Supporting Information

Phospholipid membrane fluidity alters ligand binding activity of a G protein-coupled receptor by shifting conformational equilibrium

Kouhei Yoshida[†], Satoru Nagatoishi[†,‡,*], Daisuke Kuroda[†,§], Nanao Suzuki[∥], Takeshi Murata[∥], Kouhei Tsumoto[†,‡,§,*]

*Corresponding Author

Satoru Nagatoishi

Phone: +81 3 6409 2129. Fax: +81 3 6409 2129.

E-mail: ngtoishi@ims.u-tokyo.ac.jp

Kouhei Tsumoto

Phone: +81 3 6409 2129. Fax: +81 3 6409 2129.

E-mail: tsumoto@bioeng.t.u-tokyo.ac.jp

Materials and Methods

Cloning and expression of 5-HT$_{2B}$R

Apocytochrome b$_{562}$RIL (BRIL) from *E. coli*, referred to as BRIL, was constructed from the BL21 (DE3) strain and contained thermostabilizing mutations (M7W, H102I, and R106L).[1] The gene of the human serotonin receptor 2B was obtained from HTR2B-Tango vector (Addgene) and was optimized as follows: (1) N-terminal residues 1-35 and C-terminal residues 406-481 were removed, (2) a thermostabilizing M144$^{3.41}$W mutation was introduced, (3) residues Y249-V313 within ICL3 were replaced with the thermostabilized BRIL, and (4) a 10× His tag was added at the C-terminus. The gene encoding the optimized receptor, referred to as 5-HT$_{2B}$R, was subcloned into a pFastBac1 vector (Invitrogen) using an In-Fusion HD Cloning Kit (Clontech). Recombinant baculovirus was obtained using the Bac-to-Bac Baculovirus Expression System (Invitrogen) as previously described.[2] Sf9 cells at a cell density of 3 × 10^6 cells·mL^{-1} were infected with P3 virus stock solution at 1% (v/v). Cells were harvested by centrifugation at 72 h post-infection and stored at -30 °C until use. Insect cell membranes were obtained as previously described.[2]

Cloning and expression of MSP1

Membrane scaffold protein 1 (MSP1) was expressed with a 6× His tag followed by a TEV cleavage site at the N-terminus. For biotinylation, an Avi-tag (GLNDIFEAQKIEWHE) was added at the C-terminus. TEV cleavage resulted in MSP1-Avi. Expression was performed as previously described.[3]

Protein purification

Washed membranes were resuspended in buffer containing 50 mM HEPES (pH 7.5), 200 mM NaCl, 10% glycerol, 50 μM 5-hydroxyltryptamine hydrochloride (5-HT, nacalai tesque, Inc.), and EDTA-free complete protease inhibitor cocktail tablets (Roche) and were incubated 4 °C for 1 h. The membranes were then solubilized by addition of 1% (w/v) *n*-dodecyl-β-D-maltopyranoside (DDM, Dojindo), 0.2% (w/v) cholesteryl hemisuccinate (CHS, Sigma) and incubation at 4 °C for 30 min. The supernatant, isolated by centrifugation at 210,000 × g for 30 min, was incubated with Ni-NTA agarose (QIAGEN) at 4 °C for 1 h. The resin was washed with 10 column volumes of Wash I Buffer (50 mM HEPES (pH 7.5), 800 mM NaCl, 10% (v/v) glycerol, 20 mM imidazole, 10 mM MgCl₂, 0.1% (w/v) DDM, 0.02% (w/v) CHS, and 50 μM 5-HT), followed by 5 column volumes of Wash II Buffer (50 mM HEPES (pH 7.5), 500

mM NaCl, 10% (v/v) glycerol, 50 mM imidazole, 0.05% (w/v) DDM, 0.01% (w/v) CHS, and 50 μM 5-HT). The protein was then eluted with 3-4 column volumes of Elution Buffer (50 mM HEPES (pH 7.5), 500 mM NaCl, 10% (v/v) glycerol, 250 mM imidazole, 0.05% (w/v) DDM, 0.01% (w/v) CHS, and 50 μM 5-HT). The eluted protein was subjected to size-exclusion chromatography using a HiLoad 16/600 Superdex 200-pg column (GE Healthcare) equilibrated with 50 mM HEPES (pH 7.5), 200 mM NaCl, 50 μM 5-HT, 0.02% (w/v) DDM, and 0.004% (w/v) CHS. The monodisperse peak was collected. Purification of MSP1 was carried out essentially as previously described. Proteins were frozen by immersion in liquid N_2 and stored at -80 °C until use.

Nanodisc reconstitution

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; NOF CORPORATION), 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC; Echelon Biosciences), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC; Echelon Biosciences), and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC; NOF CORPORATION) were solubilized at 50 mM in a solution containing 100 mM sodium cholate. The purified 5-HT$_{2B}$R, MSP1, and the phospholipids were incubated at 1:10:500 molar ratios at

25 °C (for samples containing DMPC) or 4 °C (for all other phospholipids) for 30 min.

Detergents DDM and sodium cholate were removed by treatment with 0.8 g·mL^{-1}

Amberlite XAD-2 (Sigma) overnight. Nanodiscs were separated from aggregated

material in a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with buffer

A (50 mM HEPES (pH 7.5) and 200 mM NaCl) and were then purified over a Ni-NTA

agarose column. Nanodiscs made of MSP1-Avi were biotinylated by treatment BirA

enzyme.[4] Empty-Nanodiscs, were Nanodiscs without 5-HT$_{2B}$R, were similarly obtained.

Kinetics and affinity measurements by SPR assay

The interaction between the 5-HT$_{2B}$R-Nanodisc and 5-HT was analyzed by SPR[5] with a

Biacore 8K instrument (GE Healthcare). The 5-HT$_{2B}$R-Nanodiscs were captured on

sample flow cells of the Series S Sensor Chip SA (GE Healthcare) via the interaction

between biotin and streptavidin. Empty-Nanodiscs were captured on reference flow

cells to evaluate nonspecific binding. The kinetics assays were carried out in buffer A

supplemented with 3.9-62.5 nM 5-HT at a flow rate of 30 μL·min^{-1}. The binding curves

were obtained by subtracting the binding responses on the reference flow cells

containing Empty-Nanodiscs from that on the sample flow cells, and data were

processed using Biacore 8K Evaluation software (GE Healthcare). Association (k_{on}) and

dissociation (k_{off}) rate constants were calculated by a global fitting analysis assuming a Langmuir binding model and a stoichiometry of 1:1. The dissociation constant (K_D) was determined from the ratio of the rate constants:[6] $K_D = k_{off} / k_{on}$. The affinity assays were carried out in buffer A supplemented with 0.9-250 nM 5-HT at a flow rate of 30 $\mu L \cdot min^{-1}$.

Affinity measurements by MST assay

Microscale thermophoresis (MST) experiments were carried out on a Monolith NT.115 (NanoTemper Technologies).[7] We prepared a series of 5-HT solutions at concentrations from 5000 nM to 0.6 nM, mixed them with 80 nM Nanodisc, and 40 nM fluorescence dye from the Monolith His-Tag Labeling kit RED-tris-NTA[8] (NanoTemper Technologies) in buffer A and incubated for 30 min at 25 °C. The samples were loaded into Monolith NT.115 Standard Capillaries (NanoTemper Technologies) and subjected to the MST analysis. Thermophoresis was measured at 25 °C by increasing the temperature 2 °C from 23 °C with an IR laser at 20% MST power. MST data was analyzed in NT Analysis software (NanoTemper Technologies), and resulting saturation binding curves were fit in Origin7 (OriginLab) using a logistic function:

$$y = \frac{A_{unbound} - A_{bound}}{1 + \left(x/x_0\right)^p} + A_{bound}$$

where x, y, p, $A_{unbound}$, and A_{bound} mean ligand concentration, F_{norm}, power, F_{norm} of apo state, and F_{norm} of holo state, respectively. x_0 is inflection point of the function, which means the center of $A_{unbound}$ and A_{bound}, referred as K_D.

Thermal stability measurements by CPM assay

The CPM assay was performed as previously described.[9] CPM dye and Nanodiscs were diluted in buffer A at concentrations of 0.0067 mg·mL^{-1} and 2 µM, respectively. The reaction was performed in a total volume of 60 µL. The sample was transferred to a Mx3000P/Mx3005P Strip Tube (Agilent Technologies) and heated in a controlled manner (1 °C·30 sec^{-1}) in a Mx3005P QPCR System (Agilent Technologies). The temperature recorded was from 25 to 90 °C. The excitation was at 350 nm, and emission was recorded at 516 nm. Each relative fluorescence unit (RFU) of the reference Empty-Nanodisc was subtracted from that of 5-HT$_{2B}$R-Nanodisc. Data analysis was performed with MxPro QPCR Software (Agilent Technologies). The data were normalized against initial fluorescence values. T_m was calculated from inflection point represented as maximal value of dRFU/dT.


Molecular dynamics simulations

Molecular dynamics (MD) simulations for phospholipid membrane and

receptor-membrane systems were performed using GROMACS 2016.3[10] with the CHARMM36m force field and the CMAP correction.[11,12] The membranes and the receptor-embedded membranes were solvated with TIP3P water in $100 \times 100 \times 100$ Å and $100 \times 100 \times 180$ Å boxes, respectively. Na or Cl ions were added to neutralize the protein charge, then further ions were added to correspond to a salt solution of concentration 0.14 M through the CHARMM-GUI.[13] Each system was energy-minimized and equilibrated with NVT ensemble (1 atm and 298 K). A production run was performed for 50 ns with NPT ensemble. The time step was set to 2 fs throughout the simulations. PyMOL, UCSF Chimera,[14] and Visual Molecular Dynamics[15] were employed to analyze and visualize the MD trajectories and to render the molecular graphics. Lateral diffusion coefficients and average distances were calculated through GROMACS based on the last 40 ns of the trajectories. The lateral diffusion coefficient D was defined as mobility of C2 atom of the phospholipid in parallel to XY plane. D was calculated from the lateral mean-squared displacement (ω) of each C2 atom:[16] $D = \frac{\omega^2}{4\Delta t}$. Each membrane thickness was measured with gmx-density program by calculating density of phospholipid C2 atoms between upper and lower leaflet. Principal component analysis was performed using ProDy and NMWiz.[17,18]
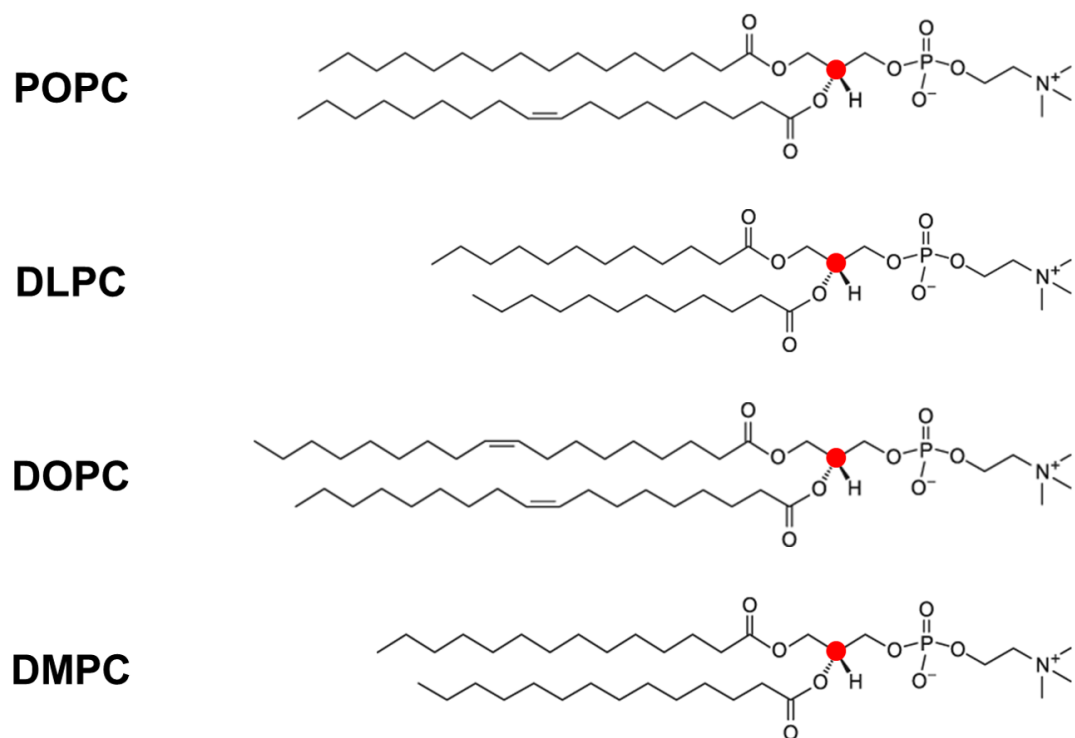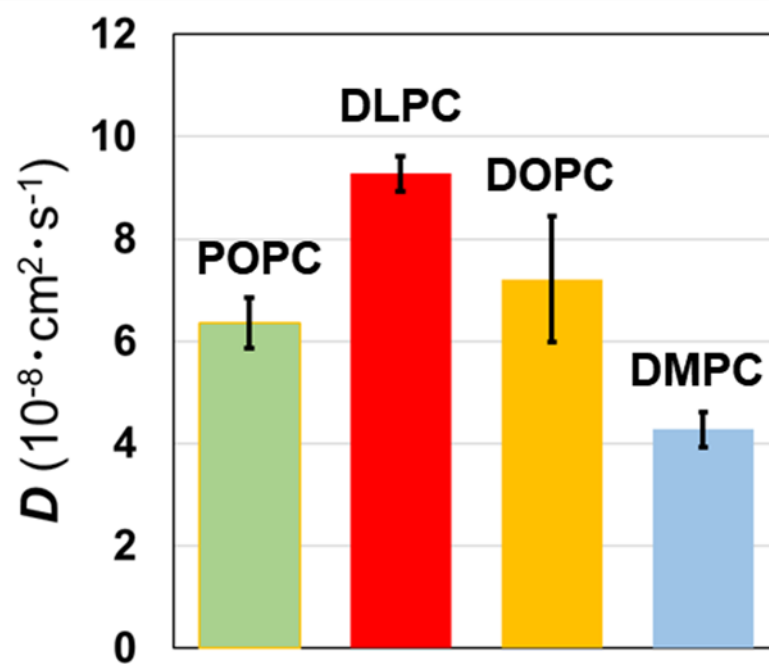
Figure S1. Chemical structures of phospholipids used for Nanodisc reconstitution. Red dots represent C2 atom of each phospholipid.
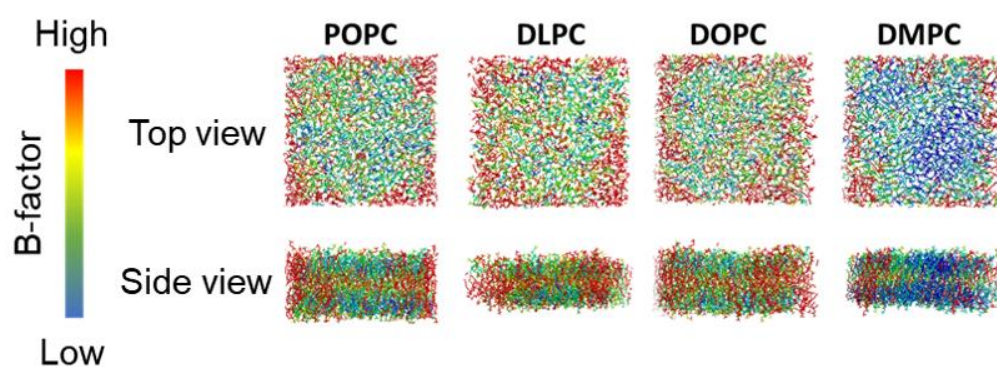
Figure S2. Fluctuation of phospholipids in membrane bilayers. Each atom of the phospholipid is colored based on the value of B-factor.

Figure S3. Lateral diffusion coefficients of phospholipid membranes in MD simulations. Error bars are standard errors (n=3).
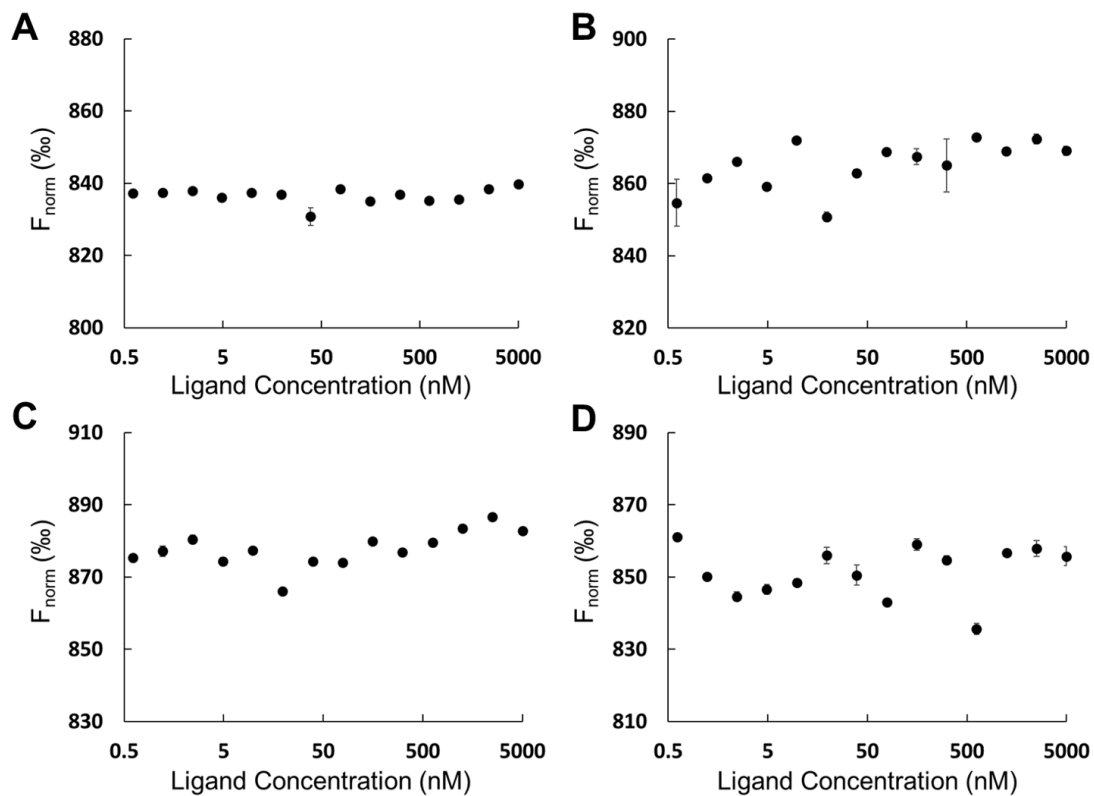
Figure S4. Plots of fluorescence of Empty-Nanodiscs made of (A) POPC, (B) DLPC, (C) DOPC, and (D) DMPC. The normalized fluorescence (F_{norm}) is plotted as a function of ligand concentration. Error bars are standard errors (n=3).
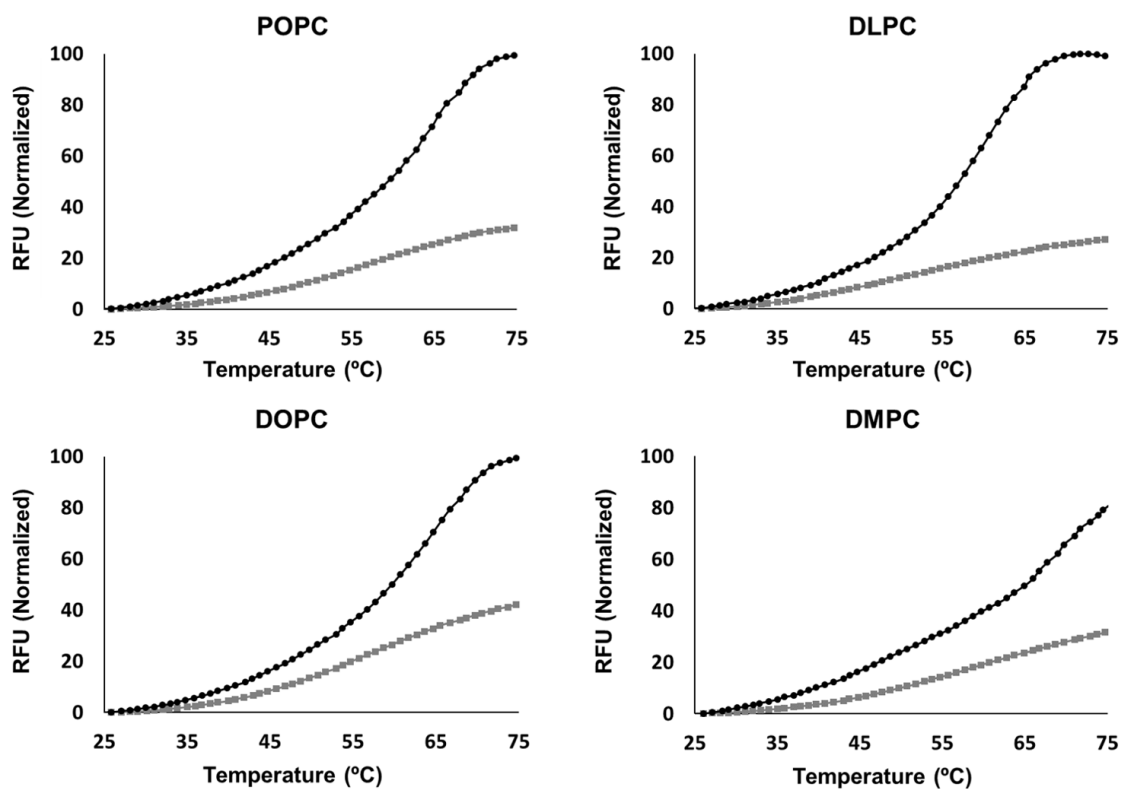
Figure S5. CPM assay of Nanodiscs in various phospholipid composition. Normalized fluorescence intensity was shown at each temperature. The fluorescence of Empty-Nanodisc and 5-HT$_{2B}$R-Nanodisc are shown in gray and black, respectively. Each curve is an average of three measurements.

A

34.0 ± 2.3 Å

B

POPC
32.7 ± 0.05 Å

DLPC
25.3 ± 0.08 Å

DOPC
32.2 ± 0.39 Å

DMPC
31.0 ± 0.10 Å
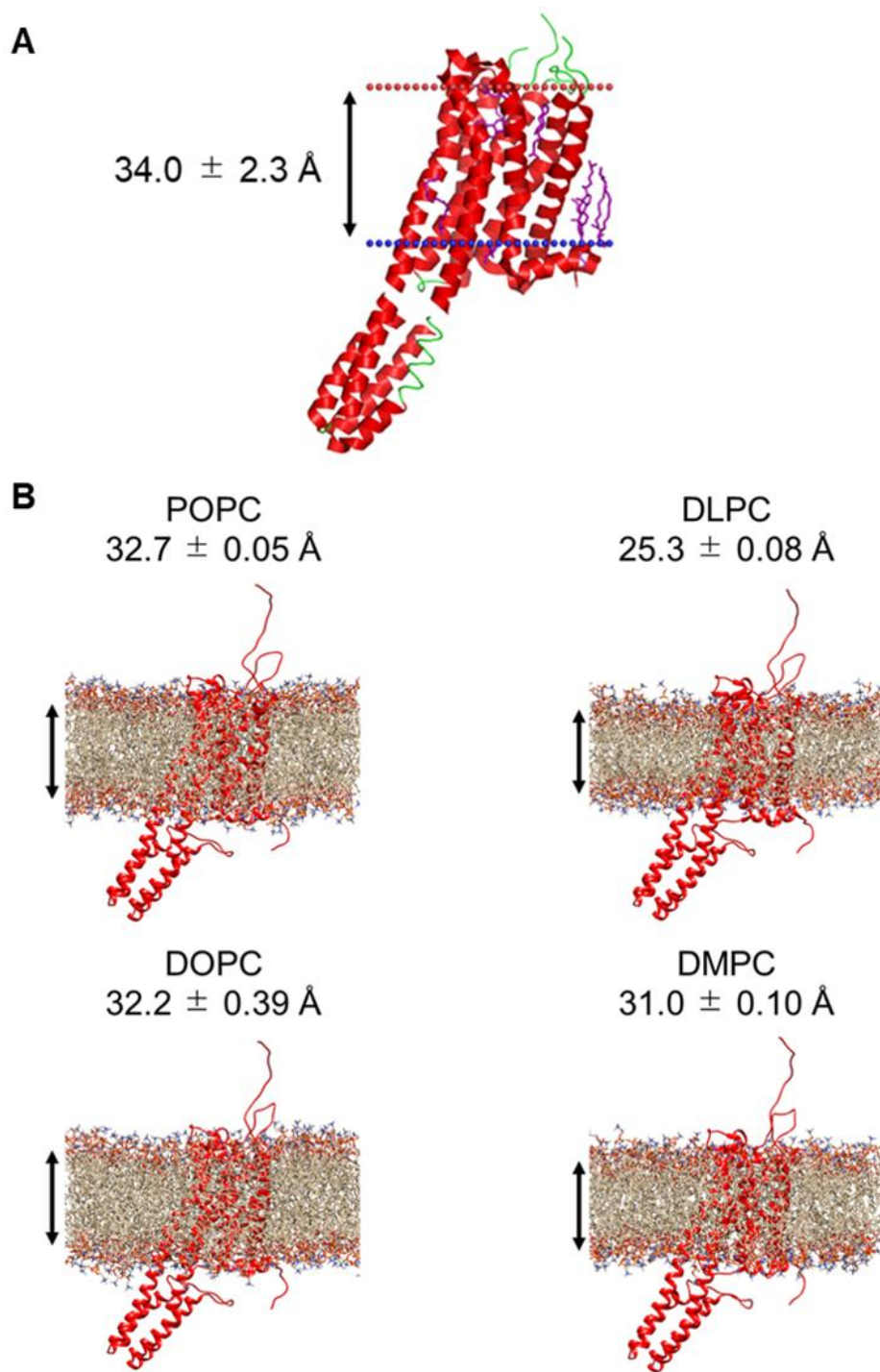
Figure S6. (A) Membrane boundary estimated from crystal structure of serotonin receptor 2B (PDB ID: 4IB4) according to Orientations of Proteins in Membranes database.[19] (B) Thickness of various phospholipid bilayers embedding 5-HT$_{2B}$R.
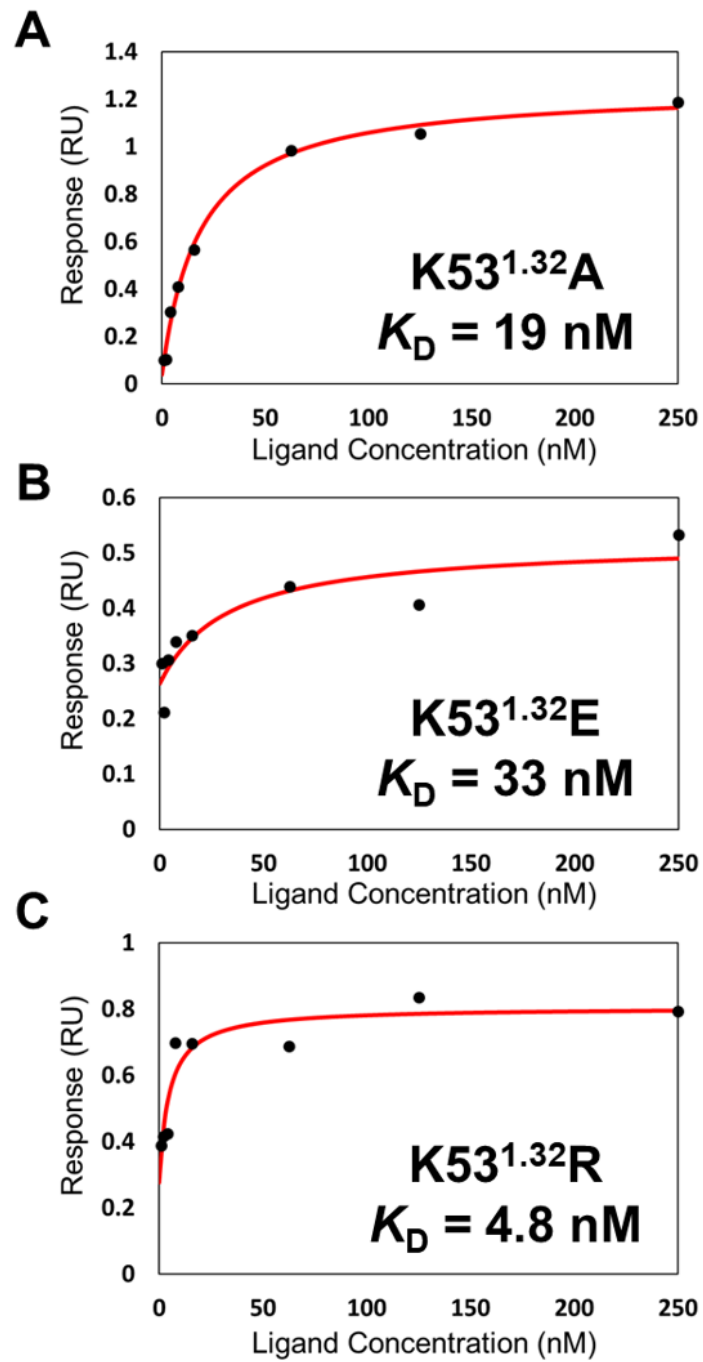
14
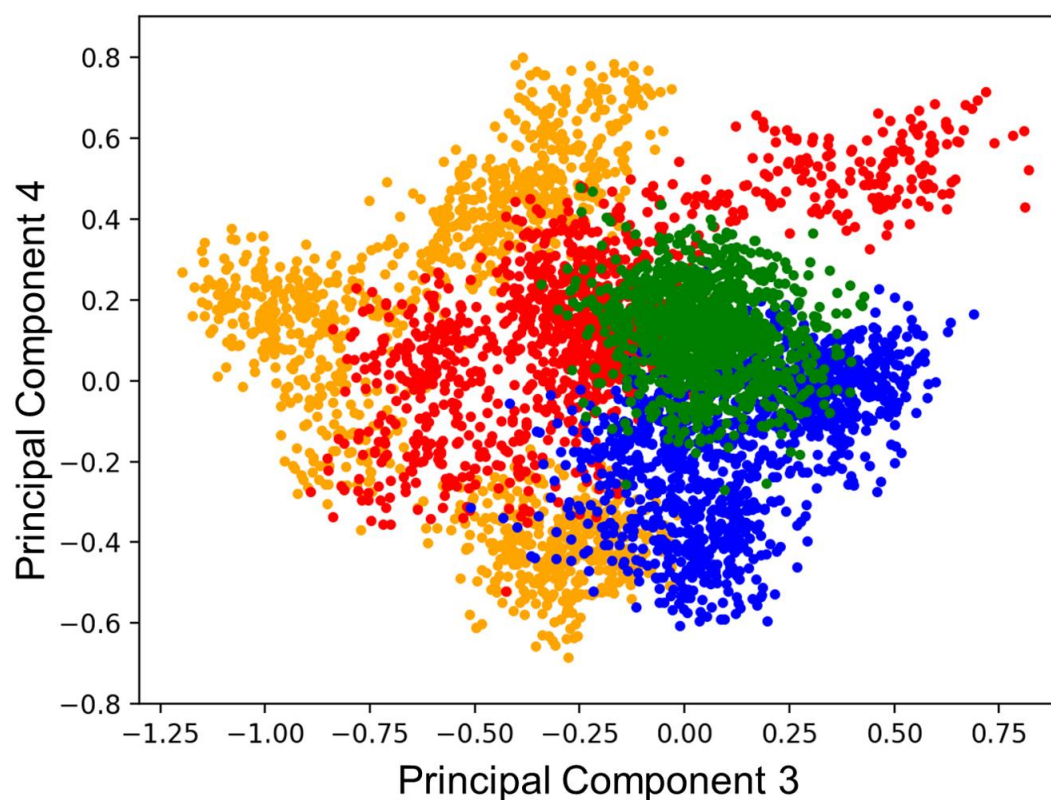
Figure S7. PCA of Cα atoms of TM helices and H8 of 5-HT$_{2B}$R. The plots of each coordinate in POPC, DLPC, DOPC, and DMPC membrane are shown in green, red orange, and blue, respectively.

Principal Component 3   Principal Component 4
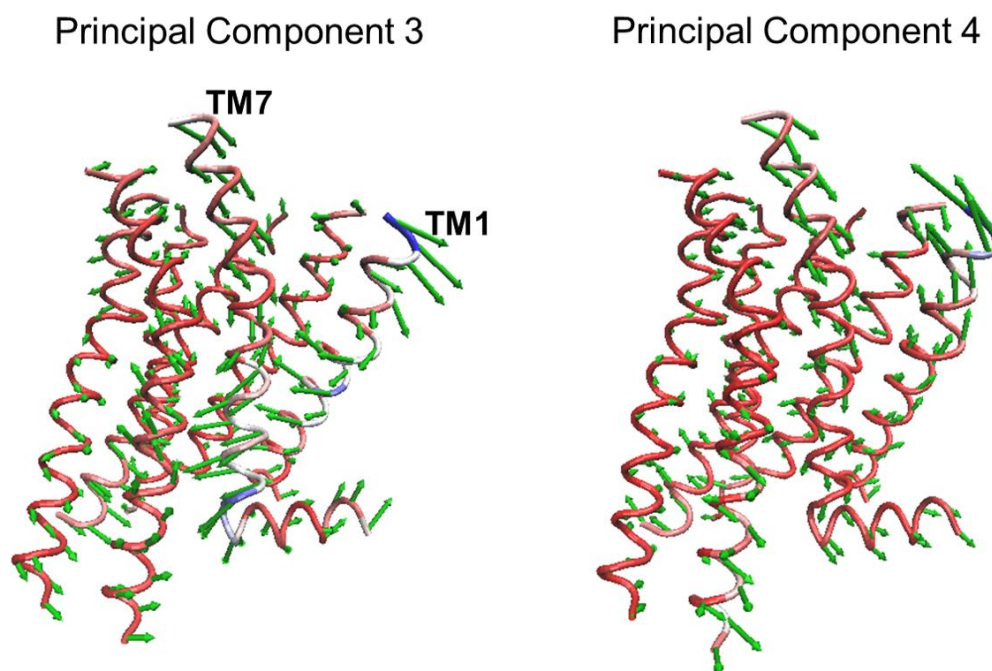
Figure S8. Conformations of principal components 3 and 4 in the MD trajectories.

Direction of mobility of each Cα atom is indicated by an arrow.

Figure S9. Ligand binding responses of mutants of the 5-HT$_{2B}$R: (A) K53$^{1.32}$A, (B) K53$^{1.32}$E, and (C) K53$^{1.32}$R. Black dots and red curves are binding response and fitted data, respectively. K_D values were obtained from the fit curves.

Figure S10. Amino acid sequence alignment of 5-HTR subtypes. Positively charged and negatively charged residues are shown in blue and red, respectively. Residues that are likely to form a hydrogen bond based on side chain identities are shown in magenta.

References

(1) Chun, E., Thompson, A. A., Liu, W., Roth, C. B., Griffith, M. T., Katritch, V., Kunken, J., Xu, F., Cherezov, V., Hanson, M. A., and Stevens, R. C. (2012) Fusion partner toolchest for the stabilization and crystallization of G protein-coupled receptors. *Structure 20*, 967–976.

(2) Wacker, D., Wang, C., Katritch, V., Han, G. W., Huang, X.-P., Vardy, E., McCorvy, J. D., Jiang, Y., Chu, M., Siu, F. Y., Liu, W., Xu, H. E., Cherezov, V., Roth, B. L., and Stevens, R. C. (2013) Structural Features for Functional Selectivity at Serotonin Receptors. *Science. 340*, 615–619.

(3) Kawai, T., Caaveiro, J. M. M., Abe, R., Katagiri, T., and Tsumoto, K. (2011) Catalytic activity of MsbA reconstituted in nanodisc particles is modulated by remote interactions with the bilayer. *FEBS Lett. 585*, 3533–3537.

(4) Gautier, A., and Hinner, M. J. (2015) Site-specific biotinylation of purified proteins using BirA. *Methods Mol. Biol. 1266*, 171–184.

(5) Yijun Tang, Xiangqun Zeng, J. L. (2011) Surface Plasmon Resonance: An Introduction to a Surface Spectroscopy Technique. *J Chem Educ 87*, 742–746.

(6) Myszka, D. G. (1997) Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors. *Curr. Opin. Biotechnol. 8*, 50–57.

(7) Jerabek-Willemsen, M., Wienken, C. J., Braun, D., Baaske, P., and Duhr, S. (2011) Molecular Interaction Studies Using Microscale Thermophoresis. *Assay Drug Dev. Technol. 9*, 342–353.

(8) Bartoschik, T., Galinec, S., Kleusch, C., Walkiewicz, K., Breitsprecher, D., Weigert, S., Muller, Y. A., You, C., Piehler, J., Vercruysse, T., Daelemans, D., and Tschammer, N. (2018) Near-native, site-specific and purification-free protein labeling for quantitative protein interaction analysis by MicroScale Thermophoresis. *Sci. Rep. 8*, 4977.

(9) Ashok, Y., and Jaakola, V. P. (2016) Nanodisc-Tm: Rapid functional assessment of nanodisc reconstituted membrane proteins by CPM assay. *MethodsX 3*, 212–218.

(10) Abraham, M. J., Murtola, T., Schulz, R., Páll, S., Smith, J. C., Hess, B., and Lindah, E. (2015) Gromacs: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. *SoftwareX 1–2*, 19–25.

(11) Huang, J., Rauscher, S., Nawrocki, G., Ran, T., Feig, M., de Groot, B. L., Grubmüller, H., and MacKerell, A. D. (2017) CHARMM36m: an improved force field for folded and intrinsically disordered proteins. *Nat. Methods 14*, 71–73.

(12) MacKerell, A. D., Feig, M., and Brooks, C. L. (2004) Improved treatment of the

protein backbone in empirical force fields. *J. Am. Chem. Soc. 126*, 698–699.

(13) Jo, S., Kim, T., Iyer, V. G., and Im, W. (2008) CHARMM-GUI: a web-based graphical user interface for CHARMM. *J. Comput. Chem. 29*, 1859–1865.

(14) Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera - A visualization system for exploratory research and analysis. *J. Comput. Chem. 25*, 1605–1612.

(15) Humphrey, W., Dalke, A., and Schulten, K. (1996) VMD: Visual Molecular Dynamics. *J. Mol. Graph. 14*, 33–38.

(16) Kim, T., Lee, K. Il, Morris, P., Pastor, R. W., Andersen, O. S., and Im, W. (2012) Influence of hydrophobic mismatch on structures and dynamics of gramicidin A and lipid bilayers. *Biophys. J. 102*, 1551–1560.

(17) Bakan, A., Dutta, A., Mao, W., Liu, Y., Chennubhotla, C., Lezon, T. R., and Bahar, I. (2014) Evol and ProDy for bridging protein sequence evolution and structural dynamics. *Bioinformatics 30*, 2681–2683.

(18) Bakan, A., Meireles, L. M., and Bahar, I. (2011) ProDy: Protein dynamics inferred from theory and experiments. *Bioinformatics 27*, 1575–1577.

(19) Lomize, M. A., Pogozheva, I. D., Joo, H., Mosberg, H. I., and Lomize, A. L. (2012) OPM database and PPM web server: Resources for positioning of proteins in membranes. *Nucleic Acids Res. 40*, 370–376.