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UP-AND-COMING SCIENTIST

Single-Molecule Insights into GPCR Conformational Landscapes

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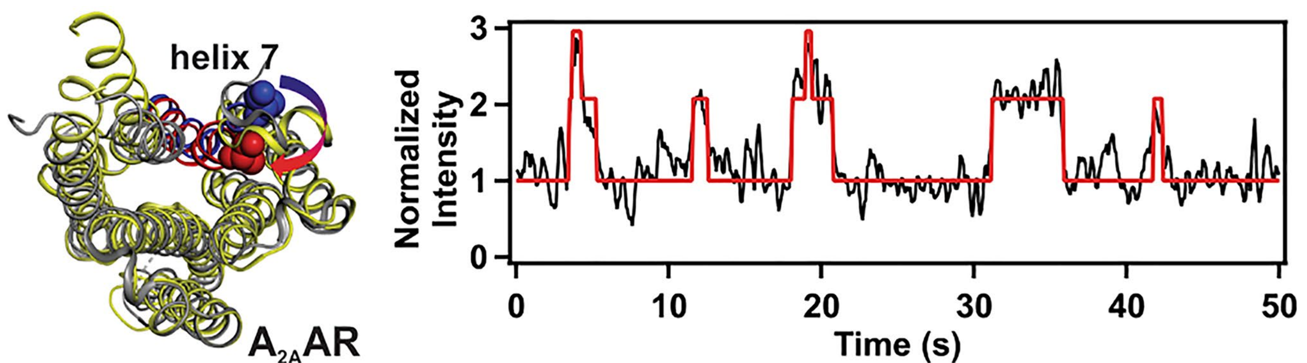
Graphical Abstract



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Foreword by Francisco Barrera, Editorial Board Member

In this edition of the Journal of Membrane Biology, it is my honor to feature the work of Dr. Rajan Lamichhane, who, at the time of the writing of these words is an Assistant Professor of Biochemistry & Cellular and Molecular Biology at the University of Tennessee. Dr. Lamichhane uses his Chemistry background (Ph.D. in Chemistry from Wayne State University under the supervision of Dr. David Rueda) to study the conformational dynamics of membrane proteins. He has a strong interest in RNA structural dynamics and nucleic acid-binding proteins, but his focus has recently shifted decisively to G protein-coupled receptors (GPCRs). These proteins are pivotal in pharmacology and represent a seemingly endless source of potential new drugs. Dr. Lamichhane began his work on GPCRs early in his postdoctoral training in the laboratory of Dr. David Millar at the Scripps Research Institute in La Jolla, CA, USA. There, he performed pioneering work where he applied the remarkable resolving power of single-molecule fluorescence to study GPCRs. His first-author manuscripts on the β2 adrenergic

receptors, a flagship member of the Class A GPCRs, highlights his groundbreaking contributions to the field (Lamichhane et al., 2015; Lamichhane et al., 2020). Dr. Lamichhane leads single-molecule efforts within the vibrant membrane community at the University of Tennessee, Knoxville. The ensuing Up-and-Coming Scientists piece highlights the latest GPCR single-molecule studies from the Lamichhane laboratory.

Introduction

G Protein-Coupled Receptors (GPCRs) are membrane proteins expressed in all human tissues and have an important role in cellular signaling. They are involved in various biological processes, including immune response, sensory perception, neurotransmission, and regulation of cellular and metabolic functions. Defects in GPCR signaling cascades are linked to numerous diseases, including Parkinson's, heart failure, and various forms of cancer (Fessl et al. 2023). There are over 800 GPCRs encoded in the human genome, and they are targets for about 35% of current US Food and Drug Administration (FDA) approved pharmaceutical drugs (Hauser et al. 2017). GPCRs are allosteric machines that bind many types of ligands (ions, small molecules, peptides) on their extracellular face and undergo conformational changes of the seven-transmembrane domain (7TM) that activate signal transduction pathways inside the cell (Deupi et al., 2010; Kobilka 2011; Rosenbaum et al. 2009). GPCRs trigger cellular signaling pathways upon ligand binding by interacting with cytoplasmic transducer proteins, such as heterotrimeric G proteins and arrestins. In G protein-mediated signaling, the process is initiated when an agonist binds to the orthosteric site of the receptor. This interaction causes conformational changes in the transmembrane helices, making it ready for G protein coupling and activation (Nygaard et al. 2013). The activated GPCR then prompts the G protein to exchange GDP for GTP on its α subunit. This exchange subsequently induces the dissociation of Gα from Gβγ subunits that activate downstream effectors to produce the physiological response (Agyemang et al. 2024). On the other hand, arrestin-mediated GPCR signaling begins with the phosphorylation of the C terminal tail of the receptor by GPCR kinases (GRKs). This phosphorylation event recruits an arrestin protein, which inhibits G protein-mediated signaling, resulting in receptor desensitization and internalization (Agyemang et al. 2024).

The activation and signaling processes in the GPCR cycle involve conformational changes and assembly–disassembly reactions that are poorly understood. Additionally, GPCRs do not function as simple on–off switches; instead, they exhibit diverse signaling responses to different ligands, depending on ligand efficacy and the specific signaling pathways activated (Keov et al. 2011; Luttrell et al., 2011; Urban et al. 2007). Despite significant advancement, the mechanistic link between the chemical structure of the ligands, the resulting functional states of the receptor, and the subsequent intracellular signaling responses remain largely unexplored. Similarly, the mechanisms by which G proteins regulate ligand binding and stabilize GPCR functional state(s) are not well understood. Moreover, it is also not clear how membrane lipids allosterically regulate the structure and dynamic behavior of GPCRs. These knowledge gaps limit our ability to develop drugs targeting GPCRs with minimal side effects.

While various methods are currently employed to study GPCR activation, few can monitor dynamic conformational changes on biologically relevant timescales while linking these changes to agonist binding and G-protein coupling. Structural data from crystallographic studies and cryo-electron microscopy (cryo-EM) of various GPCRs have provided a static snapshot of complex formation between the partners. However, these studies lack dynamic information and fail to capture the intermediate steps of the conformational changes that occur during receptor activation (Cherezov et al. 2007; Rasmussen et al. 2011; Rosenbaum et al. 2011; Zhang et al. 2017a, 2017b). Nuclear Magnetic Resonance (NMR) studies, while effective for revealing conformational exchanges, often operate on timescales that are faster than the actual activation process of most GPCRs (Horst et al. 2013; Liu et al. 2012; Nygaard et al. 2013). Similarly, ensemble fluorescence measurements can reveal conformational changes during receptor activation but cannot resolve molecular heterogeneity because these results represent average signals from thousands of molecules under given experimental conditions (Kroning et al. 2024; Soave et al. 2020; Vilardaga et al. 2003; Yao et al. 2009). A promising approach to overcome these limitations is single-molecule fluorescence (SMF), which provides quantitative information about receptor conformations and intermediate states. Moreover, it enables the resolution and quantification of distinct receptor conformational states, providing rate constants for the transitions between them on biologically relevant timescales of milliseconds to seconds (Bender et al. 2024; Bockenhauer et al. 2011; Fernandes et al. 2021; Gregorio et al. 2017; Lamichhane et al. 2015, 2020; Liu et al. 2023; Wei et al. 2023, 2022). Here, we provide a brief overview of our efforts to understand the conformational dynamics of Class A and Class B GPCRs at the molecular level.

General Mechanisms of GPCR Dynamics Among Class A GPCRs

To comprehensively understand the dynamic behavior of class A GPCRs, we investigate the conformational dynamics of the human A_{2A} adenosine receptor (A_{2A}AR), a

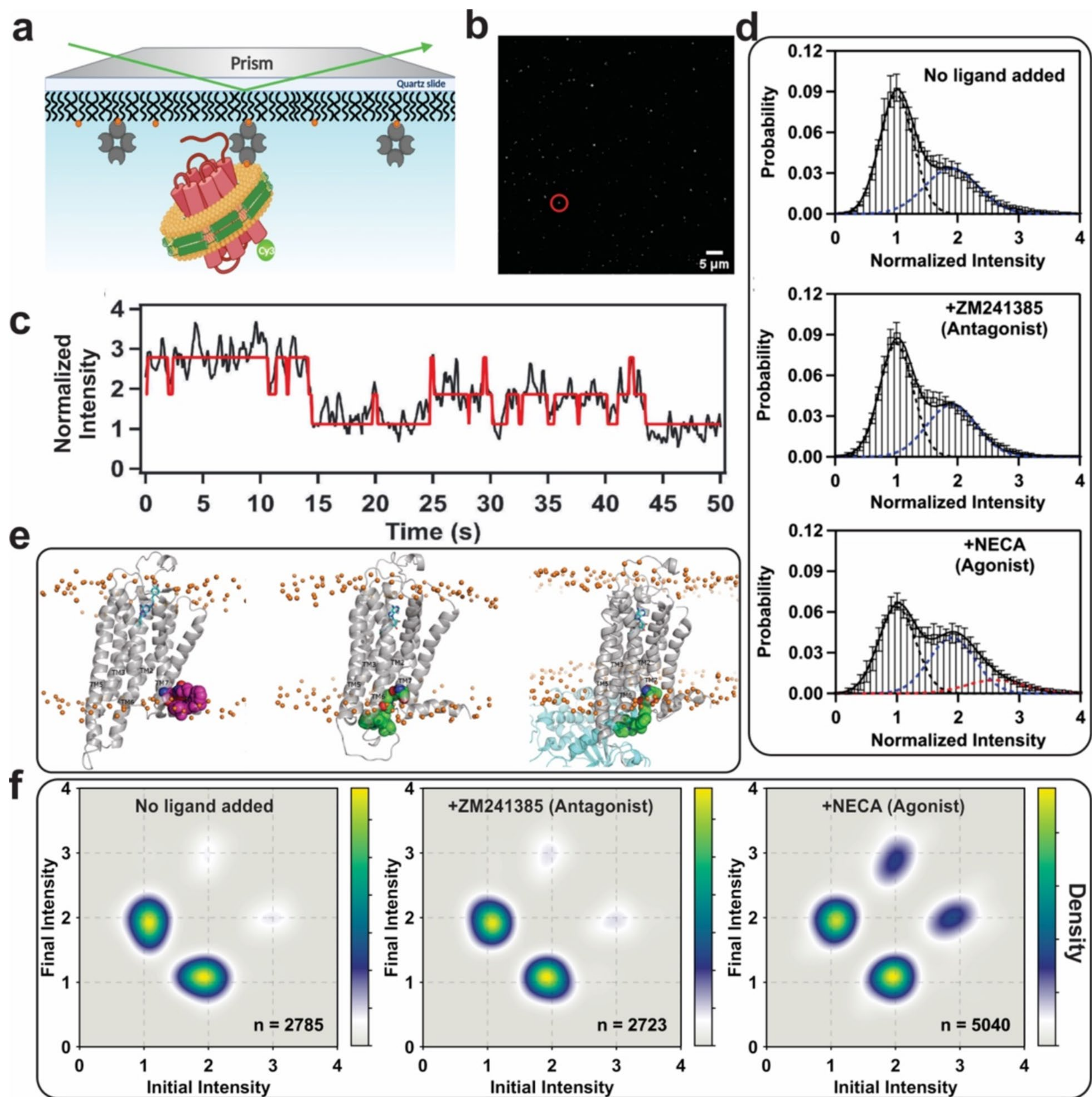

Fig. 1 Experimental system to monitor the conformational dynamics of A_{2A}AR with single-molecule fluorescence. **a** Schematic diagram of prism-based single-molecular total internal reflection fluorescence (TIRF) microscopy of A_{2A}AR in nanodisc labeled with Cy3 fluorophores. **b** A representative TIRF field-of-view image with spots representing A_{2A}AR-Cy3 molecules in nanodiscs. **c** A representative example of single-molecule fluorescence intensity trajectory for an individual A_{2A}AR-Cy3. The Cy3 intensity is normalized by the mean value of the lowest intensity state. The trajectory is in black, and the red line is a best fit from a three-state hidden Markov model. **d** Fluorescence intensity histograms compiled from many molecules at different conditions as presented. **e** The structural model of A_{2A}AR is presented in three complexes: with the antagonist ZM241385 (left), in complex with the agonist NECA (middle), and a tertiary complex with both NECA and GaS (right). A_{2A}AR is depicted as a gray ribbon, ligands shown in yellow stick, the Cy3 fluorophore covalently attached to position C289 is represented in purple and green space-filling representation, and GaS in blue ribbon. **f** The TDPs generated from the normalized initial and final intensities. ''n'' represents the number of transitions used to generate the TDPs. The TDPs of apo state (left) and antagonist-bound A_{2A}AR (middle) show that transition events predominantly occur between states 1 and 2. The TDP for A_{2A}AR in complex with the agonist NECA (right) reveals transition events occur between states 1 and 2 as well as between states 2 and 3 with measurable frequencies. The figure is modified from reference Wei et al. 2022 with permission from Structure journal) and Wei et al. 2023

prototypical class A GPCR. Since $A_{2A}AR$ is involved in many physiological and pathological processes (Armentero et al. 2011; Belardinelli et al. 1998; Collins et al. 2012; Fredholm et al. 2005; Young et al. 2016), it represents an ideal pharmacological target (Jacobson et al., 2006; Mittal et al. 2014). Unlike most structural analyses of this receptor in detergent micelles, we studied the dynamics of $A_{2A}AR$ in native-like environments of phospholipid nanodiscs (Bayburt et al., 2010; Denisov et al., 2016; Nath et al. 2007). This allows us to investigate the impact of lipid composition on receptor dynamics and provides a biologically relevant membrane context to better understand receptor behavior and dynamics.

We reconstituted $A_{2A}AR$ into lipid nanodiscs containing a 65:30:5 molar ratio mixture of POPC, POPS, and biotinylated POPE lipids, respectively (Thakur et al. 2022). The immobilized nanodiscs containing Cy3 fluorophore-labeled at position 289 in helix 7 ($A_{2A}AR$-Cy3) were then analyzed using Total Internal Fluorescence Microscope (TIRF) (Fig. 1a, b) (Wei et al. 2022). We observed three distinct conformational states for $A_{2A}AR$-Cy3 bound to the agonist NECA, corresponding to different local environments of the fluorophore (Fig. 1c, d bottom panel, and f). These three states were also observed in the apo and antagonist (ZM241385) bound complex, though the third state was sparsely populated (Fig. 1d top and middle panel). Molecular Dynamics (MD) simulations were employed to examine the orientations of Cy3 within its local environment, which modulate the conformational degrees of freedom of the dye. These modulations influence fluorescence emissions and correlate with the observed states, linked with the backbone movements of the receptor's transmembrane helices at the intracellular surface. (Fig. 1e). These observed three states are referred to as the inactive, intermediate, and active states. The observation of three states is interesting, as earlier single-molecule fluorescence studies reported only two conformational states for another class A GPCR, the β_2-adrenergic receptor (β_2AR) (Gregorio et al. 2017; Lamichhane et al. 2015, 2020). For the apo receptor, over 90% of transitions from state 1 to state 2 occur with a high frequency, characterized by a rate constant of $2.54 \pm 0.2\ s^{-1}$. Similarly, transitions from state 2 to state 3 predominantly occur with a rate constant of $2.44 \pm 0.24\ s^{-1}$ (Wei et al. 2023). The slower rate constant observed in this study aligns with the conformational state exchange rates observed in previous ^{19}F-NMR experiments of $A_{2A}AR$ in DDM and CHS detergent micelles (Sušac et al. 2018). The addition of agonist NECA shifts the equilibrium toward state 2, supporting the idea that agonists enhance the frequency of transitions to the intermediate state. Here, for the first time, we also report the reversible temporal ordering among three states, suggesting a stepwise process involving conformational changes during class A GPCR activation. This observation is similar to the single-molecule FRET study of a class C GPCR dimer, where stepwise interactions between monomers were interpreted as conformational checkpoints in the activation process (Liauw et al. 2021). We further reported that the constitutively activating mutations (CAMs) R291Q and I92N preserve the stepwise activation of $A_{2A}AR$ without inducing new conformations at the probe location on helix 7. CAMs significantly enhance the population and transition frequency to the intermediate state, whereas mutations affecting sodium sensitivity (D52N) increase transitions toward the inactive state (Gutierrez-de-Teran et al. 2013; Massink et al. 2014; White et al. 2018). Additionally, we utilized the same framework to differentiate the effects of CAMs from those of ternary complex formation with a G protein (mini-Gs). The stepwise activation model still applies in the presence of mini-Gs, as direct transitions between states 1 and 3 remain rare. Additionally, mini-Gs shift the equilibrium toward state 2, linking the intermediate state's population to the $A_{2A}AR$ function (Wei et al. 2023). These findings suggest that the impact of mutations on GPCR function can be predicted by determining whether they promote or inhibit the formation of an intermediate state. This work provides a conceptual framework for designing receptors with modified functions or developing therapies that target intermediate states.

Resolving the Dynamics of Extracellular Domain of Class B GPCR

The secretin-like class B GPCRs are drug targets for numerous diseases such as osteoporosis, obesity, diabetes, cancer, migraine, cardiovascular diseases, and some psychiatric disorders (Bortolato et al. 2014; Finan et al. 2016). Unlike class A receptors, class B receptors contain an extended N-terminus that forms a large extracellular domain (ECD). The ECD and transmembrane domains are hypothesized to work in concert to recognize and bind ligands (Hollenstein et al. 2014; Karageorgos et al. 2018; Koth et al. 2012; Pal et al. 2012; Parthier et al. 2009; Zhang et al. 2018). The glucagon receptor (GCGR), a key member of the class B GPCR family, is activated by the peptide hormone glucagon. It plays an important role in regulating glucose homeostasis and represents a significant target for treating type 2 diabetes (Ahren 2015; Bagger et al. 2011). Recent studies highlight the structural plasticity of the glucagon receptor and signaling mechanisms with a glucagon analog, emphasizing the role of conformational changes in the ECD. While numerous class B GPCR structures reveal open and closed ECD states in apo and ligand-bound forms (Fig. 2a and b), computational studies suggest the ECD can access these states even without ligands (Wu et al. 2020; Yang et al. 2015; Zhang et al. 2017a, 2018, 2017b; Zhao et al. 2019). However, the dynamics and transitions between these conformations remain poorly understood. Here, we focus on understanding the conformational

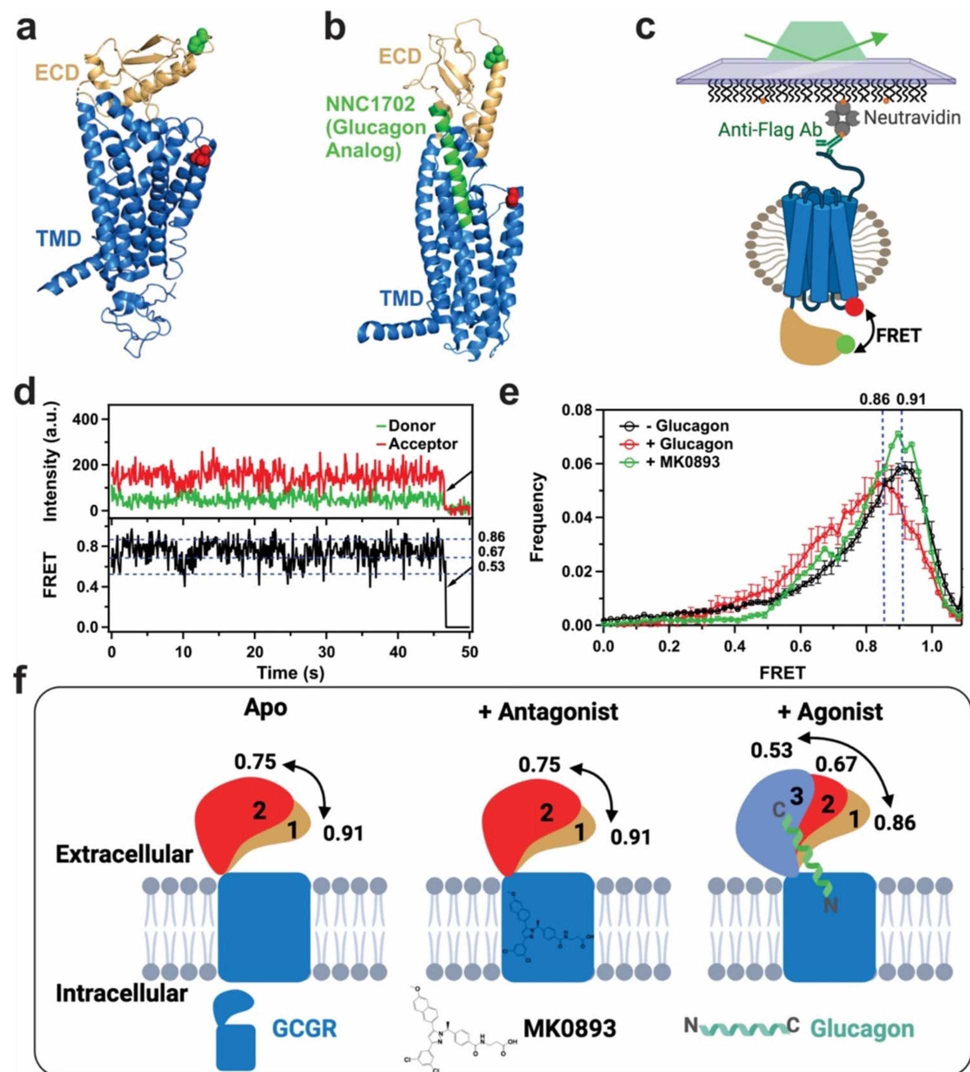

Fig. 2 Single-molecule studies of the glucagon receptor ECD. **a** Structure of closed ECD for inactive GLP-1R ECD (PDB: 6LN2). **b** Structure of open ECD for active receptor bound to glucagon analog, NNC1702 (PBD: 5YQZ). ECD: orange, TMD: blue, Ligand: green, Labeling positions and amino acids: red and green. **c** Schematic diagram of prism-based single-molecular total internal reflection fluorescence (TIRF) microscopy of glucagon receptor in detergent micelle labeled with donor and acceptor fluorophores. **d** A typical single-molecule time trace in the presence of agonist (glucagon) (top: donor green; acceptor red) and FRET (bottom) for individual glucagon receptor. The black arrows refer to the bleaching time point of the donor or acceptor traces. **e** Combined smFRET population histograms of apo (black), glucagon bound (red), and MK0893 bound (green) glucagon receptor complexes. The vertical dashed lines represent the highest FRET states observed for different complexes. **f** Schematic of the opening and closing of GCGR ECD complexes generated based on the smFRET data. The figure is modified from reference Liu et al. 2023

dynamics of the extracellular domain relative to the 7TM domains of the glucagon receptor and examine how ligand interactions modulate dynamics to drive receptor activation.

We employed single-molecule FRET measurements using site-specifically labeled GCGR with a FRET donor and acceptor at positions 49 and 287. These double-labeled receptors were immobilized on microscope slides using biotinylated anti-FLAG antibody and streptavidin and examined by TIRF microscopy (Fig. 2c) (Liu et al. 2023). A typical single-molecule time trace showed anticorrelated donor (green) and acceptor (red) fluorescence intensities (Fig. 2d, upper panel). The corresponding FRET trace (Fig. 2d, lower panel) fluctuates among multiple FRET states, highlighting the dynamic nature of the ECD. For the apo receptor, two prominent peaks at 0.91 and 0.75 FRET states were observed. In the presence of the glucagon agonist, a dominant peak emerged around the 0.86 FRET state, accompanied by additional lower FRET peaks around 0.67 and 0.53 FRET states (Fig. 2e and f). These lower FRET states correspond to the longer distances between the two fluorophores, which correlate with the ECD opening after glucagon binding. In contrast, experiments conducted in the presence of antagonist MK0893 showed FRET states at 0.91 and 0.75, similar to those observed for the apo receptor. For the first time, we observed the spontaneous opening and closing of the ECD in the glucagon receptor. Glucagon binding partially opens the receptor, allowing the glucagon N-terminus to interact with the receptor core without stabilizing the ECD in this state, aligning with the two-domain binding model (Karageorgos et al. 2018). The ECD continues to fluctuate to lower FRET states, suggesting that the ECD retains dynamic behavior. In summary, without agonist binding, the GCGR ECD oscillates between open and closed states. Glucagon binding moves the ECD away from the 7TM domain, exposing the ligand-binding pocket.

Conclusions and Future Perspectives

Our study emphasizes the critical role of single-molecule techniques in understanding receptor dynamics by comparing the conformational behaviors of A_{2A}AR and β_2AR. The distinct conformational states observed between these two class A GPCRs suggest potential differences in their activation mechanisms. The slow and sequential dynamics provide critical insights into the complex mechanisms of GPCR activation. These slow dynamics may reflect the receptor's interactions with ligands, surrounding lipids, and its coupling with intracellular signaling pathways. These findings open the door to further exploration of how receptor dynamics are linked to function, contributing to a more complete understanding of GPCR biology. Future research will expand these dynamic studies to a broader range of human GPCRs, applying integrative methodologies to study their conformational changes in cellular environments. This approach will reveal ligand-dependent patterns of conformational dynamics and activation across a wide range of GPCRs, leading to a broader understanding of GPCR function and facilitating the development of targeted therapies based on receptor-specific activation mechanisms.

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Author Contribution R.L. wrote the manuscript.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing Interests The authors declare no competing interests.

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