nature portfolio

Peer Review File

Large scale investigation of GPCR molecular dynamics data uncovers allosteric sites and lateral gateways

Corresponding Author: Dr Jana Selent

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Review nat comm

In this work, Aranda-García and coworkers present a comprehensive molecular dynamics (MD)-based study of the general dynamics of G protein-coupled receptors (GPCRs), focusing mainly on lipid/GPCR interactions. This is an impressive paper that condenses an almost millisecond-scale amount of MD simulation time and tries to distill a general and common message from this dynamic picture of the GPCRome. I really appreciate the community effort to tackle the extremely difficult problem of unraveling the dynamics of GPCRs, especially because an open, reusable and simple protocol has been established.

I am convinced that this is a very nice work and has a potential impact in the GPCR simulation community and beyond. However, I think that some of the claims are overstated and some relevant points have not been discussed given the available amount of data produced by the community.

I will try to go point by point, considering the parts of the paper that I think could be improved

-On the one hand, one of the strongest points (in my opinion) of this work is the presentation of an open protocol for the simulation of GPCRs embedded in a pure POPC membrane, ready-to-use for any user with available computing power. On the other hand, the same standardization is the main limitation of this work: all the main results are related to the motions that involve the interaction with the lipid membrane, making it the central point of the paper. Not all GPCRs function in an optimal (and more importantly, biologically relevant) state when immersed in a pure POPC membrane. Cholesterol (to mention one of the main membrane constituents) and other lipids are already known to have a dramatic effect on all the fluctuations that this paper claims to have deciphered (as an example, a 2018 paper by some of the authors of this paper: 10.1002/bab.1608).
-Given the previous point, could the author be sure that the never-before-observed configurations are not basically only generated by non-physiological simulation conditions? This point may be even more important for the previously unobserved allosteric binding pocket identified in this paper.
-The authors consider the insertion of lipids (in this case POPC) inside the receptors, but never investigate the molecular determinants of this phenomenon. Is this related to charge imbalance in the sodium binding site, as recently proposed by Pirona et al. (10.1021/acs.jcim.4c00249) for an olfactory receptor, or is it related to other reasons? Is it more common in apo/ho systems? And what about prevalence in active/intermediate/inactive systems?
-In the Methods section, the authors briefly discuss how they selected and curated the structures for this work, mentioning the role of the conserved sodium at the 2x50 position. Does this ion spontaneously bind in systems where it was not present? The role of ions is known to be important for class A GPCRs (10.1016/j.tibs.2014.03.002), and perhaps they play the same role in other classes as well.
-Can the author comment on the possibility of using structural data from machine learning-based techniques in this protocol? If I understand correctly (line 381), the experimental structures do not go through a loop reconstruction protocol, forcing the modeling on an incomplete system. To extend this point, would it be possible to use de novo derived structures that are not available in apo form in this protocol?

Minor corrections:
-Table 2: It is not clear to me what a "trajectory period" is.

-Table 2: The Kelvin symbol should be without the degree sign "°".
-Table 3: Can the authors show the distribution of the distances they use to distinguish inactive/active/intermediate receptors? This might help in understanding how meaningful these thresholds are.

(Remarks on code availability)
The protocol presented in the paper is detailed in a precise way in the GitHub repository. The README.md file is complete and helps the user in (potentially) reproducing the data shown in the manuscript or adapting the workflow for new systems.

Reviewer #2

(Remarks to the Author)
Reporting the second edition of GPCRmd alone is an important contribution that warrants publication in a high-profile journal. In this case, the task is made diffcult by the previously unkown features that have already been extracted from the simulations. The current manuscript concentrates on only three topics, which is both sensible and adequate to underline the importance of the work. I do have some suggestions, though.

1. The authors have chosen to perform three 500 ns simulations, rather than one 1500 ns run. There has been much discussion about the merits of these two approaches and I would like to see some discussion and justification of the reasons for choosing three simulations. I would have preferred one long simulation because we often observe induction times before force fields move away from initial, often X-ray based, structures. These can be missed in three shorter simulations. I am not criticizing the authors for their choice, but would like to see it justified simply because others might have done it differently.

2. The authors talk about conformations being visited in their simulations. Could they clarify what they mean by this? Are all the conformations visited really free-energy minima with a finite lifetime, or are they simply points along the way? Breathing motions are not surprizing in apo-receptors, although the timescales observed are far shorter than I would have expected. Does this depend on the force field? My feeling is that AMBER is slower than the CHARMM force field used here. In this context, analysis of the trajectories in terms of the A100 activation index (DOI 10.1021/acs.jcim.9b00604) would probably be very revealing, and would probably add new information not given by the distance measures analyzed. In this respect, I would consider the existence of stable intermediate states questionable, even if many X-ray structures have been assigned intermediate conformations.

3. The section on lipid insertion is fascinating. I must admit that we have never looked for this in our simulations. I presume that the lipid tails are acting as generic hydrophobic probes and thus detecting pockets. Is this the case? In any case, the results presented here add an additional tool to the metadynamics binding/unbinding protocols, which do not detect lateral binding paths.

All in all, this contribution richly deserves publication. Its only weakness is that selecting features from such a rich data-source is a little arbitrary and really only preempts further discoveries still hidden in the data. A speculative preview of what might still be found would be amusing.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The manuscript by Selent and colleagues developed a large MD simulation dataset of GPCRs. To build the database, MD simulations from 190 experimentally solved structures, including agonists, antagonists, negative/positive allosteric modulators, etc., were performed. The conformational flexibility and lipid insertion were analyzed and made available in a user-friendly web viewer. The database should be useful for researchers in the field. Some issues to be addressed are listed below.

1) In lines 167 – 171, it was described “Importantly, when analyzing the simulations 168 of GPCRs in complex with antagonists, inverse agonists, or NAMs, the conformational 169 sampling is significantly reduced …”. However, Fig. 2b only shows the histogram for either apo or complex form. Is the result different between agonist and antagonist? It would be nice to discuss the result in terms of different types of ligands.
2) There is an inconsistency between Fig. 2e and its legend. Please correct it.
3) In Fig. 5 c and d, the shown ligands are not included in the simulations, right? It would be nice to clarify this point in the legend.
4) I guess the CGenFF is just for ligands. Is CHARMM36 used for proteins?
5) What is the rationale behind 4 fs timestep in production runs?
6) Regarding state transition rates, it would help if the “Kaplan-Meier estimator” were explained in more detail.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you for your thorough and well-considered response to my comments. I greatly appreciate the time and effort you invested in addressing each point in such detail and providing additional context to strengthen the manuscript. Your revisions have significantly enhanced the clarity and depth of the work, and I am pleased with the improvements made. I have no further concerns and believe this version is well-suited for publication.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The authors have taken my review suggestions into account in the revised manuscript. They have (for my comments and those of the other referees) retained their laudable reluctance to speculate excessively and have provided balanced comments throughout.

Two points that do not necessarily require revision are that

1. I am unconvinced that stable intermediate states exist. The current simulations cannot clear up this point, and so it is better not to speculate.

2. On the point that cholesterol has not been included in the membrane: It is very likely that cholesterol would be an even better probe for unknown binding sites that the lipid tails (examples are know experimentally). This point could be mentioned, but wouzld not wrrant further review.

I recommend publication.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors answered all of my concerns satisfactory.

(Remarks on code availability)

Dear Editor and Reviewers,

We are pleased to submit the revised version of our manuscript “**Large-scale investigation of GPCR molecular dynamics data uncovers receptor plasticity, novel allosteric sites, and lateral gateways**”.

We sincerely thank the reviewers for their time and thoughtful feedback, which have been critical for enriching and improving the manuscript.

We provide a detailed point-by-point response to the reviewers' comments below. Our responses are highlighted in **blue** for clarity, and any new additions or modifications to the main manuscript or supplementary information are marked in **yellow** for easy reference.

Sincerely,
The Authors

Reviewer #1

Remarks to the Author

In this work, Aranda-García and coworkers present a comprehensive molecular dynamics (MD)-based study of the general dynamics of G protein-coupled receptors (GPCRs), focusing mainly on lipid/GPCR interactions. This is an impressive paper that condenses an almost millisecond-scale amount of MD simulation time and tries to distill a general and common message from this dynamic picture of the GPCRome. I really appreciate the community effort to tackle the extremely difficult problem of unraveling the dynamics of GPCRs, especially because an open, reusable and simple protocol has been established.

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I will try to go point by point, considering the parts of the paper that I think could be improved

1. On the one hand, one of the strongest points (in my opinion) of this work is the presentation of an open protocol for the simulation of GPCRs embedded in a pure POPC membrane, ready-to-use for any user with available computing power. On the other hand, the same standardization is the main limitation of this work: all the main results are related to the motions that involve the interaction with the lipid membrane, making it the central point of the paper. Not all GPCRs function in an optimal (and more importantly, biologically relevant) state when immersed in a pure POPC membrane. Cholesterol (to mention one of the main membrane constituents) and other lipids are already known to have a dramatic effect on all the fluctuations that this paper claims to have deciphered (as an example, a 2018 paper by some of the authors of this paper: 10.1002/bab.1608).

We appreciate the reviewer's insightful comments regarding the use of a pure POPC membrane in our GPCR simulations. Our choice of POPC stems from the abundance of such diacyl-phosphatidylcholine lipids in animal cell membranes (40-60% of total cellular phospholipids, DOI: 10.1016/j.bbamem.2017.04.006) and its role as a well-characterized lipid in computational studies. We acknowledge that this simplified membrane composition does not capture the full complexity of biological membranes, particularly the absence of cholesterol and other lipids known to affect GPCR dynamics significantly (DOI: 10.1146/annurev-pharmtox-010919-023411; DOI: 10.1371/journal.pcbi.1007818; DOI: 10.1016/j.bpj.2020.03.008). On the other hand, our findings demonstrate that a pure POPC membrane setup effectively reproduces key lipid GPCR interactions, including lipid insertions and lateral ligand gateways across a large diversity of GPCR types (**Figure 4c**). Our future research will expand on these findings by systematically incorporating cholesterol and additional lipids to assess the effects of complex membrane environments on GPCR dynamics.

We now acknowledge these considerations in our discussion section:

*"In our study, we employ a membrane model composed entirely of POPC. This choice is motivated by the prevalence of diacyl-phosphatidylcholine lipids in animal cell membranes, where they constitute 40-60% of total cellular phospholipids[55], and by its status as a well-characterized lipid in computational research. We recognize that this simplified membrane composition does not fully capture the complexity of biological membranes, notably lacking cholesterol and other lipids that significantly influence GPCR dynamics[50,56–59]. Nonetheless, our results show that a pure POPC membrane setup effectively replicates essential lipid interactions, including lipid insertions and lateral ligand gateways, across a broad range of GPCR types (**Figure 3c**). Future research will expand on these findings by systematically incorporating cholesterol and additional lipids to assess the effects of complex membrane environments on GPCR dynamics"*

2. Given the previous point, could the author be sure that the never-before-observed configurations are not basically only generated by non-physiological simulation conditions? This point maybe even more important for the previously unobserved allosteric binding pocket identified in this paper.

We value the reviewer's concern regarding the physiological relevance of the novel configurations we observed and the newly identified allosteric binding pocket. Despite using a simplified POPC membrane model, we believe our findings remain valuable for several reasons:

2a. **Physiological relevance of receptor configurations**: We agree with the reviewer that the type of lipid and membrane composition will impact the receptor configuration as well as the overall conformational ensemble. For instance, this has been recently assessed for the $A_{2A}R$ through an experimental study of phospholipids using NMR [PMID: 36781870]. In this study, the authors find that the $A_{2A}R$ is able to couple to miniGs mediated via an induced fit mechanism in a pure POPC membrane. Such observations support that POPC alone can serve as a reasonable lipid environment for studying physiologically relevant receptor conformations.

2b. **Physiological relevance of lipid insertions**:

We would like to highlight that a pure POPC membrane successfully replicated 46 out of 49 lipid insertions identified in experimental structures, produced by lipid-like detergents and other molecules used in the crystallization of a membrane protein (see **Figure 3c,d**). This provides credibility that the selected setup is a valuable approach to capturing such events.

2c. **Physiological relevance of detected allosteric pockets (including lateral entrance gateways)**: The high recovery rate of known and experimentally validated allosteric sites (Figure 5) or lateral entrance gateways in our study provides us with additional confidence that the applied simulation conditions yield realistic results. Unfortunately, the experimental validation of all newly detected sites is out of the scope of this paper. Nevertheless, we provide a large arsenal of pockets that can be tested by the research community. In this respect, a strength of our work is a comprehensive dynamic description of detected pockets which can facilitate the search for novel allosteric modulators.

Altogether, the positive benchmarking across many different receptor types provides confidence that the outcomes reflect biologically relevant configurations. Nevertheless, we are fully aware that our setup is limited in catching effects induced by other lipid molecules (e.g. cholesterol) and needs to be transmitted to the readers. Done as follows:

"*We recognize that this simplified membrane composition does not fully capture the complexity of biological membranes, notably lacking cholesterol and other lipids that significantly influence GPCR dynamics*[50,56–59]*. Nonetheless, our results show that a pure POPC membrane setup effectively replicates essential lipid interactions, including lipid insertions and lateral ligand gateways, across a broad range of GPCR types (**Figure 3c**). Future research will expand on these findings by systematically incorporating cholesterol and additional lipids to assess the effects of complex membrane environments on GPCR dynamics*"

3. The authors consider the insertion of lipids (in this case POPC) inside the receptors, but never investigate the molecular determinants of this phenomenon. Is this related to charge imbalance in the sodium binding site, as recently proposed by Pirona et al. (10.1021/acs.jcim.4c00249) for an olfactory receptor, or is it related to other reasons? Is it more common in apo/ho systems? And what about prevalence in active/intermediate/inactive systems?

We thank for the author's suggestions to further explore the reasoning behind lipid insertions.

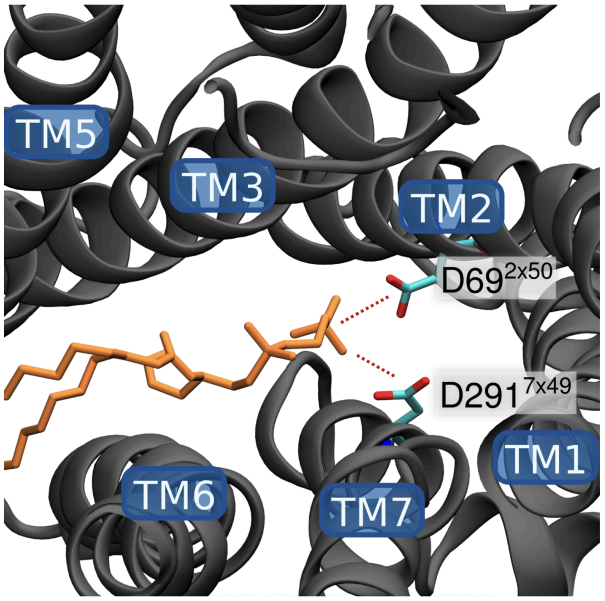
a) **Double charge imbalance in the sodium binding site: D2.50 and E3.39.** The proposed mechanism for lipid insertion in the human olfactory receptor OR51E2 mediated by a double negative charge composed of D2.50 and E3.39 (Pirona et al.) is very interesting. However, we noticed that such double negative charge is rarely conserved in non-olfactory class A receptors (except CCR1, CCR3, CCRL2) and none of those systems is in our data set.

   Nevertheless, building upon this intriguing observation, we explored whether the positively charged head group of POPC, in the absence of ligand binding, could penetrate the receptor core to interact with the negatively charged residue D2x50. Of note, our simulation data revealed one intriguing example involving the leukotriene receptor 1. Our structural analysis shows that the positively charged head of POPC was indeed attracted by two negatively charged residues, D2x50 and D7x49, mirroring a similar interaction observed in the olfactory receptor OR51E2.

   We have now included this interesting finding in our manuscript:

   "Interestingly, the majority of observed lipid insertions into the transmembrane domain of GPCRs are mediated by the hydrophobic tail of membrane lipids. However, we also observe one event in which the polar lipid head opens a lateral channel into the receptor interior, as seen in the cysteinyl leukotriene receptor 1 (PDB id: 6RZ4, GPCRmd id: 738). Thereby, the positively charged choline group of POPC penetrates the receptor between TM5 and TM6, attracted by two negatively charged residues: the allosteric ion binding site D^{2x50} *and* D^{7x49} *(**Suppl. Figure 4**).*

Notably, such a mechanism has been previously reported for the olfactory receptor OR51E2, which features a dual negatively charged site (D^{2x50}, E^{3x39}) to coordinate divalent ions[31]. In this case, the POPC molecule infiltrates between TM6 and TM7, with its choline group interacting with D^{2x50} and E^{7x49}. Overall, these results suggest that core penetration by positively charged lipid heads is facilitated by a dual unbalanced negative charge within the receptor."



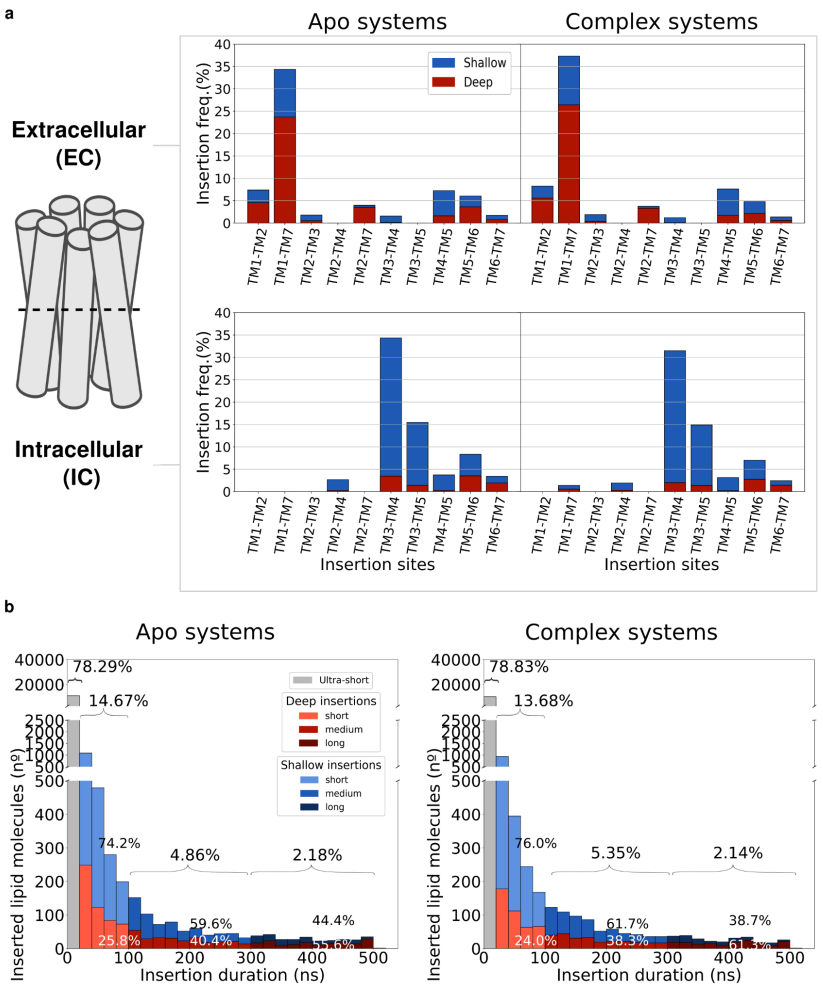
Supplementary Fig. 4 | Lipid insertion into the cysteinyl leukotriene receptor 1. *A deep and very stable lipid insertion (orange licorice) is observed in all apo form trajectories of the cysteinyl leukotriene receptor 1 (gray cartoon). The insertion occurs at TM5-$TM6_{IC}$, and is stabilised by interactions between the inserted POPC's positively charged choline head and two deprotonated aspartate residues, $D69^{2x50}$ and $D291^{7x49}$ (cyan licorice). PDB id: 6RZ4, GPCRmd id: 738.*

b) **Lipid Insertion in Apo vs. Complex Systems:** In response to the reviewer's suggestion, we have analyzed the frequency and distribution of lipid insertions in simulated apo and complex systems (***Suppl. Figure 9***). Our findings indicate that, when averaging across all simulation systems, there are no substantial differences in receptor insertion sites (***Suppl. Figure 9a***) or in the number and duration of lipid insertions (***Suppl. Figure 9b***). However, we acknowledge that some specific systems may exhibit differences, particularly when a deep lipid insertion in the apo form overlaps with the ligand binding site as already described for rhodopsin in the results section.

These observations are included in the discussion section as follows:

"*Another important question is whether the observed pattern of lipid insertions changes under certain conditions, such as ligand binding. In this respect, our data indicate that the lipid insertion profile in apo receptors closely resembles that of those in complex with ligands, both in terms of insertion sites and the number and duration of lipid insertions (**Suppl. Figure 9a-b**). However, differences may arise in specific*

cases where deep lipid insertions overlap with the ligand binding site. Such lipid insertions are likely to be reduced in the presence of a ligand, as observed in the rhodopsin receptor (see Results section).”



***Supplementary Fig. 9** | Comparative distribution of lipid insertions between apo and complex form systems. **a**, Frequency of lipid insertions indicating TM helices and GPCR side (either Extracellular (EC) or Intracellular (IC)). Frequency values represent dataset-wide averages for each pocket in apo (left) and complex (right) systems. Insertions are classified as deep (red) or shallow (blue) (see Figure 3 for further details on insertion criteria) **b**, Comparative quantification of lipid insertions in the dataset apo and complex simulations. Insertions are classified according to depth (blue vs red) and duration in simulation (color intensity).*

c) Lipid insertion in active vs inactive receptors.

The reviewer raises an interesting question regarding the effect of receptor state on lipid insertions. Unfortunately, a comprehensive analysis of this effect is not feasible with our current dataset. We lack cases where the same receptor has been simulated in both inactive and active states. Additionally, we have not gathered sufficient active-like frames from

simulations of receptors transitioning from inactive to active states to fully analyze the differences in lipid insertion patterns.

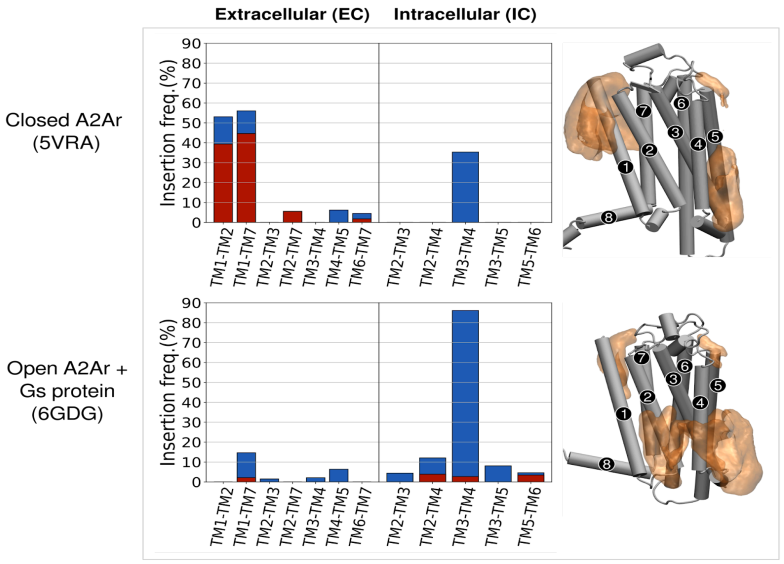
Nonetheless, we have attempted to explore this question for a specific receptor, the adenosine A2A receptor ($A_{2A}R$). To this end, we conducted an additional simulation using the structure of Gs protein-bound $A_{2A}R$ (PDB ID: 6GDG, GPCRmd ID: 1967). In this setup, the bound G protein stabilizes an open conformation, allowing lipids to thoroughly sample an active receptor state. When comparing this open $A_{2A}R$ state with the simulated inactive $A_{2A}R$ (which remained mostly inactive during MD, PDB ID: 5VRA, GPCRmd ID: 1011), we observe remarkable differences for the lipid insertion pattern (see **Suppl. Figure 10**).

An important observation is that the closed state reveals only one insertion at TM3-TM4 at the intracellular (IC) side. In contrast, in the open state, this insertion becomes more pronounced, along with several new insertions at TM2-TM3, TM2-TM4, TM3-TM5, and TM5-TM6. These new insertions can be attributed to the open IC core that results from G protein binding. Additionally, we observe fewer insertions on the extracellular (EC) side (e.g., TM1-TM7) in the active $A_{2A}R$, indicating tighter transmembrane (TM) packing in these regions. This is likely due to activation-related rearrangements of the EC side in response to the core opening at the IC receptor side.

In summary, the $A_{2A}R$ case reveals very distinct lipid insertion patterns in different receptor activation states. Further investigation will be required to explore this phenomenon in other GPCRs. We acknowledge the reviewer for proposing this possibility which we will continue to research in the future.

This interesting information has been included in the manuscript as follows:

*"It is tempting to speculate that the receptor state also impacts the lipid insertion pattern. Unfortunately, we cannot investigate this in our dataset, as we lack data for the same receptor simulated in both inactive and active states. Nevertheless, to address this question, we focused on a specific receptor, the $A_{2A}R$, by including an active-state simulation for direct comparison with the inactive-state simulation (**Suppl. Figure 10**). In this active state, we observe several new lipid insertion sites on the intracellular side. This likely results from less tightly packed transmembrane helices which are connected to the core opening upon G protein binding. Our findings indicate that lipid insertion patterns indeed differ between active and inactive receptor states, highlighting an important area for further investigation in future studies."*



Supplementary Fig. 10 | Lipid insertion for inactive and active adenosine A_{2A} receptors ($A_{2A}R$). ***a,*** *Lipid insertion in a closed (inactive) $A_{2A}R$ (PDB id: 5VRA, GPCRmd id: 1011). Left: Lipid insertion frequencies at specific receptor sites (red: deep, blue: shallow). Right: structural depiction of the $A_{2A}R$, including an occupancy map that highlights lipid insertion sites occurring with at least 5% frequency during the simulation.* ***b,*** *Lipid insertion for an open (active) $A_{2A}R$ (PDB id: 6GDG, GPCRmd id: 1967). Left: Lipid insertion frequencies at specific receptor sites (red: deep, blue: shallow). Right: structural depiction of the $A_{2A}R$ including an occupancy map that highlights lipid insertion sites occurring with at least 5% frequency during the simulation.*

4. In the Methods section, the authors briefly discuss how they selected and curated the structures for this work, mentioning the role of the conserved sodium at the 2x50 position. Does this ion spontaneously bind in systems where it was not present? The role of ions is known to be important for class A GPCRs (10.1016/j.tibs.2014.03.002), and perhaps they play the same role in other classes as well.

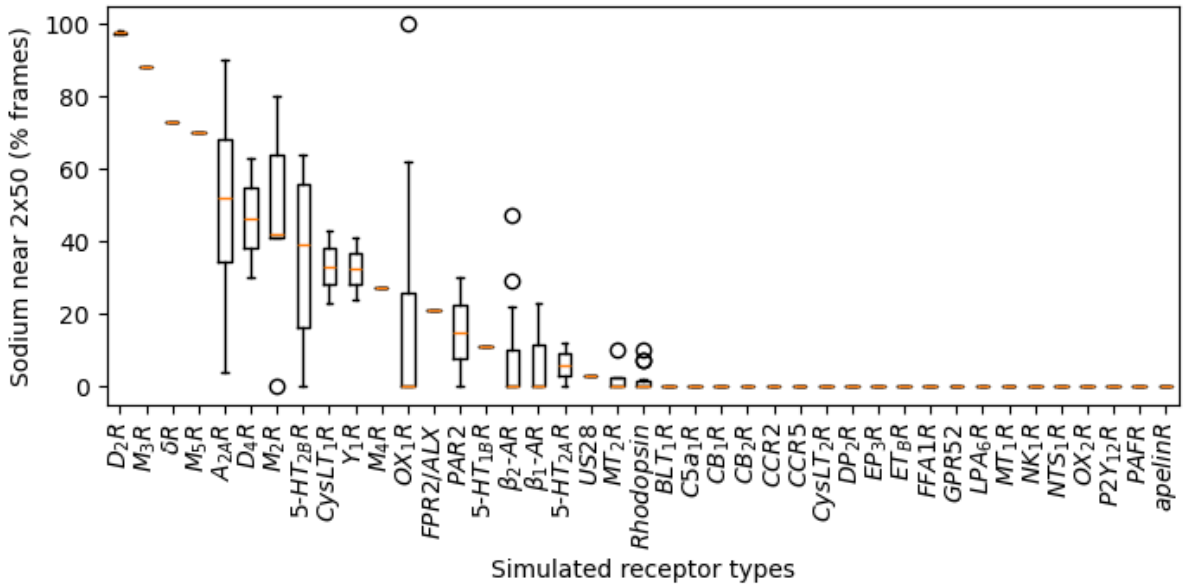
The reviewer brings attention to a highly relevant question regarding the binding of allosteric sodium ions to the D2x50 site in class A receptors. In fact, the sodium binding event has been previously studied by our consortium across a large set of different receptor types (DOI: 10.1038/s41592-020-0884-y) by simulating the apo receptor with D2x50 in its deprotonated state. In the current study, we have also applied the same setup which allows us to monitor sodium binding. We observe varying degrees of 2x50 sodium binding (**Suppl. Figure 11**) including receptors where the ion rapidly binds and stays for the majority of the simulation time (e.g.: Dopamine 2, Muscarinic 3 and delta-opioid receptors). The D2R is a prime example for sodium ion binding with an open extracellular entrance gateway and intramolecular channel towards the allosteric site D2x50 (**Suppl. Figure 12a**). This channel makes the receptor core very accessible from the extracellular space, thus greatly enabling the binding of a sodium ion to 2x50 in this receptor as confirmed in our simulations.

However, we also observe receptor types where sodium binding is not detected within 3 x 500 ns simulations. Examining a selection of these systems, we find that either (i) the extracellular entrance is largely occluded (**Suppl. Figure 12b-d**) or (ii) the extracellular side is open, yet no continuous channel is formed within the receptor interior (**Suppl. Figure 12e**) in contrast to D2R (**Suppl. Figure 12a**). The occluded extracellular entrance in receptors like Rhodopsin, FFA1R, and MT1R aligns with the fact that these receptors are regulated by hydrophobic endogenous ligands, which access the receptor core through lateral membrane-facing channels (DOI: 10.1038/s41586-019-1141-3, 10.1073/pnas.1718084115, 10.1101/2023.08.20.553924). In contrast, while CCR2 is characterized by an open extracellular entrance, the interior channel guiding the ion to D2x50 is obstructed, likely slowing the kinetics of sodium binding. This may explain why we do not observe this event within the 3 x 500 ns simulations.

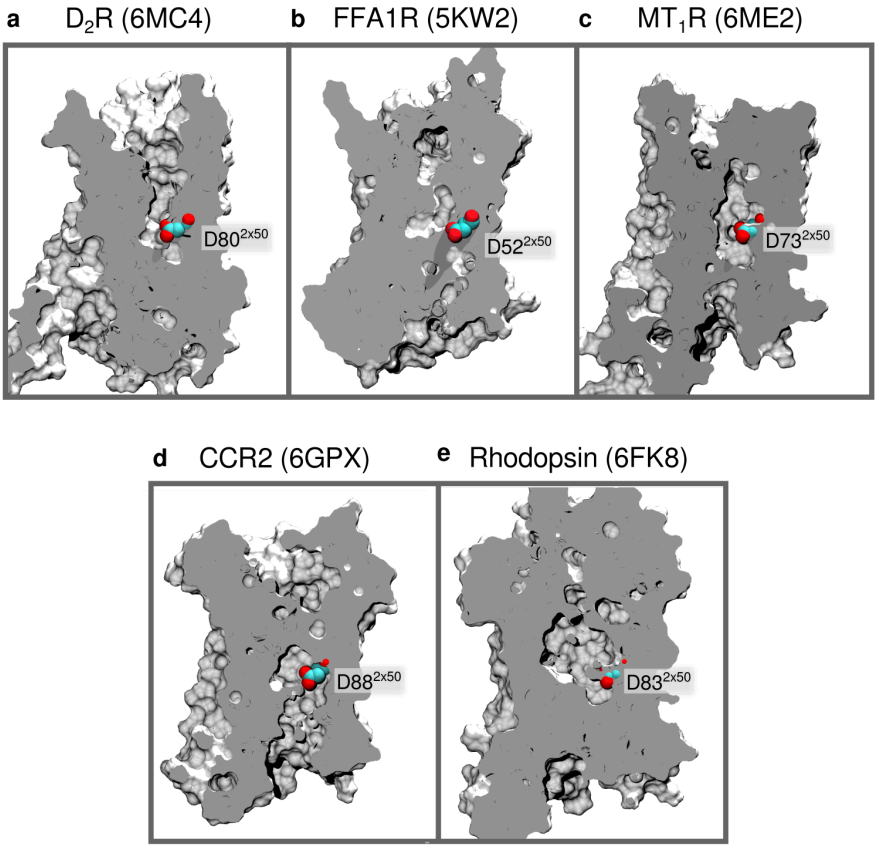
In summary, the degree of sodium-2x50 binding in GPCRs varies significantly depending on the structural properties of the extracellular entrance and the intramolecular intramolecular channel towards the allosteric site D2x50.

We have included this information into our manuscript.

*"In addition to allosteric sites revealed by membrane lipids, we also observe sodium ions allosterically binding in the transmembrane bundle of class A GPCRs - a well-recognized phenomenon[46]. Our data unveil a diverse range of sodium binding profiles, including rapid, delayed, or absent binding at D^{2x50} (Suppl. Figure 11). Consistent with previous findings[14], the structural characteristics of the extracellular entrance and the intramolecular channel guiding sodium to the allosteric site D^{2x50} appear to modulate the degree of ion binding (**Suppl. Figure 12**).*



***Supplementary Fig. 11 | Percentage of sodium binding at the 2x50 residue in apo systems**. Systems are grouped according to their receptor type (x-axis). In the y-axis is represented the percentage of simulated frames, in each individual system, where a sodium ion is found near 5 Å of its 2x50 residue. Only class A systems are included.*



Supplementary Fig. 12 | Cross section for 5 different GPCR types, *showing the accessibility of the allosteric sodium binding site 2x50 residues (van der Waals).* ***a****, The Dopamine D2 (D_2R) receptor with the highest rate of ion binding (see* ***Suppl. Figure 11****) shows an open entrance and channel towards D^{2x50}. In contrast,* ***b,*** *FFA1R,* ***c,*** *MT1R,* ***d,*** *CCR2 and* ***e,*** *rhodopsin with low or null ion sodium-2x50 binding rates reveal a closed entrance and/or disrupted channel towards D^{2x50}.*

5. Can the author comment on the possibility of using structural data from machine learning-based techniques in this protocol? If I understand correctly (line 381), the experimental structures do not go through a loop reconstruction protocol, forcing the modeling on an incomplete system. To extend this point, would it be possible to use de novo derived structures that are not available in apo form in this protocol?

a) **Loop reconstruction**. The reviewers' question about loop reconstruction made us realize we have not presented this information properly in the corresponding "Structure Selection and Curation" methods section. The structures simulated were obtained from GPCRdb, and this web server applies an automatic refinement protocol designed for GPCR structures to reconstruct and remodel missing or incomplete loops. These refined structures were then curated manually by GPCRmd community experts, who revised the adequacy of GPCRdb's automated reconstructions and modelings, and applied any new ones as deemed necessary. We have added a more explicit description of the curator's roles in this field to ensure the understandability of our protocol.

b) **De novo derived structures.** The GPCRmd simulation protocol is highly flexible to accommodate any type of GPCR structure in different states including de-novo structures. In fact, we are currently working on a project to simulate all available GPCR structures from the AlphaFold database using this standardized simulation protocol.

Minor corrections:

1. Table 2: It is not clear to me what a "trajectory period" is.

There was a small mistake referring to the units used in the “trajectory period” parameter, and we thank the reviewer for making us aware of it. The trajectory period is an internal parameter of ACEMD3, our simulation software, regarding the interval at which frames are saved into the output trajectory (specified in simulation steps). We have added this description below Table 2 for further clarification.

2. Table 2: The Kelvin symbol should be without the degree sign "°".

We have corrected this mistake.

3. Table 3: Can the authors show the distribution of the distances they use to distinguish inactive/active/intermediate receptors? This might help in understanding how meaningful these thresholds are.

We thank the reviewer for this comment. In fact, distribution plots for Table 3 can be found in Figure 2a (generated from source data 2). We have now included a small reference to figure 2a and source data 2 in the Table 3 caption.

Reviewer #2

Remarks to the Author

Reporting the second edition of GPCRmd alone is an important contribution that warrants publication in a high-profile journal. In this case, the task is made diffcult by the previously unkown features that have already been extracted from the simulations. The current manuscript concentrates on only three topics, which is both sensible and adequate to underline the importance of the work. I do have some suggestions, though.

1. The authors have chosen to perform three 500 ns simulations, rather than one 1500 ns run. There has been much discussion about the merits of these two approaches and I would like to see some discussion and justification of the reasons for choosing three simulations. I would have preferred one long simulation because we often observe induction times before force fields move away from initial, often X-ray based, structures. These can be missed in three shorter simulations. I am not criticizing the authors for their choice, but would like to see it justified simply because others might have done it differently.

We thank the reviewer for raising this interesting point. Indeed when considering MD protocols one has to balance sampling diversity against continuous trajectory length.

Running three independent 500 ns simulations provides the advantage of sampling different regions of conformational space. Each simulation can explore distinct pathways, potentially capturing different conformations or interactions that may be missed in a single long run. This increases the statistical robustness of the results by allowing comparison between independent trajectories, ensuring that observed results are not dependent on the peculiarities of one specific trajectory. Previous studies by Knapp et al. (DOI: 10.1021/acs.jctc.8b00391) prove that results derived from single-trajectory simulations are often hard to reproduce. Furthermore, in a single, continuous 1500 ns simulation, there is a risk that the system could get "trapped" in a local energy minimum, limiting the exploration of the full conformational space. By restarting the simulations (i.e., running multiple independent simulations), the system can escape from such minima and explore alternative conformations, enhancing the likelihood of capturing rare events or transitions between states. We have added the following explanation in the text this decision:

"Running three independent replicates provides the advantage of sampling different regions of conformational space and enhances results replicability[68]*"*

As a final consideration, the breathing motions observed at the intracellular side of the receptor over 3 x 500 ns (Figure 2) would suggest that the induction times required for force fields to deviate from the initial structure are generally overcome. Nevertheless, we hope that future improvements in hardware and software will enable extended simulation times in our dataset.

2. The authors talk about conformations being visited in their simulations. Could they clarify what they mean by this? Are all the conformations visited really free-energy minima with a finite lifetime, or are they simply points along the way? Breathing motions are not surprising in apo-receptors, although the timescales observed are far shorter than I would have expected. Does this depend on the force field? My feeling

is that AMBER is slower than the CHARMM force field used here. In this context, analysis of the trajectories in terms of the A100 activation index (DOI 10.1021/acs.jcim.9b00604) would probably be very revealing, and would probably add new information not given by the distance measures analyzed. In this respect, I would consider the existence of stable intermediate states questionable, even if many X-ray structures have been assigned intermediate conformations.

a) **Sampled Conformational States:** We appreciate the reviewer's insightful questions regarding our conformational analysis. By "conformations being visited," we refer to states observed during the simulations, which may not necessarily correspond to stable free-energy minima. These states could indeed be transient along the conformational landscape, as our classification of conformations is based on structural metrics and does not necessarily indicate thermodynamic stability.

We have included this important consideration into the manuscript.

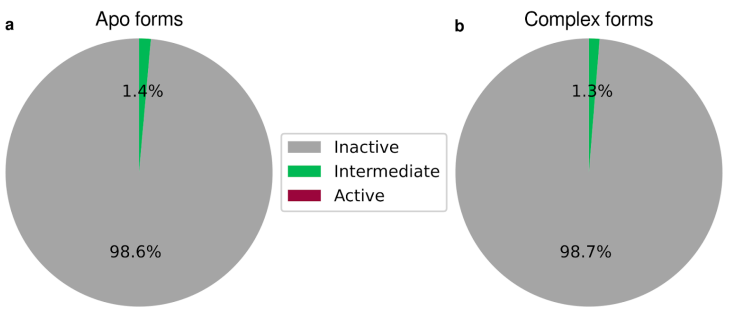
"*Of note, the described conformational states may not always correspond to low-energy conformations and could represent transient intermediates, as our classification relies on structural metrics that do not reflect thermodynamic stability.*"

b) **Timescale of breathing motions**. The observed breathing motions in apo receptors at shorter timescales than expected could be influenced by our choice of the CHARMM force field. We acknowledge that force field selection can impact the speed of conformational changes, and AMBER might indeed show slower transitions. This is an important point for future comparative studies.

c) **A100 activation index**. We thank the reviewer for his suggestion on using the A100 activation index. In fact, we had applied this method at the beginning. However, its results showed that during a 3x500ns simulation receptors rarely reach the full active state (**Suppl. Figure 8**). Therefore, we refer to our findings as "breathing motions of the receptor core" and not "receptor in(activation)".

We have included this information as follows:

*"Not surprisingly, open receptor cores typically do not reach fully active states based on the A100 index (**Suppl. Figure 8**) - an index that integrates various metrics of receptor activation[36]. Achieving these states likely requires a longer timescale and/or agonist binding or G protein coupling."*



Supplementary Fig. 8 | Classification of inactive, intermediate and active states for class A receptors *according to the Activation Index A100.* ***a,*** *Conformational classification in apo systems (i.e. ligand was removed from original PDB, 100 PDBs) and* ***b****, complex systems co-solved with an antagonist, inverse agonist or NAM ligand; and identified as inactive by GPCRdb (100 PDBs). The overall percentage for active, intermediate and inactive states was obtained by computing the activation state for each frame (i.e., each 0.2 ns) in the 3 x 500 ns simulation data across all 100 simulation systems. This data indicates that GPCRs sample at times intermediate states but no active states based on the Activation Index A100.*

3. The section on lipid insertion is fascinating. I must admit that we have never looked for this in our simulations. I presume that the lipid tails are acting as generic hydrophobic probes and thus detecting pockets. Is this the case? In any case, the results presented here add an additional tool to the metadynamics binding/unbinding protocols, which do not detect lateral binding paths.

We appreciate the reviewer's interest in our findings on lipid insertions. Indeed, in our simulations, lipid tails effectively act as generic hydrophobic probes, uncovering potential binding pockets and lateral pathways in GPCRs (interesting examples are shown in Figures 4 and 5 and Supplementary Figures 3 and 4). We believe that this approach can complement existing computational methods by providing a straightforward and effective means of identifying potential binding sites and pathways, particularly for lipophilic molecules. Such insights could be especially valuable in early-stage drug discovery efforts targeting GPCRs, shedding light on potential binding sites and mechanisms of action for lipophilic compounds.

4. All in all, this contribution richly deserves publication. Its only weakness is that selecting features from such a rich data-source is a little arbitrary and really only preempts further discoveries still hidden in the data. A speculative preview of what might still be found would be amusing.

We thank the reviewer for the suggestion. We have included this idea as follows:

"*As a final remark, our study focuses on a selection of highly relevant GPCR features, providing valuable insights into GPCR function. However, the dataset we present extends far beyond these findings, offering significant potential for uncovering yet-to-be-explored features. These could include the discovery of cryptic (sub)pockets within the receptor interior or a systematic GPCR-wide analysis of intramolecular allosteric communication to advance our understanding of GPCR (sub)type-, or class-specific functions among others.*"

Reviewer #3

Remarks to the Author

The manuscript by Selent and colleagues developed a large MD simulation dataset of GPCRs. To build the database, MD simulations from 190 experimentally solved structures, including agonists, antagonists, negative/positive allosteric modulators, etc., were performed. The conformational flexibility and lipid insertion were analyzed and made available in a user-friendly web viewer. The database should be useful for researchers in the field. Some issues to be addressed are listed below.

1. In lines 167 – 171, it was described "Importantly, when analyzing the simulations 168 of GPCRs in complex with antagonists, inverse agonists, or NAMs, the conformational 169 sampling is significantly reduced …". However, Fig. 2b only shows the histogram for either apo or complex form. Is the result different between agonist and antagonist? It would be nice to discuss the result in terms of different types of ligands.

We appreciate the reviewer's interest in our findings and their valuable suggestion. The idea of comparing the breathing motions of agonist- and antagonist-bound systems was indeed explored and yielded intriguing results. However, one limitation is the imbalance in our dataset (Figure S12): agonist or allosteric agonist complexes (24 PDBs) are underrepresented compared to antagonist, inverse agonist, or NAM complexes (106 PDBs). Despite this limitation, the data suggest that agonist or allosteric agonist complexes more frequently sample intermediate states compared to antagonist, inverse agonist, or NAM complexes. This observation aligns with the notion that agonists or allosteric agonists shift the receptor state from a closed, inactive-like conformation toward more intermediate states.

We have added a mention of this in the result and discussion, highlighting that it is only a preliminary indication and that future studies with a more balanced dataset will be necessary to validate this observation.

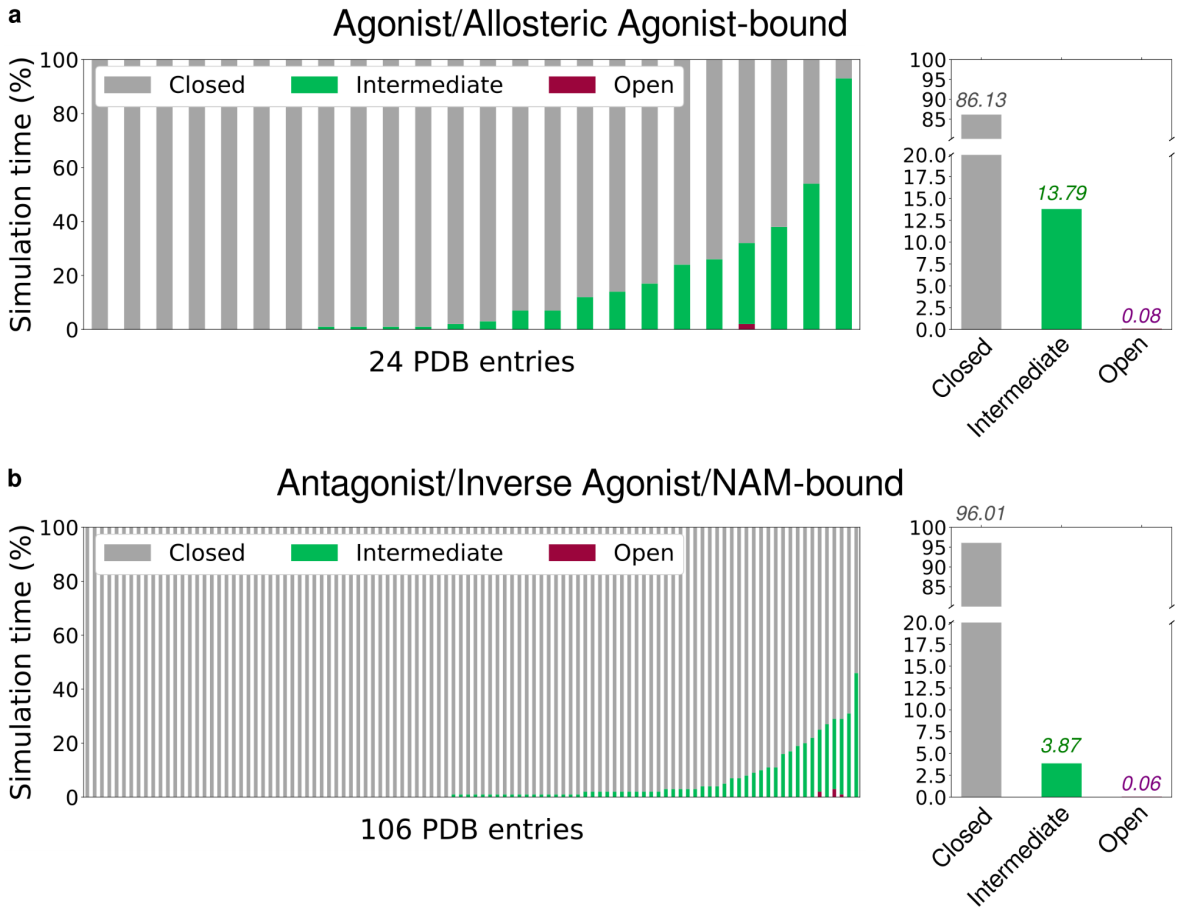
Result section

"*We also investigated whether breathing motions differ when GPCRs are bound to agonists or allosteric agonists (26 PDBs) compared to antagonists, inverse agonists, or NAMs (106 PDBs) (**Suppl. Figure 1**). Although this analysis provides only a preliminary perspective due to the underrepresentation of agonist or allosteric agonist complexes, the data suggest that these complexes more frequently sample intermediate states compared to antagonist, inverse agonist, or NAM complexes. This observation aligns with the notion that agonists or allosteric agonists shift the receptor state from a closed, inactive-like conformation toward more intermediate states within 3 × 500 ns simulations. We further speculate that simulations over longer timescales could potentially reveal increased sampling of open (active-like) states for agonists or allosteric agonists.*"

Discussion Section

"*Furthermore, our data provide preliminary evidence that the binding of agonists or allosteric agonists destabilizes the closed (inactive-like) state, promoting a shift toward intermediate*

*states (**Suppl. Figure 1**). However, future studies with a more balanced dataset will be necessary to confirm this observation."*



Supplementary Fig. 1 | Receptor core flexibility of agonists or allosteric agonists (24 PDBs) versus antagonists, inverse agonists, or NAMs (106 PDBs). Systems started from initially closed (inactive) states belonging to classes A or B1. **a,** Percentage of simulation time spent in each state by initially inactive systems simulated in complex to an agonist or allosteric agonist ligand. Percentages are represented for each system individually (left) or cumulatively for the whole subset (right). **b,** Same concept, applied to systems bound to antagonists, inverse agonists or NAMs.

2. There is an inconsistency between Fig. 2e and its legend. Please correct it.

We thank the reviewer for noticing this mistake. The correct version of this figure, that matches the legend's content, has been added.

3. In Fig. 5 c and d, the shown ligands are not included in the simulations, right? It would be nice to clarify this point in the legend.

We thank the reviewer for this comment.

Indeed, ligands are not part of the displayed system (Figure 5c and d), which is an apo receptor. To generate this figure, the ligand of the corresponding complexed system was superimposed onto the apo structure. This approach is intended to facilitate the identification and localization of the allosteric pocket within the apo structure. The figure legend has been updated to clarify this.

4. I guess the CGenFF is just for ligands. Is CHARMM36 used for proteins?

We apologize for this mistake. The forcefield used is indeed CHARMM36, in which CGenFF is included. The corresponding table has been changed to describe it properly.

5. What is the rationale behind 4 fs timestep in production runs?

ACEMD, the MD simulation software used in this study (DOI: 10.1021/ct9000685), incorporates a hydrogen-mass repartitioning (HMR) scheme. This method redistributes mass from bonded heavy atoms to hydrogen atoms, increasing the mass of hydrogens and thereby reducing the fastest vibrational frequencies of hydrogen-involved bonds. As a result, a larger timestep (e.g., 4 fs) can be employed without compromising simulation stability. In contrast, without HMR, the timestep is typically limited to 2 fs due to the rapid motions of hydrogen atoms. We adopted this algorithm to enhance the efficiency of our simulation protocol (DOI: 10.1038/s41592-020-0928-3).

6. Regarding state transition rates, it would help if the “Kaplan-Meier estimator” were explained in more detail.

We thank the reviewer for this suggestion in this regard. We have added the following explanations in the methods section to better clarify this concept:

“*The Kaplan-Meier formula calculates the still-in-closed-state “survival” probability at time t by multiplying the probabilities of survival at each time point when a transition occurs, calculating these individual probabilities as (number of trajectories surviving - number of events) divided by (number of trajectories at risk) at each time point*”

Response to Reviewer #2

Reviewer #2 (Remarks to the Author):

The authors have taken my review suggestions into account in the revised manuscript. They have (for my comments and those of the other referees) retained their laudable reluctance to speculate excessively and have provided balanced comments throughout.

We sincerely thank Reviewer #2 for recognizing the improvements made to our manuscript and for the recommendation for publication.

Two points that do not necessarily require revision are that

1. I am unconvinced that stable intermediate states exist. The current simulations cannot clear up this point, and so it is better not to speculate.

We fully agree with the reviewer's point regarding the existence of stable intermediate states. This may not have been clearly communicated in our previous response.

In our study, we employ distance metrics to classify receptor conformations as ‘closed’, ‘intermediate’, or ‘open’. It is important to note that these metrics and classifications do not indicate thermodynamic stability. Therefore, we refrain from making conclusions about the thermodynamic stability of the observed states. This clarification has been included in our previous revision process as follows:

“*Of note, the described conformational states may not always correspond to low-energy conformations and could represent transient intermediates, as our classification relies on structural metrics that do not reflect thermodynamic stability.*”

2. On the point that cholesterol has not been included in the membrane: It is very likely that cholesterol would be an even better probe for unknown binding sites that the lipid tails (examples are know experimentally). This point could be mentioned, but would not warrant further review.

We fully agree with the reviewer. Cholesterol (as well as other membrane components) would serve as valuable probes which will be explored in future studies.