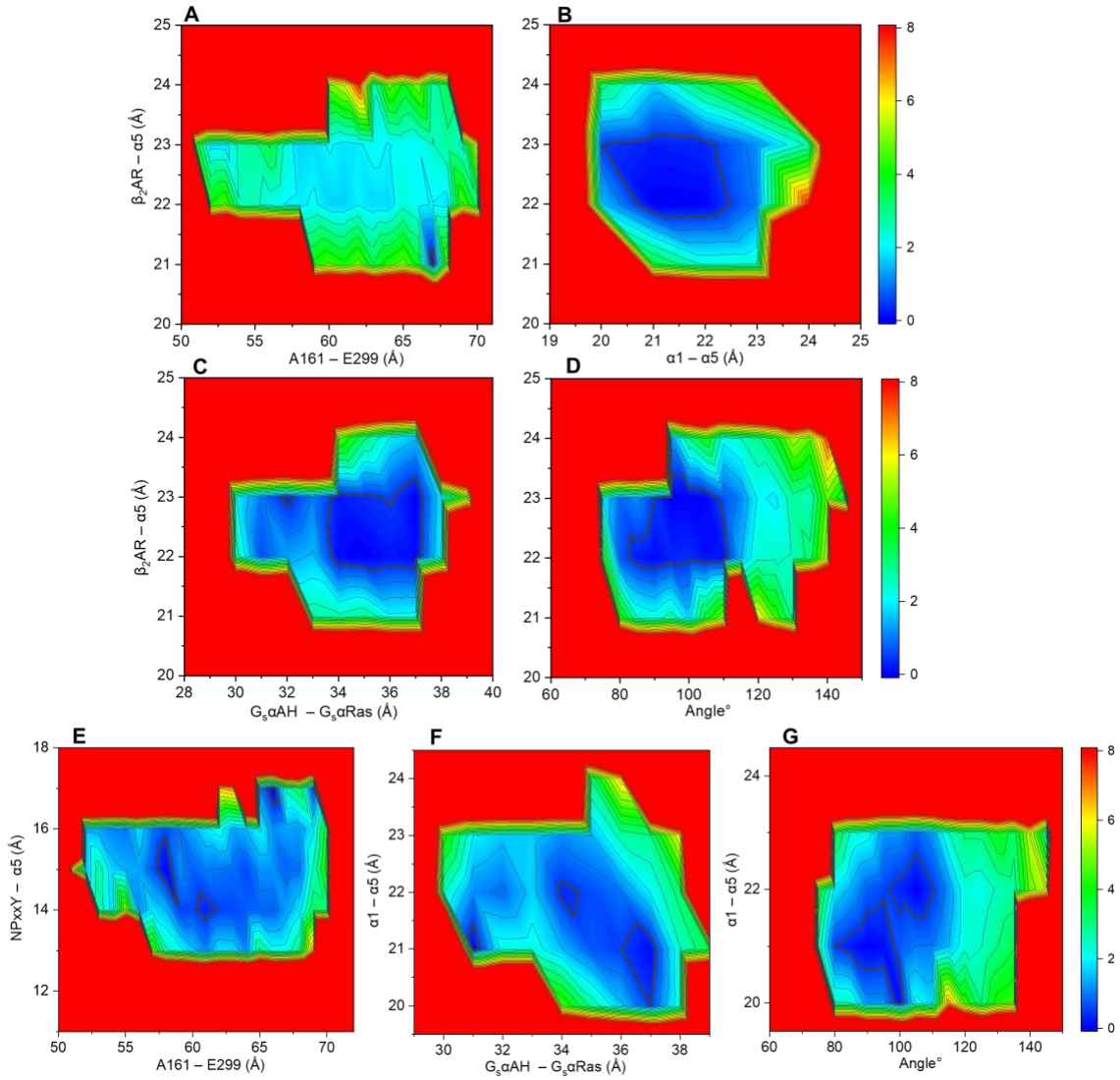

Fig. S11. 2D potential of mean force (PMF) or free energy profiles (in kcal/mol) based on $G_s\alpha$ conformation and its possible partial dissociation from β_2AR from all-atom GaMD simulations of the active state of the human β_2AR-G_s complexes with bound NE(+): **(A)** A161 to E299 distance indicating $G_s\alpha$ open or closed conformation is shown as *X*-axis. Distance between $G_s\alpha$ α5 and β_2AR indicating possible partial β_2AR-G_s dissociation is shown as *Y*-axis. **(B)** $G_s\alpha$ α1 to α5 distance is shown as *X*- axis. Distance between α5 and β_2AR indicating possible partial β_2AR-G_s dissociation is shown as *Y*-axis. **(C)** Distance between $G_s\alpha$AH and $G_s\alpha$Ras is set as *X*-axis. Distance between $G_s\alpha$ α5 and β_2AR indicating possible partial β_2AR-G_s dissociation is shown as *Y*-axis. **(D)** Angle between two vectors, one from $G_s\alpha$AH and the other from $G_s\alpha$Ras, is set as X-axis (shown in Figure 4C). Distance between α5 and β_2AR indicating possible partial β_2AR-G_s dissociation is shown as *Y*-axis. **(E)** Distance between $G_s\alpha$ A161 and E299 is shown as *X*-axis. β_2AR NPxxY to $G_s\alpha$ α5 distance is shown as *Y*-axis. **(F)** Distance between $G_s\alpha$AH and $G_s\alpha$Ras is set as *X*-axis. Distance between $G_s\alpha$ α5 and α1 is shown as *Y*-axis. **(G)** Angle between two vectors, one from $G_s\alpha$AH and the other from $G_s\alpha$Ras (shown in Figure 4C), is set as *X*-axis. Distance between G_s α5 and α1 is shown as *Y*-axis. All data are from GaMD simulations. (The geometric centers were used for the distance and angle measurements.)

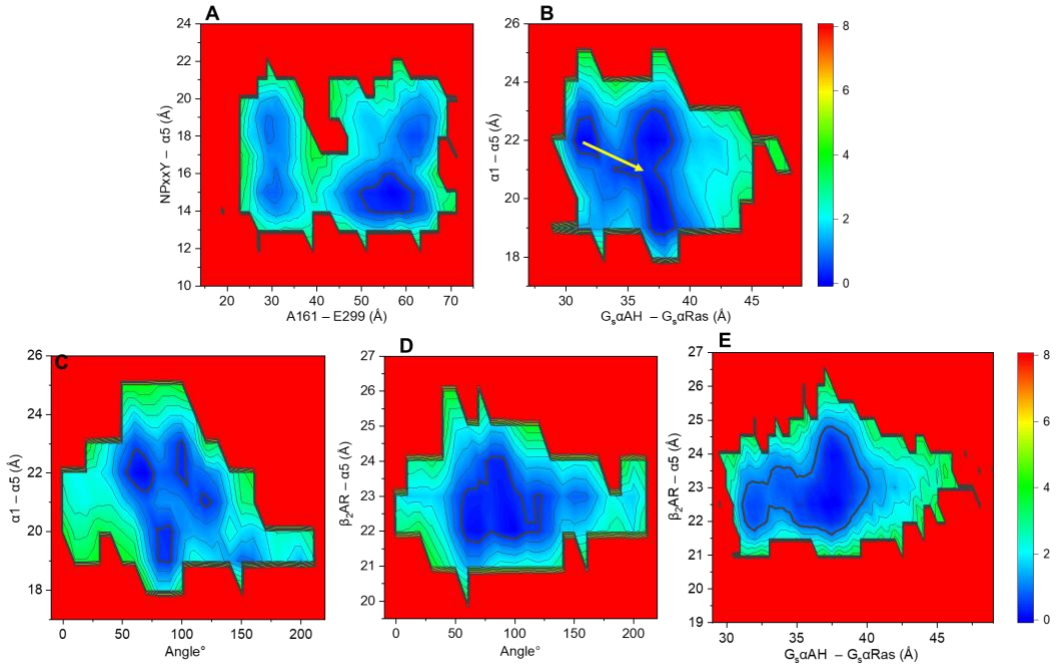

Fig. S12. 2D potential of mean force (PMF) or free energy profiles (in kcal/mol) from all-atom Anton 2 MD simulations of the active state of the human β_2AR-G_s complexes with bound NE(+) . **(A)** Distance between $G_s\alpha$ A161 and E299 is shown as *X*-axis. β_2AR NPxxY to $G_s\alpha$ α5 distance is shown as *Y*-axis. **(B)** Distance between $G_s\alpha$AH and $G_s\alpha$Ras is set as *X*-axis. Distance between $G_s\alpha$ α5 and α1 is shown as *Y*-axis. **(C)** Angle between two vectors, one from $G_s\alpha$AH and the other from $G_s\alpha$Ras, is set as *X*-axis. Distance between $G_s\alpha$ α5 helix and α1 helix is shown as *Y*-axis. **(D**) Angle between two vectors, one from $G_s\alpha$AH domain and the other from $G_s\alpha$Ras domain, is set as *X*-axis; distance between Gsα α5 and β_2AR is shown as *Y*-axis. **(E)** Distance between $G_s\alpha$AH and $G_s\alpha$Ras domains is set as *X*-axis; distance between α5 and β_2AR is shown as *Y*-axis. (The geometric centers were used for the distance and angle measurements.)

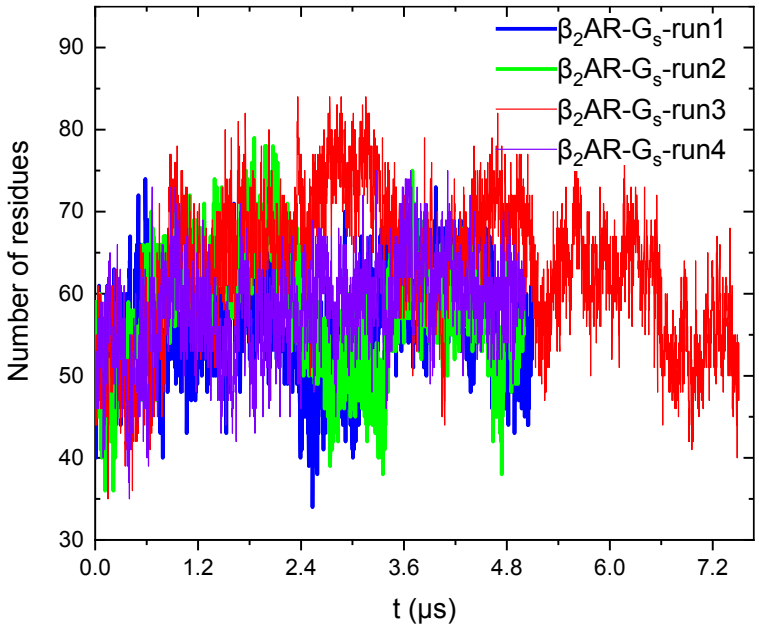

Fig. S13. Time series of the number of amino acid residues (AAs) in the binding interface between β_2AR and G_s from all-atom Anton 2 MD simulations of β_2AR-G_s-NE(+) system. The AAs in the binding interface were defined as those within 3 Å of either β_2AR or G_s.

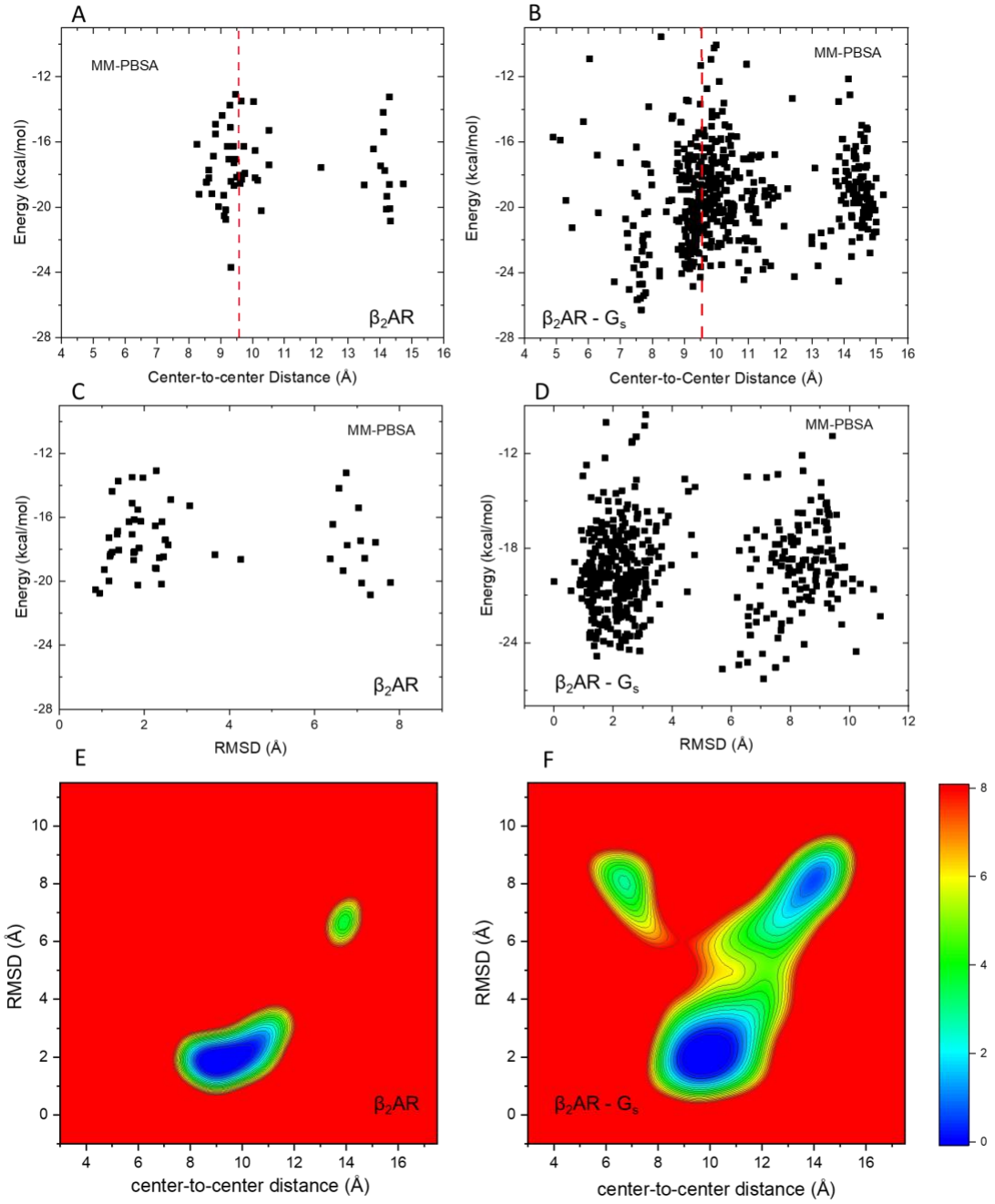

Fig. S14. **(A)** Scatter plot of MM-PBSA binding energies between NE(+) and β_2AR with their center-to-center distances in β_2AR only system. **(B)** Scatter plot of MM-PBSA binding energies between NE(+) and β_2AR with their center-to-center distances in β_2AR-G_s system. **(C)** Scatter plot of MM-PBSA binding energies between NE(+) and β_2AR with RMSDs of NE(+) in β_2AR only system. **(D)** Scatter plot of MM-PBSA binding energies between NE(+) and β_2AR with RMSDs of NE(+) in β_2AR-G_s system. **(E)** 2D PMF based on RMSD of NE(+) and center-to-center distance between NE(+) and β_2AR captured in the β_2AR only system. **(F)** 2D PMF based on RMSD of NE(+) and center-to-center distance between NE(+) and β_2AR captured in the β_2AR-G_s systems. All plots are based on Anton 2 simulations, the vertical red dashed line in panels A and B indicates the initial center-to-center distance between NE(+) and β_2AR. (The geometric centers were used for the distance measurements.)