G-protein-coupled receptors (GPCRs) constitute the largest family of membrane receptors in eukaryotes. Crystal structures have provided insight into GPCR interactions with ligands and G proteins\textsuperscript{3,4}, but our understanding of the conformational dynamics of activation is incomplete. Metabotropic glutamate receptors (mGluRs) are dimeric class C GPCRs that modulate neuronal membrane domain via a cysteine-rich domain, and LBD closure which contains the ligand-binding site, is coupled to the trans-membrane domain. A ‘clamshell’ ligand-binding domain (LBD), which contains the ligand-binding site, is coupled to the trans-membrane domain via a cysteine-rich domain, and LBD closure seems to be the first step in activation\textsuperscript{5,6}. Crystal structures of isolated mGLUR LBD dimers led to the suggestion that activation also involves a reorientation of the dimer interface from a ‘relaxed’ to an ‘active’ state\textsuperscript{5,6}, but the relationship between ligand binding, LBD closure and dimer interface rearrangement in activation remains unclear. Here we use single-molecule fluorescence resonance energy transfer to probe the activation mechanism of full-length mammalian group II mGluRs. We show that the LBDs interconvert between three conformations: resting, activated and a short-lived intermediate state. Orthosteric agonists induce transitions between these conformational states, with efficacy determined by occupancy of the active conformation. Unlike mGluR2, mGluR3 displays basal dynamics, which are Ca\textsuperscript{2+}\textsuperscript{7}\textsuperscript{,8}-dependent and lead to basal protein activation. Our results support a general mechanism for the activation of mGluRs in which agonist binding induces closure of the LBDs, followed by dimer interface reorientation. Our experimental strategy should be widely applicable to study conformational dynamics in GPCRs and other membrane proteins.

Single-molecule fluorescence resonance energy transfer (smFRET) spectroscopy is a powerful tool for high-resolution probing of protein conformational change\textsuperscript{9,10,12}, and was recently applied to study membrane proteins\textsuperscript{10–12}. To visualize ligand-induced rearrangements of full-length mGluRs, we used previously described amino-terminal SNAP- or CLIP-tagged proteins (Fig. 1a), permitting the selective and orthogonal introduction of either a FRET donor or an acceptor fluorophore into each subunit of the dimer, near the LBD\textsuperscript{13,14}. Electrophysiological recordings in cells co-expressing the G-protein-gated inward rectifier potassium channel (GIRK) showed that these constructs were physiologically functional (Extended Data Fig. 1a). SNAP–mGluR2 and CLIP–mGluR2 were expressed in HEK293T cells and labelled with FRET donor (DY-547) and acceptor (Alexa-647) fluorophores, respectively (Methods and Extended Data Fig. 1b). Glutamate induced a concentration-dependent decrease in ensemble FRET (Extended Data Fig. 1c, d), as previously shown\textsuperscript{15}. For the smFRET assay, we used single-molecule pull-down\textsuperscript{16} with an anti-carboxy-terminal antibody for in situ immunopurification of labelled receptors from HEK293T cell lysate, followed by total internal reflection fluorescence microscopy (Fig. 1b and Extended Data Fig. 2a). The pull-down was specific, mGluR2 remained a dimer after pull-down (Extended Data Fig. 2b, c), and there was no cross labelling between the SNAP and CLIP tags (Extended Data Fig. 2d).

In the absence of glutamate, the smFRET efficiency was \(\sim 0.45\) (Fig. 1c, top), and saturating glutamate (1 mM) shifted this to \(\sim 0.2\) (Fig. 1c, bottom), consistent with ensemble FRET (Extended Data Fig. 1c). Both the 0 and 1 mM glutamate states were stable within our time resolution (30 ms), with few transitions to other FRET levels. However, at intermediate glutamate concentrations, mGluR2 displayed rapid transitions between three distinct states: the 0.45 (high) FRET level seen in 0 mM glutamate, the 0.2 (low) FRET level seen in 1 mM glutamate, and a short-lived 0.35 (medium) FRET level (Fig. 1d, e and Extended Data Fig. 3a). The competitive antagonist LY341495 produced a similar FRET histogram to that seen in 0 mM glutamate: a major high FRET peak (0.45) and a minor medium FRET peak (0.35) (Fig. 1e, bottom). About 20% of individual FRET trajectories showed visits to the low FRET state in 0 mM glutamate (Extended Data Fig. 3b), but these transitions were rare and brief and, thus, almost undetectable in the FRET histograms (Fig. 1e, top). Control experiments with an antibody against the mGluR2 N terminus instead of the C terminus showed identical histograms (Extended Data Fig. 2e, f). Moreover, the application of GTP or apyrase, to favour receptor association or dissociation from G proteins, respectively, did not alter the smFRET histograms (Extended Data Fig. 2g), indicating that G proteins are not co-immunoprecipitated with mGluR2.

Because mGluR2 did not induce G-protein signalling in the presence of 0 mM glutamate or LY341495 (Extended Data Fig. 3c), we proposed that the high and medium FRET states represent functionally inactive conformations, and that the low FRET state corresponds to the active state. Consistent with this interpretation, the low FRET state glutamate concentration-dependence had a half-maximum effective concentration (EC\textsubscript{50}) value of 5.7 \(\pm\) 0.3 \(\mu\text{M}\) (mean \(\pm\) s.e.m.) (Fig. 1f and Extended Data Fig. 3d), corresponding to the concentration-dependence of GIRK current activation in HEK293T cells (3.2 \(\pm\) 0.3 \(\mu\text{M}\) (Extended Data Fig. 3e). Moreover, glutamate had no effect on the FRET histogram in the glutamate-insensitive mutant mGluR2-YADA (Tyr216Ala, Asp295Ala)\textsuperscript{17} (Extended Data Fig. 3g). Finally, the addition of LY341495 to glutamate abolished the low FRET state (Extended Data Fig. 3h). These observations confirm the assignment of the low FRET peak to the active conformation.

We next quantified the glutamate-induced fluctuations with cross-correlation and dwell-time analyses. The cross-correlation amplitude of donor and acceptor signals increased with glutamate concentration, reaching a maximum near the EC\textsubscript{50} value, and decreased at high glutamate concentrations (Fig. 1g). Anti-correlation between donor and acceptor was almost abolished in saturating glutamate (1 mM), confirming the stabilization of the active state. Kinetic analysis of individual traces showed an \(\sim 84\)-ms active state dwell time that was nearly independent of glutamate concentration (Fig. 1h and Extended Data Fig. 3i), suggesting that the active state dwell time reflects the glutamate dissociation rate and the inherent stability of the active state.

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conformation. Two-dimensional histograms obtained from synchronized transitions into or out of the low FRET state showed a short dwell at the medium FRET value of \( \sim 0.35 \), providing additional evidence that this state is an intermediate in the activation pathway (Fig. 1i and Extended Data Fig. 3).

Next, we investigated the relationship between ligand efficacy and receptor conformation. We studied two group II mGluR agonists, the efficacies of which differ from that of glutamate\(^1\) (Fig. 2a). While DCG-IV increased the occupancy of the low FRET state, even at saturating concentrations \((\geq 100 \mu M)\), the low FRET state was not fully occupied and \(\sim 30\%\) of the distribution remained in the high and medium FRET states (Fig. 2b). Single-molecule traces in saturating DCG-IV showed recurrent transitions out of the low FRET state (Fig. 2c, d and Extended Data Fig. 4a). By contrast, the full-agonist LY341495 showed a large decrease in the glutamate-free GIRK current (Fig. 3b, c; Extended Data Fig. 6a) and underwent a glutamate-dependent decrease in ensemble FRET (Extended Data Fig. 5), further supporting the role of active state occupancy in determining agonist efficacy, and confirming that the immobilized receptors retain TMD function and coupling to the LBD.

We next wondered whether the properties of mGluR2 apply to other mGluRs. We turned to the other group II mGluR, mGluR3, which possesses \(\sim 70\%\) sequence identity with mGluR2. SNAP- or CLIP-tagged mGluR3 constructs were physiologically functional (Extended Data Fig. 6a) and underwent a glutamate-dependent decrease in ensemble FRET similar to mGluR2, but with a lower EC\(_{50}\) value \((0.5 \pm 0.2 \mu M)\). Notably, single-molecule trajectories of mGluR3 in 0 mM glutamate exhibited frequent transitions between the three FRET states (Fig. 3a, top), resulting in \(\sim 30\%\) occupancy of the low FRET active state (Extended Data Fig. 6b, c), which was eliminated by LY341495 (Fig. 3a, bottom; Extended Data Fig. 6b, c). LY341495 produced a large decrease in the glutamate-free GIRK current (Fig. 3b, c and Extended Data Fig. 6d) and ensemble FRET (Extended Data

Figure 1  A single-molecule FRET assay reveals three conformations of the mGluR2 activation pathway. a, Crystal structures of mGluR1 in the ‘relaxed’ (Protein Data Bank (PDB) accession 1EWK) and ‘active’ states (PDB code 1EWK) show an increase in the distance between N termini after activation. Red and green ovals show the approximate positions of SNAP and CLIP tags, respectively. b, Schematic of single-molecule FRET measurements. Ab, antibody; Donor (green) and acceptor (red) intensity time traces and FRET trace (blue) in the absence (top) or presence (bottom) of 1 mM glutamate show a decrease in FRET in the presence of saturating glutamate. a.u., arbitrary units. c, Representative smFRET traces at 4 \(\mu M\) glutamate in the absence of glutamate (black) or in saturating glutamate (1 mM, green), but enhanced dynamics at intermediate concentrations (8 \(\mu M\), magenta). d, Representative smFRET traces at 4 \(\mu M\) glutamate reveal rapid dynamics between three states. A three-state fit obtained from hidden Markov analysis is overlaid over the filtered raw data. e, smFRET histograms in the presence of a range of glutamate concentrations or a competitive antagonist (LY341495). Blue lines show global three-component Gaussian fits that show the high (\(\sim 0.45\)), medium (\(\sim 0.35\)) and low (0.2) FRET states. The sum of all three components is shown in red. f, Titration curve for the low FRET peak. \(n_E\), Hill coefficient. g, Cross-correlation plots show limited dynamics in the absence of glutamate (black) or in saturating glutamate (1 mM, green), but enhanced dynamics at intermediate concentrations (8 \(\mu M\), magenta). Solid lines show single exponential fits. h, Concentration dependence of low FRET dwell times obtained from dwell time analysis. i, FRET density plots constructed from synchronized transitions from the high to low FRET states show a short dwell at the medium FRET level (yellow box). Error bars are s.e.m.
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Fig. 2 | Conformational basis of partial agonism of mGluR2. a, Ensemble FRET titrations in HEK293T cells expressing mGluR2 in the presence of glutamate (red), DCG-IV (blue) or LY379268 (black). FRET values in each condition are normalized to the response to 1 mM glutamate. b, smFRET histogram for DCG-IV shows the same three states as seen for glutamate (dotted lines), with dose-dependent occupancy of the low FRET state. c, Representative smFRET trace shows transitions out of the low FRET state in saturating DCG-IV. d, Cross-correlation plots for saturating agonist reveal dynamics = DCG-IV > glutamate > LY379268. Solid lines show single exponential fits. e, smFRET histograms for saturating agonist; occupancy of the low FRET state = DCG-IV < glutamate < LY379268 (inset). *P = 0.008, two-tailed t-test. f, g, At concentrations that result in comparable population of the active state, LY379268 induces slower dynamics than DCG-IV and glutamate as shown in cross-correlation and fit to a single exponential function (f) and representative smFRET traces (g). h, LY379268 induces significantly longer low FRET state dwell times than glutamate and DCG-IV (two-tailed t-test, *P = 0.0084). Error bars are s.e.m.

Fig. 3 | mGluR3 has high basal structural dynamics and activity. a, Representative mGluR3 smFRET traces show basal dynamics in the absence of glutamate (top) that are abolished by the competitive antagonist LY341495 (bottom). b, In HEK293T cells co-expressing GIRQ channels, mGluR3 has basal activity in the absence of glutamate, which is blocked by LY341495. c, Basal activity ([I_{LY341495}]/([I_{LY341495}] + [I_{glutamate}]) for mGluR2 and mGluR3. Values in parentheses indicate number of cells tested. *P = 0.0097 (unpaired two-tailed t-test). d, smFRET histograms for mGluR3 show occupancy of the low FRET state that is abolished by the removal of Ca\(^{2+}\) or introduction of the Ser152Asp mutation. e, Representative smFRET traces for mGluR3 in the absence of Ca\(^{2+}\) with either 0 (top) or saturating (bottom) glutamate. f, Cross-correlation plots fit to a single exponential function for mGluR3. g, h, Dwell time of the active state for mGluR3 in the presence of glutamate (100 nM) or Ca\(^{2+}\) (2 mM) compared to mGluR2 (4 μM glutamate) (unpaired two-tailed t-test, *P = 0.00055). Error bars are s.e.m.
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Figure 4 | A three-state model of mGluR activation. a, b. Mutation of residue Lys240 at the lower lobe LBD dimer interface (blue marker) (a) decreases occupancy of the low FRET state at saturating (10 mM) glutamate (b). WT, wild type. c. Representative smFRET traces for Lys240A in the absence of glutamate show transitions out of the low FRET state. d, e. Heterodimers of wild-type mGluR2 and the glutamate-insensitive mGluR2 (YADA) mutant (d) show enhanced occupancy of the medium FRET state (e). f, Representative smFRET traces for WT/YADA heterodimers show transitions between the high and medium FRET states with limited visits to the low FRET state. g. Three-state structural model of mGluRs based on intra-subunit (closed ‘C’ to open ‘O’) and inter-subunit (relaxed ‘R’ to active ‘A’) conformational changes. Error bars are s.e.m.

active conformation of mGluR3 is more stable than in mGluR2. Consistent with this, the time scale of donor and acceptor cross-correlation for mGluR3 in all conditions was much slower than for mGluR2 (Extended Data Fig. 9c). Overall, smFRET measurements revealed that mGluR3 transitions between the same three FRET states as mGluR2. However, unlike mGluR2, mGluR3 is Ca²⁺-sensitive and therefore basally active under physiological conditions.

Having observed three ligand-dependent FRET states in the LBDs of both mGluR2 and mGluR3, we sought to identify the underlying conformational rearrangements in the activation pathway. The available structures of mGluR LBDs have been characterized as either ‘relaxed’, with the lower lobes of the LBD far apart, or ‘active’, with the lower lobes of the LBD closer to one other (Fig. 4a and Extended Data Fig. 10a). We proposed that electrostatic interactions between charged residues in the lower lobe, including a conserved lysine (Lys240 in mGluR2), stabilize the active conformation. Indeed, neutralizing Lys240 (Lys240Aa) decreased the apparent affinity for glutamate in both ensemble FRET and GIRK activation assays in cells (Extended Data Fig. 10b). In the smFRET assay, even at saturating glutamate concentrations (10 mM), this mutant populated the low FRET state less than wild-type mGluR2 (Fig. 4b). Single-molecule trajectories showed frequent transitions out of the low FRET state in saturating glutamate (Fig. 4c and Extended Data Fig. 10c), consistent with destabilization of the active state. These results support the idea that the lower lobes of mGluR2 come into close proximity in the active state and help to stabilize it.

We next investigated the conformations corresponding to the high (0.45) and medium (0.35) FRET states. Considering the comparatively small distance change between high and medium FRET states (~4 Å) compared to medium and low FRET states (~8 Å), and the observation that the medium FRET state seems to be inactive, we proposed that the medium state corresponds to a relaxed conformation in which only one LBD has closed. If this is true, an mGluR2 heterodimer composed of a wild-type subunit and a YADA subunit (wild-type/YADA) is expected to bind glutamate only in the wild-type LBD, and therefore populate the middle FRET state more than the wild-type homodimer. smFRET analysis showed that at near-saturating concentrations for the wild-type subunit, wild-type/YADA had an ~55% occupancy of the medium FRET state, whereas wild-type/wild-type had a maximal medium FRET occupancy of ~25% (Fig. 4d, e and Extended Data Fig. 10d–f). Unlike the mono-phasic concentration-dependence of occupancy of the low FRET state seen in wild-type mGluR2 (Fig. 1f), wild-type/YADA showed a biphasic distribution (Extended Data Fig. 10g), resembling the previously reported dose-response of effector activation by the wild-type/YADA heterodimer of mGluR5 (ref. 19), and supporting the assignment of the low smFRET conformation to the active state. smFRET traces at 50 μM glutamate showed numerous transitions between the high and medium FRET states with rare and brief visits to the low FRET state (Fig. 4f and Extended Data Fig. 10h), consistent with the activity of this heterodimer, and possibly due to occasional binding of glutamate to the YADA subunit or spontaneous closure of the YADA subunit in the absence of glutamate.

Our kinetic, mutational and functional analyses indicate that mGluR2 and mGluR3 undergo ligand-dependent fluctuations between three conformations: a resting and inactive O–O/R conformation, an active C–C/A conformation and an intermediate inactive short-lived C–O/R conformation (in which ‘C’ denotes ‘closed’, ‘O’ denotes ‘open’, ‘R’ denotes ‘relaxed’ and ‘A’ denotes ‘active’) (Fig. 4g). The relative instability of the intermediate conformation may explain why crystal structures have not been obtained in the C–O/R state.

Electrostatic interactions at the lower lobe LBD interface stabilize the active state, suggesting that mGluR activation requires closure of both LBDs followed by rearrangement of the dimer interface (Fig. 4g) and consistent with findings that activation requires downstream reorientation at the inter-subunit interfaces between cysteine-rich domains and TMDs25–26. We observed occupancy times of tens of milliseconds to seconds—on the timescale of G protein signalling56—for both the O–O/R and C–C/A conformations. This is longer than the sub-millisecond fluctuations seen in isolated LBDs using diffusion-based FRET57, suggesting that the TMD of the intact receptor stabilizes the LBDs in the R and A states, giving the receptors a wide dynamic range of activity. Our findings suggest that the fractional occupancy of the C–C/A conformation determines agonist efficacy, consistent with isolated LBD single-molecule spectroscopy58 and crystal structures, in
which the degree of closure is similar for full and partial agonists. Finally, we revealed kinetic differences between mGluR2 and mGluR3 and found that mGluR3 has a more stable active state and is activated by physiological concentrations of external Ca\(^{2+}\), resulting in considerable basal G-protein signalling in cells. Our study provides a framework for investigating the activation mechanisms of other bi-lobed, clamshell LBDs, such as in the GABAB receptor and ionotropic neurotransmitter receptors.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Rasmussen, S. G. et al. Crystal structure of the \(\beta_2\) adrenergic receptor-Gs protein complex. Nature 477, 549–555 (2011).
2. Katritch, V., Cheerezov, V. & Stevens, R. C. Structure-function of the G protein-coupled receptor superfamily. Annu. Rev. Pharmacol. Toxicol. 53, 531–556 (2013).
3. Conn, P. J. & Pin, J. P. Pharmacology and functions of metabotropic glutamate receptors. Annu. Rev. Pharmacol. Toxicol. 37, 205–237 (1997).
4. Niswender, C. M. & Conn, P. J. Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annu. Rev. Pharmacol. Toxicol. 50, 295–322 (2010).
5. Knaazef, J. et al. Locking the dimeric GABA\(\beta\)-G protein-coupled receptor in its active state. J. Neurosci. 24, 370–377 (2004).
6. Kumar, J. & Mayer, M. L. Functional insights from glutamate receptor ion channel structures. Annu. Rev. Physiol. 75, 313–337 (2013).
7. Kunishima, N. et al. Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. Nature 407, 971–977 (2000).
8. Tsuchiya, D., Kunishima, N., Kamiya, N., Jingami, H. & Morikawa, K. Structural views of the ligand-binding cores of a metabotropic glutamate receptor complexed with an antagonist and both glutamate and Gd\(^3+\). Proc. Natl Acad. Sci. USA 99, 2663–2668 (2002).
9. Roy, R., Hohng, S. & Ha, T. A practical guide to single-molecule FRET. Nature Methods 5, 507–516 (2008).
10. Zhao, Y. et al. Single-molecule dynamics of gating in a neurotransmitter transporter homologue. Nature 465, 188–193 (2010).
11. Bockenhauer, S., Furstenberg, A., Yao, X. J., Koblika, B. K. & Moerner, W. E. Conformational dynamics of single G protein-coupled receptors in solution. J. Phys. Chem. B 115, 13328–13338 (2011).
12. Morrison, E. A. et al. Antiparallel EmrE exports drugs by exchanging between asymmetric structures. Nature 481, 45–50 (2011).
13. Keppler, A. et al. A general method for the covalent labeling of fusion proteins with small molecules in vivo. Nature Biotechnol. 21, 86–89 (2003).
14. Doumazane, E. et al. A new approach to analyze cell surface protein complexes reveals specific heterodimeric metabotropic glutamate receptors. FASEB J. 25, 66–77 (2011).
15. Doumazane, E. et al. Illuminating the activation mechanisms and allosteric properties of metabotropic glutamate receptors. Proc. Natl Acad. Sci. USA 110, E1416–E1425 (2013).
16. Jain, A. et al. Probing cellular protein complexes using single-molecule pull-down. Nature 473, 484–488 (2011).
17. Kniazeff, J.-F. et al. Closed state of both binding domains of homodimeric mGlu receptors is required for full activity. Nature Struct. Mol. Biol. 11, 706–713 (2004).
18. Jin, R., Banke, T. G., Mayer, M. L., Traynelis, S. F. & Gouaux, E. Structural basis for partial agonist action at ionotropic glutamate receptors. Nature Neurosci. 6, 803–810 (2003).
19. Yamashita, T., Kai, T., Terakita, A. & Shichida, Y. A novel constitutively active mutation in the second cytoplasmic loop of metabotropic glutamate receptor. J. Neurochem. 91, 484–492 (2004).
20. Yanagawa, M., Yamashita, T., Shichida, Y. Activation switch in the transmembrane domain of metabotropic glutamate receptor. Mol. Pharmacol. 76, 201–207 (2009).
21. Kubo, Y., Miyashita, T. & Murata, Y. Structural basis for a Ca\(^2+\)-sensing function of the metabotropic glutamate receptors. Science 279, 1722–1725 (1998).
22. Nash, M. S., Saunders, R., Young, K. W., Challiss, R. A. & Nahorski, S. R. Reassessment of the Ca\(^2+\) sensing property of a type I metabotropic glutamate receptor by simultaneous measurement of inositol 1,4,5-trisphosphate and Ca\(^2+\) in single cells. J. Biol. Chem. 276, 19286–19293 (2001).
23. Tateyama, M., Abe, H., Nakata, H., Saito, O. & Kubo, Y. Ligand-induced rearrangement of the dimeric metabotropic glutamate receptor 1alpha. Nature Struct. Mol. Biol. 11, 637–642 (2004).
24. Huang, S. et al. Interdomain movements in metabotropic glutamate receptor activation. Proc. Natl Acad. Sci. USA 108, 15480–15485 (2011).
25. Hlavackova, V. et al. Sequential inter- and intrasubunit rearrangements during activation of dimeric metabotropic glutamate receptor 1. Sci. Signal. 5, ra59 (2012).
26. Xue, L. et al. Major ligand-induced rearrangement of the heptahedral domain interface in a GPCR dimer. Nature Chem. Biol. 11, 134–140 (2015).
27. Lohse, M. J., Maielaro, I. & Calebier, D. Kinetics and mechanism of G protein-coupled receptor activation. Curr. Opin. Cell Biol. 27, 87–93 (2014).
28. Olofsson, L. et al. Fine tuning of sub-millisecond conformational dynamics controls metabotropic glutamate receptors agonist efficacy. Nature Commun. 5, 5206 (2014).
29. Muto, T., Tsuichiya, D., Morikawa, K. & Jingami, H. Structures of the extracellular regions of the group II/III metabotropic glutamate receptors. Proc. Natl Acad. Sci. USA 104, 3759–3764 (2007).

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to E.I.Y. (ehud@berkeley.edu).
METHODS

No statistical methods were used to predetermine sample size. Experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment.

Cell culture. HEK293T cells were cultured in DMEM with 5% FBS on poly-L-lysine-coated glass coverslips. HEK293T cells were obtained from the UC Berkeley MCB tissue culture facility, authenticated by DDC Medical, and tested negative for mycoplasma contamination. Previously described HA–SNAP and Flag–CLIP-tagged rat mGluR2 DNA were provided by J. P. Pin. DNA plasmids were transfected into cells using lipofectamine 2000 (Sigma). For electrophysiology experiments, cells were transfected with wild-type-mGluR2 or wild-type-mGluR3, GIRQ1-F137S, and yellow fluorescent protein (YFP) (as a transfection marker) at a 7:7:1 ratio with 0.7 μg plasmid per well for receptor and channel. For FRET experiments, cells were transfected with SNAP and CLIP-tagged constructs at a ratio of 1:2 with 0.3 μg of SNAP–mGluR2 DNA per well.

Patch clamp electrophysiology. Whole-cell patch clamp recordings from single isolated cells were performed 24–48 h after transfection in a high potassium extracellular solution containing (in mM): 120 KCl, 29 NaCl, 2 CaCl2, 1 MgCl2, 50 HEPES, pH 7.4. Cells were voltage clamped to −60 mV using an Axopatch 200B amplifier (Axon Instruments) and membrane currents were recorded. Glass pipettes of resistance between 3 and 8 MΩ were filled with intracellular solution containing (in mM): 140 KCl, 10 HEPES, 3 Na2ATP, 0.2 Na2GTP, 200B amplifier (Axon Instruments) and membrane currents were recorded. Glass pipettes of resistance between 3 and 8 MΩ were filled with intracellular solution containing (in mM): 140 KCl, 10 HEPES, 3 Na2ATP, 0.2 Na2GTP, 5 EGTA and 3 MgCl2, pH 7.4. Data were acquired with a 2 kHz acquisition rate and filtered with the amplifier 4-pole Bessel filter at 1 kHz. Data acquisition and analysis were performed using pCLAMP 10 software (Axon Instruments).

Ensemble FRET. Approximately 24–48 h after transfection, cells were labelled while attached to poly-L-lysine-coated coverslips. Culture media was removed and coverslips were washed and transferred to extracellular solution containing (in mM): 135 NaCl, 5.4 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, pH 7.4. Cells were labelled at 37 °C with 2.5 μM benzylguanine Alexa-647 (NEB) for 45 min followed by 5 μM benzylcytosine DY-547 (NEB) for 45 min. The fluorophores were diluted in extracellular solution and coverslips were washed in between labelling with donor and acceptor. After labelling, cells were mounted on an upright, scanning confocal microscope (Zeiss LSM 780) and imaged with ×20 objective. Donor excitation was performed using a 561-nm laser and images were taken in the donor and acceptor channels at 1 Hz. Clusters of cells were analysed together and FRET was calculated as FRET = (I0)/(I0 + Ia), in which I0 is the fluorescence donor intensity, and Ia is the fluorescence acceptor intensity. For individual traces, FRET was normalized to the basal FRET value observed before application of any drugs. FRET changes calculated for dose–response curves were normalized to saturating glutamate (1 mM) and dose–response curves were obtained from multiple cell clusters and averaged from at least three experiments. Fitting of dose–response curves was performed using Prism (Graphpad). All drugs were purchased from Tocris and delivered with a gravity-driven perfusion system.

smFRET measurements. To inhibit nonspecific protein adsorption, flow cells for single-molecule experiments were prepared as previously described using mPEG (Laysan Bio) passivated glass coverslips (VWR) and doped with bee B PEGG3. Before each experiment, coverslips were incubated with NeutrAvidin (Thermo), followed by 10 nM biotinylated secondary antibody (donkey anti-rabbit, Jackson Immunoresearch). For receptor immunopurification, 10 nM anti-mGluR2 primary antibody (Cell Signaling, 12056) or 10 nM anti-mGluR3 secondary antibody (abcam, ab26228) was incubated in the chamber (Fig. 1c). Between each conjugation step, the chambers were flushed to remove free reagents. The antibody dilutions and washes were done in T50 buffer (50 mM NaCl, 10 mM Tris, pH 7.5).

For single-molecule experiments, fresh cells expressing tagged mGluR constructs were labelled as described above. After labelling, cells were recovered from coverslips by incubating with Ca2+−free PBS buffer for 20–30 min followed by gentle pipetting. Cells were then pelleted and lysed in the lysis buffer consisting of 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail (Thermo Scientific) and 1.2% IGEPAL (Sigma) or 1% n-dodecyl-B-D-maltoside (DDM) (Anatrace). After 1 h incubation at 4 °C, cells were centrifuged at 16,000g for 20 min and supernatant was collected and kept on ice. The cell lysate was diluted to achieve sparse immobilization of labelled receptors on the surface (ranging from 5× to 50× dilution depending on the expression and labelling efficiency) and applied to coverslips. After achieving optimum surface immobilization (~400 molecules in a 2,000 μm2 imaging area), unbound receptors were washed out of the flow chamber and the flow cells were then washed extensively (up to 5× the cell volume). Finally, receptors were imaged in the imaging buffer consisting of (in mM) 3’Trolsox, 120 KCl, 29 NaCl, 2 CaCl2, 1 MgCl2, 50 HEPES, 0.04% IGEPAL and an oxygen scavenging system (0.8% dextrose, 0.8 mg ml−1 glucose oxidase, and 0.02 mg ml−1 catalase), pH 7.4. Reagents were purchased from Sigma and were all UltraPure grade (purity >99.99%). All buffers were made in UltraPure distilled water (Invitrogen). For the experiments done in the absence of Ca2+, 10 mM EGTA and 1 mM MgCl2 were added to the imaging buffer. Catalase was diluted in T50 buffer and passed through a spin column 3% (BioRad). To ensure lack of glutamate contamination for the experiments done in the absence of glutamate further, the oxygen scavenging solution was treated with glutamic-pyruvic transaminase (Sigma) in the presence of 2 mM sodium pyruvate (Gibco) to remove possible trace levels of glutamate.

Samples were imaged with a ×60 objective (Olympus) on a total internal reflection fluorescence microscope with 30 ms time resolution unless stated otherwise. Lasers 532 nm (Cobolt) and 632 nm (Melles Griot) were used for donor or acceptor excitation, respectively. FRET efficiency was calculated as (I0−0.1Ib)/ (Ib + Ia), in which Ib and Ia are the donor and acceptor intensity after background subtraction.

smFRET data analysis. Single-molecule intensity traces showing single-donor and single-acceptor photobleaching with a stable total intensity for longer than 5 s were collected (20–30% of total molecules per imaging area). Individual traces were smoothed using a nonlinear filter from ref. 30 with filter parameters: window = 2, M = 2 and P = 15. Each experiment was repeated at least five times independently to ensure reproducibility of the results and one data set for each condition is presented. FRET histograms were compiled from at least 200 molecules per condition (30 ms time resolution). Error bars in the histograms represent the standard error from at least six independent movies. To ensure that traces with different length contribute equally, histograms from individual traces were normalized to one before compiling. Fitting to histograms was done in Origin Pro. Synchronized density plots were constructed from manually selected transitions that were synchronized to the data point where FRET signal drops below (forward transition) or above (reverse transition) the threshold of 0.3. The proportion of dynamic traces was calculated manually from the raw smFRET traces. Each molecule that exhibited at least one FRET transition (defined by anti-correlated changes in donor and acceptor signals) and lasted for at least two data points was counted as having dynamics. Dwell-time analysis was performed using unfiltered traces to avoid smoothing artefacts. First, donor and acceptor signals were each idealized to a two state signal with the value of idealized intensity obtained from the two main peaks in the intensity histogram. Idealized FRET was then calculated from idealized donor and acceptor signals and further analysed to calculate the active state dwell time. Owing to time resolution limitations, dwell-time measurement represents an upper limit estimate. All transitions were individually inspected to make sure the FRET transitions were real based on anti-correlation of the donor and acceptor intensity signals at the transition. The number of traces used in dwell-time analysis for mGlu2 with glutamate, LY379268 and DCG–IV are 53, 61 and 41 obtained from at least six independent movies and error bars represent the s.e.m. More than 300 transitions in each case were analysed. The hidden Markov fit in Fig. 2 was done using HaMMy41. The cross-correlation (CC)42 of donor and acceptor intensity times at time t is defined as CC(t) = dI(t)Δδd(t) + d(t − I(t)) + I(t)), in which δd(t) = I(t)− <I(t)> and δI(t) = I(t)−<I(t)}. δ(t) and <I(t)> are time average donor and acceptor intensities, respectively. The cross-correlation calculation was performed on the same traces that were used for the histogram. We fit the cross-correlation data to a single exponential function to obtain two parameters: the characteristic time of the exponential (τ) and the amplitude of the exponential.

Because of the low anisotropy of the donor and acceptor fluorophores when attached to these SNAP and CLIP tags43, and taking into account their spectral overlap, a Förster radius of 52 Å was used to estimate the distances.

30. Haran, G. Noise reduction in single-molecule fluorescence trajectories of folding proteins. Chem. Phys. 307, 137–145 (2004).
31. McKinney, S. A., Joo, C. & Ha, T. Analysis of single-molecule FRET trajectories using hidden Markov modeling. Biophys. J. 91, 1941–1951 (2006).
32. Zhou, R. et al. SSB functions as a sliding platform that migrates on DNA via reptation. Cell 146, 222–232 (2011).
Extended Data Figure 1 | Fluorophore labelling is specific and FRET constructs are functional in HEK293T cells. a, SNAP–mGluR2 shows glutamate-induced currents in cells co-expressing GIRK channels. b, Treatment with 2.5 μM benzylguanine Alexa-647 (for SNAP) followed by 5 μM benzylcytosine DY-547 (for CLIP) produces specific and orthogonal labelling of SNAP and CLIP–mGluR2 constructs in HEK293T cells. All conditions were imaged with identical settings in both the red (excitation = 635 nm) and green (excitation = 561 nm) channels. c, Ensemble FRET measurements from HEK293T cells. Top, image of cells expressing SNAP and CLIP–mGluR2 labelled with donor (DY-547, green) and acceptor (Alexa-647, red). Bottom, representative trace showing dose-dependent, reversible decrease in FRET after glutamate application. Glutamate was washed out between applications. d, Ensemble FRET glutamate titration in HEK293T cells. Error bars are s.e.m.
Extended Data Figure 2 | Control experiments verifying specificity of the smFRET assay. a, Representative TIRF image showing single receptors in donor and acceptor channels during donor excitation with a 532 nm laser. b, Single-molecule pull-down of SNAP–mGluR2 on a passivated surface is specific. Left, representative images of individual molecules in the absence or presence of an anti-mGluR2 antibody. Right, quantification of the number of molecules pulled down for each condition. c, Photobleaching step analysis shows that mGluR2 remains a dimer in single-molecule pull-down. Left, representative single-molecule bleaching steps for mGluR2–GFP. Right, histogram of bleaching step counts for all molecules. Dotted red line shows the predicted proportions for an 80% GFP maturation rate. d, Pulldown of lysate from cells expressing only SNAP–mGluR2 (c) or CLIP–mGluR2 (d), and labelled with both donor and acceptor fluorophores confirms labelling specificity at the single-molecule level. e, f, Pulldown of SNAP– and CLIP–mGluR2 via an antibody against an N-terminal HA-tag (e) leads to very similar smFRET histograms (f, filled circles) in the absence (black) or presence of 1 mM glutamate (green) compared to pull-down with a C-terminal antibody (f, open circles). g, Application of either GTP, to remove any co-assembled G proteins, or apyrase, to lock any G proteins onto mGluR2, does not alter smFRET histograms.
Extended Data Figure 3 | Further analysis of glutamate-induced smFRET and functional properties of mGluR2. a, Representative smFRET traces for mGluR2 in the presence of 4 µM or 8 µM glutamate. b, Quantification of the percentage of single-molecule traces showing at least one transition to the active state at different glutamate concentrations. c, In HEK293T cells co-expressing mGluR2 and GIRK, LY341495 prevents glutamate-induced inward currents without altering the baseline current. d, Glutamate titration curves produced from fitting FRET histograms to the sum of three Gaussian distributions. e, f, Glutamate induces inward currents via mGluR2 in a dose-dependent manner (n = 9 cells). g, The glutamate-insensitive mGluR2-YADA (Tyr216Ala, Asp295Ala) shows no smFRET response to 50 µM or 1 mM glutamate. h, smFRET histograms showing that application of the competitive antagonist LY341495 reverses the FRET change induced by glutamate. i, Dwell time analysis of mGluR2 for sub-saturating glutamate concentrations. Solid lines show single exponential fits to the data. j, FRET density plots constructed from synchronized transitions from the low to high FRET states show a short dwell at the medium FRET level (yellow box). Error bars are s.e.m.
Extended Data Figure 4 | Further analysis of the effects of orthosteric agonists on mGluR2 smFRET. a, Cross-correlation plot for mGluR2 in the presence of DCG-IV shows concentration-dependent dynamics. b, c, smFRET histogram (b) and cross-correlation plots (c) for mGluR2 in the presence of the full agonist LY379268. d, smFRET histograms in the presence of 1 μM glutamate, 100 nM DCG-IV and 2 nM LY379268 yields comparable occupancy of the active state. e, f, Representative smFRET traces for mGluR2 in the presence of 0.1 μM DCG-IV (e) or 2 nM LY379268 (f), which are the concentrations used for dwell-time analysis. g, h, FRET density plots constructed from the average transitions from the low to high states show a short dwell at the medium FRET level (yellow boxes) for mGluR2 in the presence of DCG-IV (g) or LY379268 (h).
Extended Data Figure 5 | TMD mutations that introduce basal activity and a positive allosteric modulator increase the affinity and efficacy of a partial agonist. a, Crystal structures of the mGluR1 TMD bound to a negative allosteric modulator (PDB code 4OR2) showing the location of conserved residues in TM4 and TM6 previously shown to be sensitive to mutations that induce basal activity. b, Ensemble FRET titrations showing that mutations Gln679Val and Cys770Ala increase the affinity and efficacy of DCG-IV compared to wild-type mGluR2. c, d, smFRET histograms for TMD mutants show population of the same three FRET states as the wild type, but with greater occupation of the low FRET state at either sub-saturating (c) or saturating (d) concentrations of DCG-IV. e, Binding of PAM LY487379 to the TMD of mGluR2 (top) increases the apparent affinity and efficacy of DCG-IV in ensemble FRET measurements in HEK293T cells. All values were normalized to the response to 1 mM glutamate. b, smFRET histograms showing a LY487379-induced shift in the response to 1 μM DCG-IV. Error bars are s.e.m.
Extended Data Figure 6 | Characterization of basic ensemble and smFRET properties of mGluR3 as compared to mGluR2.  a, Activation of GIRK by SNAP–mGluR3 in HEK293T cells. b, smFRET histograms for mGluR3 show glutamate-independent low FRET population. c, Cross-correlation plots for mGluR3 show glutamate-independent dynamics. d, Unlike mGluR3, mGluR2 shows zero or minimal current response to the antagonist LY341495 in the absence of glutamate. e, f, Ensemble FRET in HEK293T cells shows a robust antagonist LY341495-induced FRET increase in mGluR3 (e) but not in mGluR2 (f). g, smFRET histograms for mGluR3 with or without GTP treatment to dissociate any G proteins that may be coupled to the receptor. The time resolution for this data is 100 ms. Error bars are s.e.m.
Extended Data Figure 7 | Calcium sensitivity of mGluR3. a, b, Representative smFRET traces for mGluR3 in the absence (a) or presence (b) of 2 mM Ca^{2+}. c, smFRET histograms for mGluR3 in the presence of various concentrations of calcium. d, Cross-correlation plots for mGluR3 in the presence of various concentrations of calcium. e, FRET density plot for showing that Ca^{2+}-induced synchronized transitions show a similar intermediate in mGluR3, as observed in mGluR2. f, g, smFRET histograms (f) and cross-correlation plots (g) for mGluR2 in the presence of various concentrations of calcium.
Extended Data Figure 8 | mGluR3(Ser152Asp) shows decreased basal FRET and calcium sensitivity. a, Ensemble FRET glutamate titrations for mGluR3 and mGluR3(Ser152Asp). b, Representative ensemble FRET trace for mGluR3(Ser152Asp) shows no response to LY341495. c, Summary of basal FRET for mGluR3, mGluR2 and mGluR3-S152D. Basal FRET = \([\Delta\text{FRET}_{\text{LY341495}}]/(\Delta\text{FRET}_{\text{LY341495}} + \Delta\text{FRET}_{\text{Glu}})\). d, Representative smFRET traces for mGluR3(Ser152Asp) in the absence (left) or presence (right) of 2 mM Ca\(^{2+}\). e, smFRET histograms for mGluR3(Ser152Asp) in the absence or presence of Ca\(^{2+}\) or saturating glutamate. f, Cross-correlation plots for mGluR3(Ser152Asp). Inset shows the percentage of traces showing dynamics in different ligand conditions. Error bars are s.e.m.
Extended Data Figure 9 | Glutamate induced smFRET dynamics of mGluR3 in the absence of calcium. a, Representative smFRET traces for mGluR3 in the absence of Ca\(^{2+}\) and the presence of sub-saturating glutamate. b, smFRET histogram showing dose-dependent response of mGluR3 to glutamate in the absence of Ca\(^{2+}\). c, Cross-correlation plots showing glutamate-induced smFRET dynamics. mGluR2 (green) at its maximum dynamics shows a smaller cross-correlation amplitude compared to mGluR3.
Extended Data Figure 10 | Further characterization of mGluR2(Lys240Ala) and wild-type/YADA heterodimers. a, Crystal structures of mGluR1 in the relaxed (top; PDB code 1EWT) and active states (bottom; PDB code 1EWK) show a reorientation of the dimer interface that brings charged residues of the lower lobe in close proximity. Conserved negatively charged residues are shown in red, and Lys260 (Lys240 in mGluR2) is shown in blue. b, Glutamate titrations show a decreased apparent affinity for mGluR2(Lys240Ala) in ensemble FRET (top) and GIRK current activation (bottom). c, Cross-correlation plots in the presence of saturating glutamate show increased dynamics for mGluR2(Lys240Ala) relative to wild type. d, smFRET histogram showing distributions for wild-type/YADA heterodimers at a range of glutamate concentrations. e, Concentration-dependence of low FRET population in wild-type/YADA heterodimers produced from fitting FRET histograms to the sum of three Gaussian distributions. The EC50 for each phase of the distribution is shown. f, Concentration-dependence of medium FRET population in wild-type/YADA heterodimers and wild-type homodimers produced from fitting FRET histograms to the sum of three Gaussian distributions. g, Three-state fit to FRET histogram for wild-type/YADA heterodimers in the presence of 100 μM glutamate shows substantial population of the medium FRET (0.35) peak. h, Cross-correlation plots for wild-type/YADA heterodimers. Error bars are s.e.m.