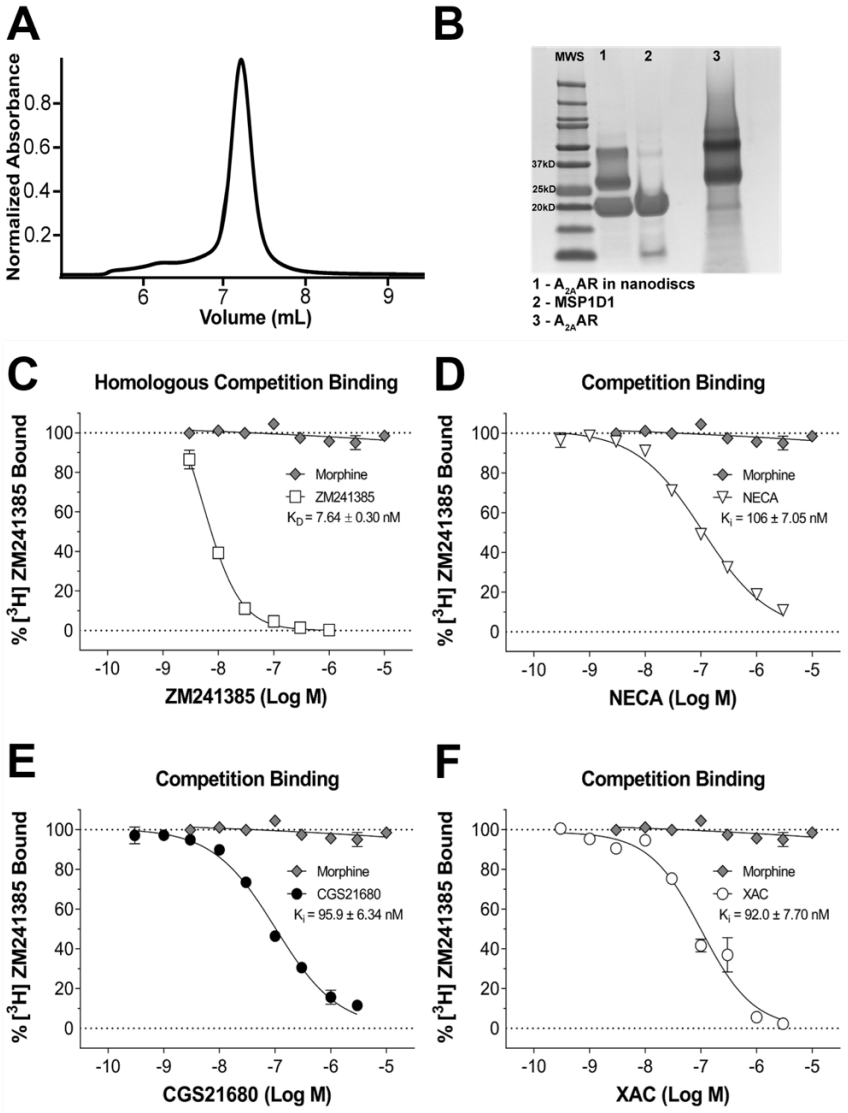

Figure S1, Related to Figure 1. Sample preparation characterization and pharmacological validation of A_{2A}AR[A289C]-Cy3 in lipid nanodiscs.

(A) Representative analytical size exclusion chromatogram of A_{2A}AR-Cy3 in lipid nanodiscs shows a single homogenous preparation.

(B) Image of a representative SDS-PAGE characterization of A_{2A}AR preparations. The lane labeled "MWS" contains a standard molecular weight ladder with three different molecular weights annotated. The lanes labeled "1", "2", and "3" contain purified A_{2A}AR in lipid nanodiscs, the MSP1D1 scaffold protein only and A_{2A}AR purified in detergent micelles, respectively.

(C–F) Radioligand binding activity measurements of A_{2A}AR-Cy3 in lipid nanodiscs. Homologous competition binding with (C) the antagonist ZM241385, and competition binding experiments with (D) the agonist NECA, (E) agonist CGS21680 and (F) antagonist XAC. Morphine was included in each experiment as a negative control. The measured K_D or K_i values are indicated in each panel. All error bars indicate s.e.m for 3 independent trials.

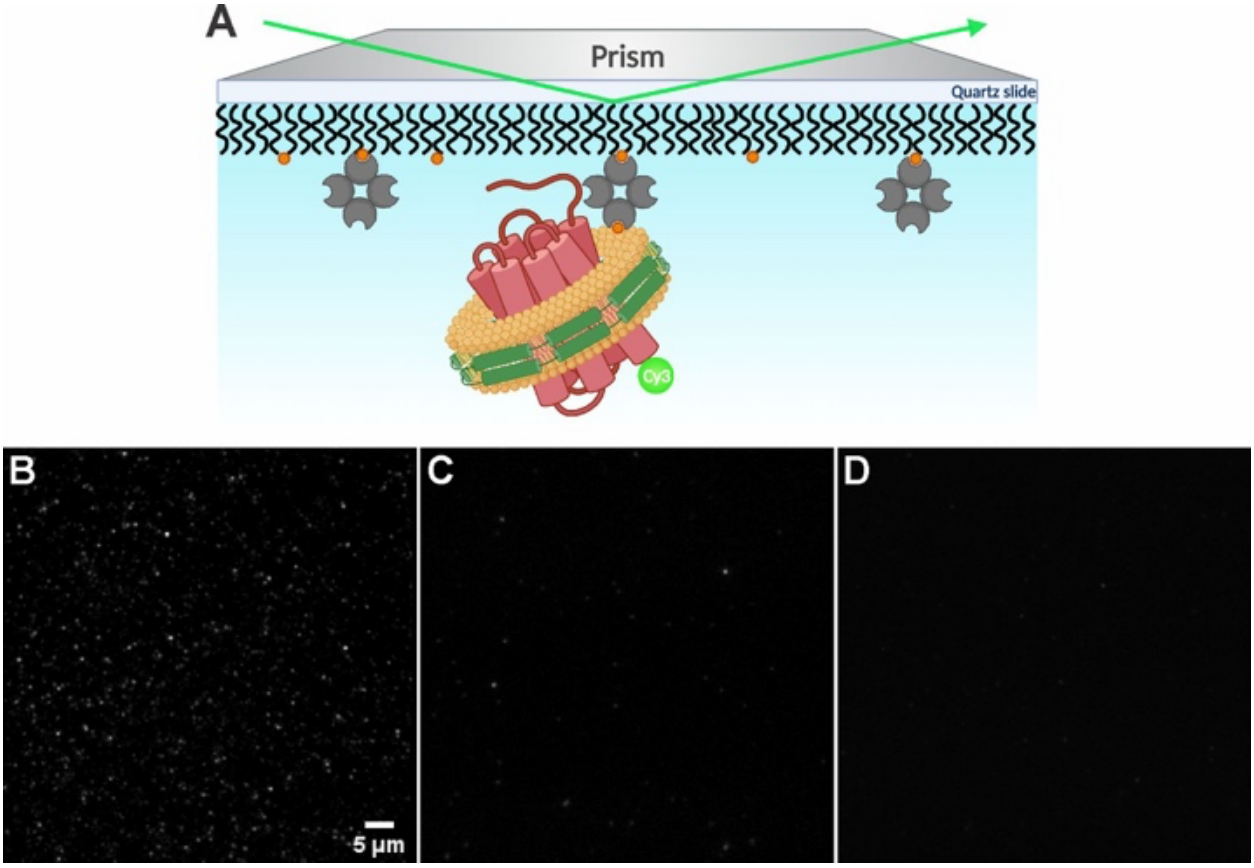

Figure S2, Experimental design to study conformational dynamics of A_{2A}AR by single-molecule fluorescence and representative TIRF field-of-view images, Related to Figure 1. (A) Schematic showing Cy3 (green sphere)-labeled A_{2A}AR (red cylinders) in a nanodisc (lipids shown in orange and membrane scaffold protein shown in green) immobilized to a microscope slide using biotin (orange circles) and streptavidin (grey semi-circles). The microscope slides were passivated with poly-ethylene glycol (black wavy lines) to decrease non-specific interactions between the surface and the labeled nanodiscs. A 532 nm laser (green line) was used to excite the Cy3 fluorophore indirectly by creating the evanescent wave from the total internal reflection of the laser beam. This image was prepared with BioRender.
(B) A representative TIRF field-of-view image from immobilized nanodiscs containing A_{2A}AR-Cy3. Each bright spot corresponds to the fluorescence emission from a single Cy3-labeled A_{2A}AR molecule in lipid nanodiscs.
(C) Corresponding TIRF field-of-view image from a control experiment without bound streptavidin. Significantly fewer fluorescent spots are observed as compared to (B).
(D) A TIRF field-of-view image from a control experiment of a sample containing only imaging buffer.

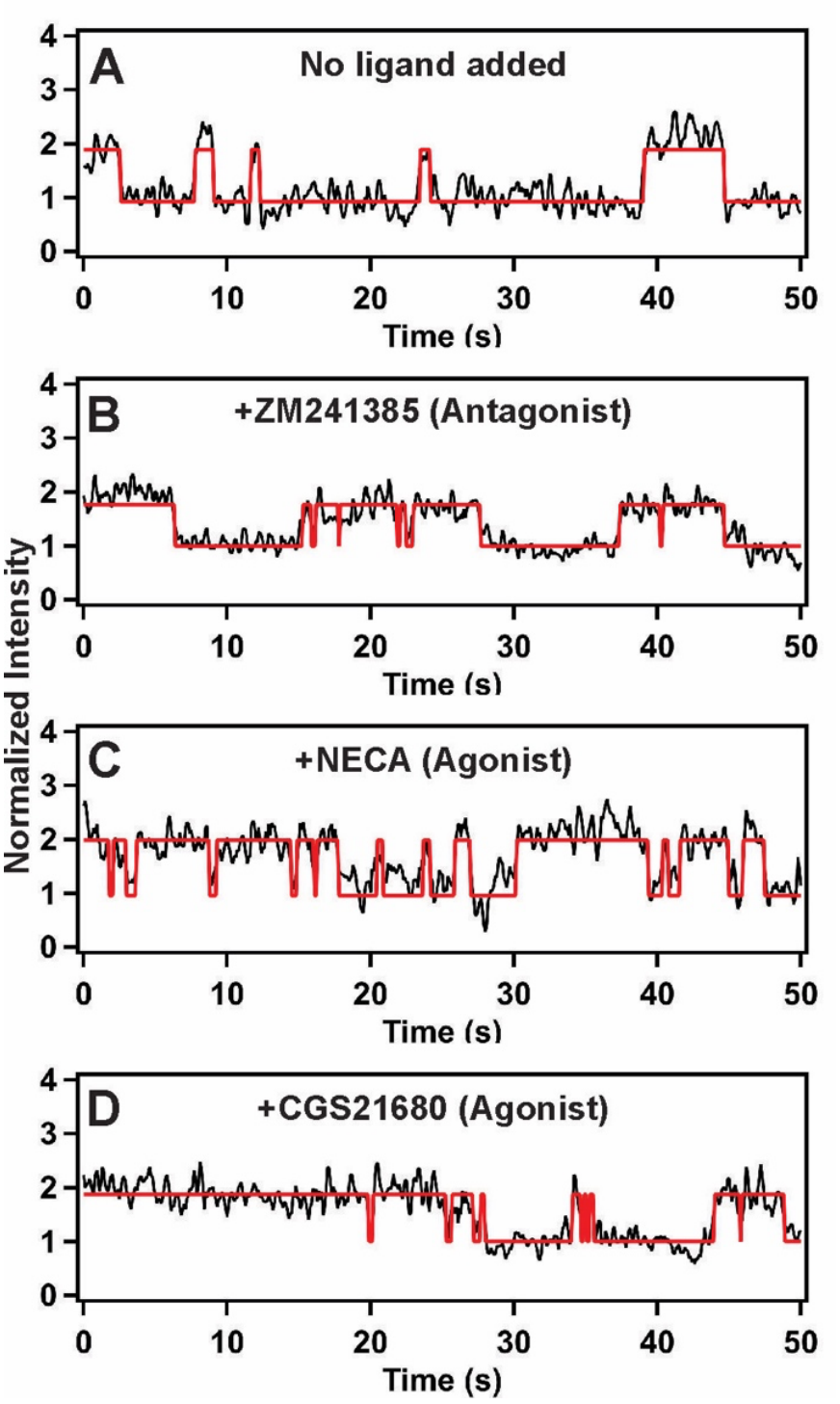

Figure S3, Representative fluorescence intensity trajectories from individual Cy3-labeled A_{2A}AR single molecules in complexes with different ligands as annotated and with no ligand added (apo), Related to Figure 2. Example trajectory fits showing reversible transitions between two states are presented.

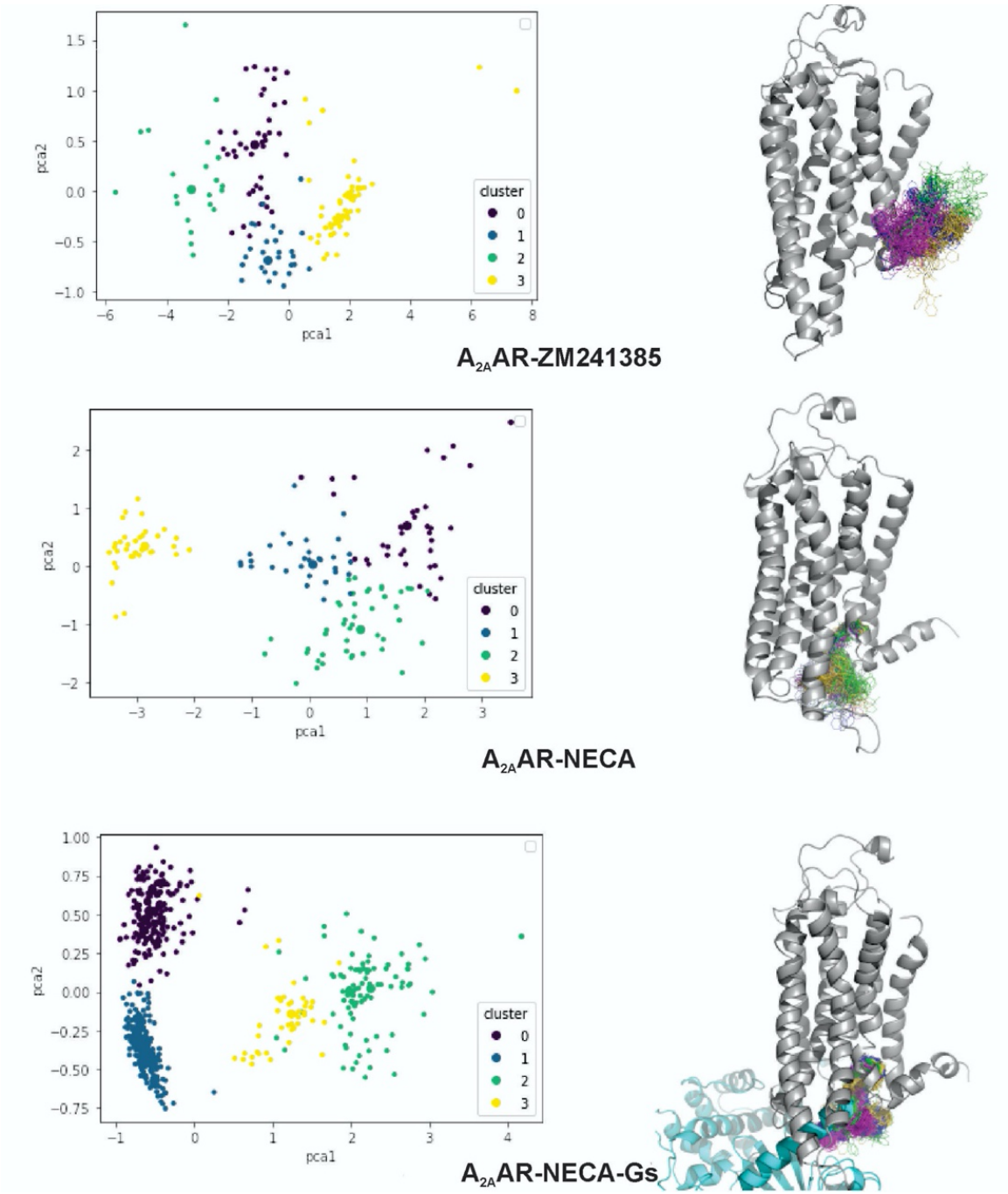

Figure S4. Principal component analysis (PCA) and cluster analysis, Related to Figure 2. PCA and cluster analysis (Affinity Propagation method, preference=-25, damping=.9) of the configurations visited by the dye during the pulled triplicate MD simulations on each relevant state (total simulation time 1.5 μS for each state). Analysis was performed with MDtraj. Four well defined clusters are observed in each case (left panels), and the 3D representation of these configurations on the starting structure of the receptor (grey ribbon representation) is shown on the corresponding right panels.

Table S1. Percentage of time spent by A_{2A}AR-Cy3 molecules in one state before transitioning to a different state, Related to Figure 3. In each column, the percent time spent in state i before transitioning to state j was calculated by dividing the time spent at each transition state by the total time spent in all states for all molecules.

A_{2A}AR Sample	State Transitions					
	1 → 2	2 → 1	2 → 3	3 → 2	1 → 3	3 → 1
Apo	59.1%	28.8%	6.1%	5.3%	0.4%	0.3%
ZM241385	51.7%	35.7%	5.4%	5.7%	0.8%	0.7%
NECA	42.5%	31.1%	10.9%	13.7%	0.5%	1.3%
CGS21680	44.5%	28.8%	12.7%	11.7%	1.1%	1.2%

Table S2: Comparing State three transition events and the distributions of molecules showing the two and three states, Related to Figure 3.

$A_{2A}AR$ Sample	State 3 transition events[a]		Distribution of molecules[b]	
	Average time spent (S)	Percentage of number of events	State 2	State 3
Apo	1.1	10.6%	69%	31%
ZM241385	1.2	10.3%	70%	30%
NECA	1.4	17.4%	38%	62%
CGS21680	1.1	18.3%	40%	60%

[a]Represents the transitions to State 3 events
[b]Number of molecules showing either only two or all three states.