Figure S1.

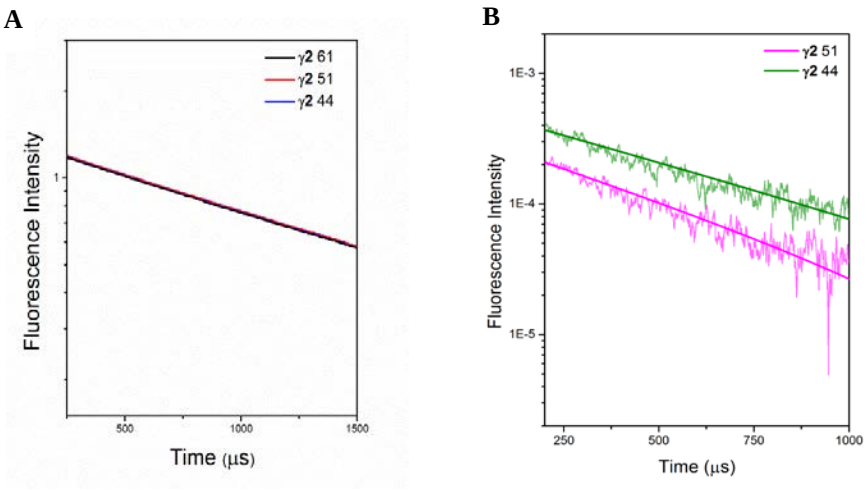

Figure S1. Measurement of AMPA receptor-stargazin distances, related to Figure 2. **A)** Donor-only measurements between the GluA2*Y128C and stargazin sites 44, 51 and 61. **B)** LRET lifetimes between GluA2 amino-terminal domain site 128 of the AMPA receptor in the desensitized (1mM glutamate bound) state and sites 44 (green) and 51 (magenta) in the extracellular region of stargazin (γ2) are shown. The donor fluorophore used was Terbium chelate and the acceptor fluorophore was fluorescein (R_0= 45 Å). From the lifetimes of 535.44 μs for site 44, and 446.29 μs for site 51, the distances between the two fluorophores determined *via* the Förster equation are 39.0 ± 0.04 Å for site 44, and 37.4 ± 0.03 Å for site 51.

Table S2.

Lifetimes and distances from LRET measurements between AMPA receptor amino-terminal domain and stargazin, related to Figure 2:

Site on γ2 extracellular domain	Donor-only Lifetime (μs)	Donor-Acceptor Lifetime (μs)	Distance (Å)
44	1792.9 ± 0.5	584.7 ± 9.8	39.9 ± 0.1
51	1796.2 ± 0.5	304.7 ± 2.8	34.5 ± 0.1
61	1793.9 ± 0.5	304.6 ± 4.5	49.9 ± 0.1

Table S2. Distance measurements from Y128C on the amino terminal domain of GluA2 homotetramer to specific sites on the extracellular domain of stargazin (γ2) are shown. The donor fluorophore used was Terbium chelate for all measurements, while the acceptor fluorophore was fluorescein (R_0= 45) for sites 44 and 51, and Alexa 555 (R_0= 65) for site 61. The error in the LRET lifetimes is the S. E. of the fit. Distances were calculated using the Förster Equation and the error was determined by the propagation of the lifetime errors (Eq. 1).

Figure S3.

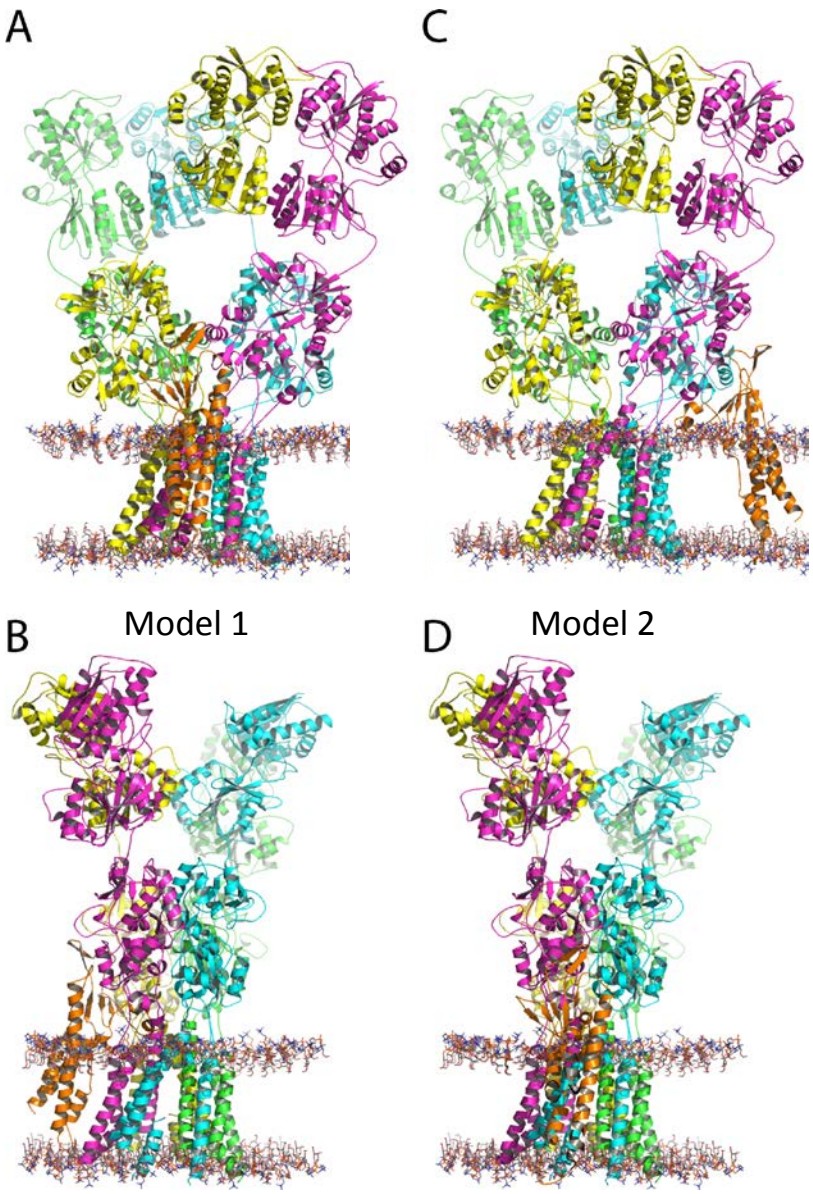

Figure S3. Potential poses of the AMPA receptor-stargazin interaction based on LRET measurements, related to Figure 2. Poses shown have stargazin placed near the B subunit at the A-B interface (Model 1, panels A, B), or near the A subunit at the A-D interface (Model 2, panels C, D). When placing Stargazin relative to the AMPA receptor, steric clashes, FRET-based distance constraints, and feasible orientations of the transmembrane helices of stargazin were all considered. While model 2 satisfies these parameters, note the large distance between the transmembrane helices of stargazing and the AMPA receptor, which may not be optimal for their functional relationship. Thus, Model 1 is our proposed model of the AMPA receptor-stargazin interaction based on our measurements of intact complexes in HEK-293 cells.

Figure S4.

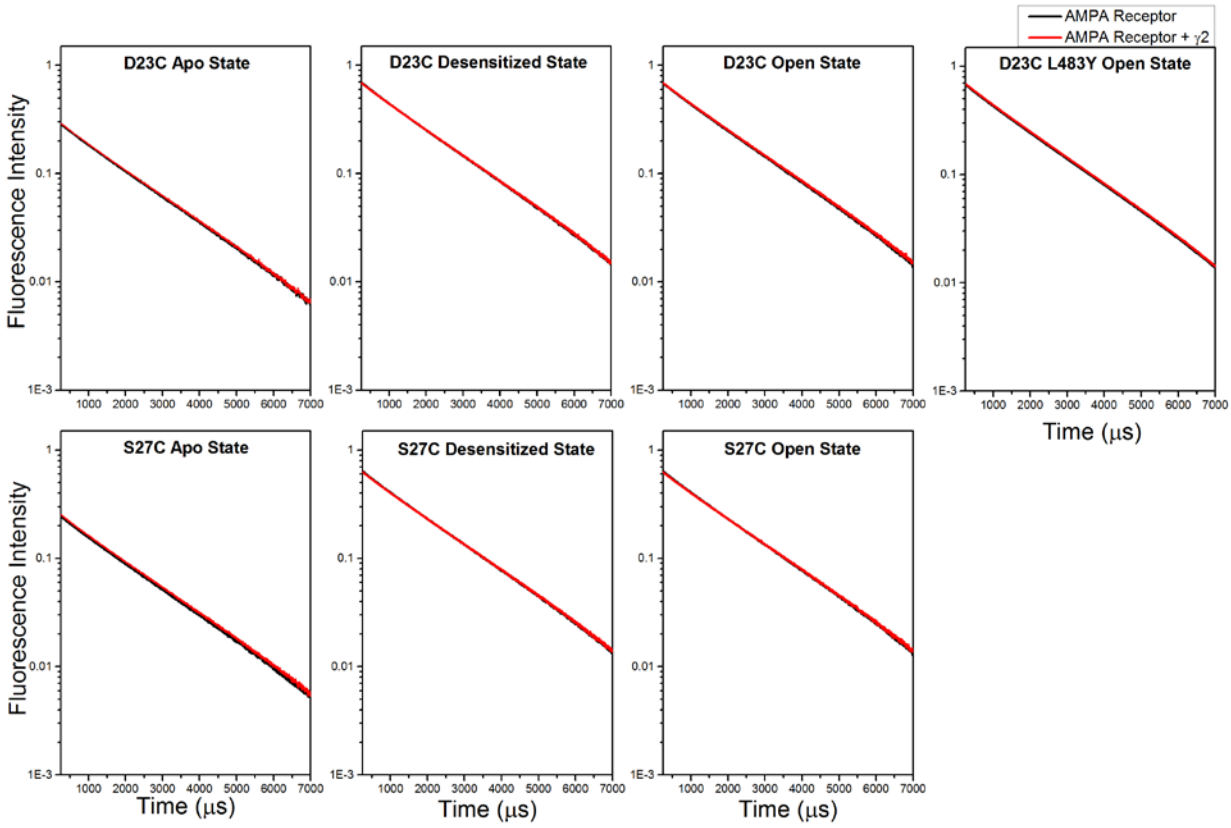

Figure S4. Donor-only measurements at sites 23 (top panel) and 27 (bottom panel) in the apo (resting) state, desensitized (1mM glutamate bound) state, and open (1mM glutamate + 100 μM cyclothiazide bound) state, related to Figure 2.

Table S5.

Lifetimes and distances from LRET measurements between AMPA receptor amino-terminal domain subunits in the presence and absence of γ2, related to Figure 3:

Sites between subunits B and D at the ATD	Ligand Conditions	Donor-only Lifetime (μs)	Donor-Acceptor Lifetime (μs)	Distance (Å)
D23 without γ2	Resting	1785.2 ± 0.6	536.4 ± 4.3	39.1 ± 0.1
	Desensitized	1768.4 ± 0.6	645.8 ± 4.9	41.0 ± 0.1
	CTZ Open	1773.1 ± 0.6	456.6 ± 4.3	37.7 ± 0.1
	L483Y Open	1753.1 ± 0.5	453.9 ± 1.2	37.8 ± 0.02
D23 with γ2	Resting	1794.2 ± 0.6	412.5 ± 1.1	36.8 ± 0.02
	Desensitized	1788.3 ± 0.5	459.1 ± 1.7	37.7 ± 0.02
	CTZ Open	1791.7 ± 0.5	407.1 ± 2.5	36.7 ± 0.04
	L483Y Open	1765.0 ± 0.5	412.1 ± 1.2	36.9 ± 0.02
S27 without γ2	Resting	1781.6 ± 0.6	602.1 ± 2.4	40.23 ± 0.003
	Desensitized	1777.8 ± 0.5	806.1 ± 3.7	43.6 ± 0.04
	CTZ Open	1777.1 ± 0.5	548.5 ± 1.8	39.3 ± 0.02
S27 with γ2	Resting	1795.4 ± 0.6	538.2 ± 2.7	39.1 ± 0.03
	Desensitized	1797.5 ± 0.5	588.3 ± 1.8	39.9 ± 0.02
	CTZ Open	1805.9 ± 0.5	499.5 ± 2.4	38.3 ± 0.03

LRET distance measurements between the proximal subunits at the amino-terminal domain (ATD) of GluA2 homotetramer in the presence and absence of γ2 are shown. The desensitized condition was obtained in the presence of 1 mM glutamate and open condition in the presence of 1 mM glutamate + 100 μM CTZ. The donor fluorophore

used was Terbium chelate and the acceptor fluorophore was fluorescein (R_0= 45). The error in the LRET lifetimes is the S. E. of the fit. Distances were calculated using the Förster Equation (Eq. 1), and error was calculated by propagation of the lifetime errors.

Figure S6.

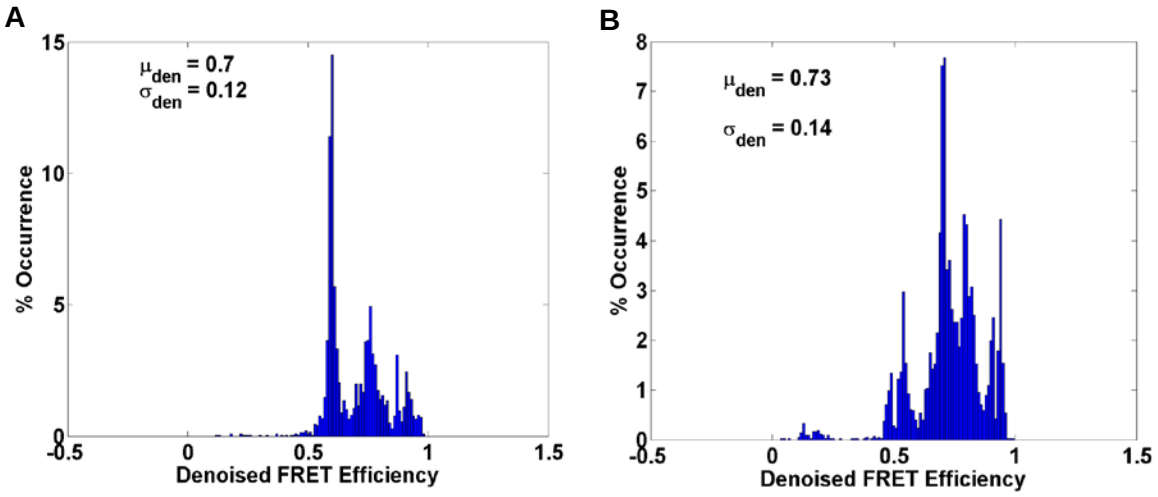

Figure S6. Denoised FRET efficiency for the AMPA receptor alone (GluA2*-D23C) (panel A) and the tandem AMPA receptor with stargazin (GluA2*-D23C/γ2) (panel B), both in the desensitized (1 mM glutamate bound) state.

Table S7.

FRET efficiency, corresponding FRET distance and occupancy of the significant states obtained by smFRET, related to Figure 4:

	States obtained	Occupancy %	Efficiency	FRET Distance (Å)	Cα-Cα Structure Distance (Å)
AMPA Receptor (D23C) Desensitized	State 1	17.0	0.902	35	
	State 2	35.8	0.754	42	44 (EMD 2687); 42 (PDB ID 4U4F)
	State 3	44.4	0.597	48	48 (EMD 2686)
	State 4	2.1	0.443	53	
AMPA Receptor (D23C) Open	State 1	68	0.894	36	
	State 2	30	0.789	41	
	State 3	2	0.501	51	
AMPA Receptor/γ2 (D23C/γ2) Desensitized	State 1	15.8	0.923	34	
	State 2	29.0	0.808	40	
	State 3	38.5	0.701	44	
	State 4	15.0	0.524	50	

smFRET measurements between site 23 of the proximal subunits at the amino-terminal domain of the GluA2*-D23C homotetramer and tandem GluA2*-D23C/γ2 homotetramer under desensitized condition (presence of 1 mM glutamate) are shown. The FRET efficiency was calculated using Eq. 2 and the distances were calculated using the Förster Equation (Eq. 3).

Methods:

Flow Chamber Preparation:

Glass coverslips (22 × 22 mm No. 1) were plasma-cleaned and functionalized (aminosilanized) through Vectabond treatment (Vectabond in acetone 2% vol/vol; Vector Laboratories, Burlingame, CA). Silicone templates, with an elliptical opening at the center, were used to treat a small section on the coverslips, with PEG-BioPEG solution containing 5kDa biotin terminated PEG (2.5% w/w in molecular biology grade (MB) water, NOF Corp.), 5kDa mPEG succinimidyl carbonate (25% w/w in MB water, Laysan Bio Inc.) and sodium bicarbonate (Sigma-Aldrich). The coverslips with PEG-BioPEG solution were allowed to incubate in a dark and moist environment overnight. Prior to protein immobilization on the day of experiment, the coverslips were treated with an extra round of PEGylation with a short chain 333Da NHS-ester PEG (Thermo Scientific) and incubated for 2-3 h. Excess PEG was washed off and the coverslips were dried with a mild flow of Nitrogen. Custom hybriwell chambers (Grace bio-Labs) with dual silicon press-fit tubing connectors (Grace bio-Labs) were placed atop the coverslips to construct a flow chamber.

Protein immobilization:

The flow chamber was filled with Streptavidin in buffer solution (phosphate buffered saline containing 1 mM n-dodecyl-β-D-maltoside (DDM) and 0.2 mM cholesteryl hemisuccinate (CHS) detergents with protease inhibitor (ThermoScientific)) and incubated for 10 min. 10 nM of biotinylated goat-Anti-Mouse IgG (H+L) secondary antibody (Jackson Immuno Research Laboratories, Inc.) was flowed into the chamber and incubated for 20-30 min. 10 nM of the primary antibody (Anti GluA2 mouse monoclonal - Biolegend) was flowed in. After each antibody addition, the chamber was flushed with 10x PBS buffer (Gibco) to get rid of the unbound antibodies. All dilutions were made in 10x PBS buffer with 1 mg/mL Bovine serum albumin (Sigma-Aldrich). Finally the protein solution is passed through the chamber in three 60 μL shots and incubated for 20-30 min, before flushing the chamber with the above detergent containing buffer.

smFRET data acquisition:

For smFRET data acquisition the sample was excited at both 532 nm and 637 nm to co-localize the proteins having an acceptor tag and exhibiting FRET. All single molecule FRET measurements were acquired using a custom-built confocal microscope described previously in Landes et. al. (Nat. Chem. Bio., 2011). The sample was excited either with a continuous wave 532 nm laser (Compass 315M-100SL; Coherent) or a 637 nm laser (OBIS-FP 637 LX; Coherent) and focused through an oil immersion objective (100 X 1.3 NA; Carl Zeiss) on the sample. The power density at the sample was ~50 W/cm^2. The sample position was controlled through a scanning x-y-z piezo stage (P-517.3CL; Physik Instrumente). Emission signal from the sample was collected back through the same objective and separated via a 640 nm high-pass dichroic mirror (640 DCXR; Chroma Technology) to collect the donor and acceptor emission signals separately at two avalanche photodiodes (SPCM-AQR-15; PerkinElmer). The emission signal was tuned to 570 and 670 nm with band-pass filters (NHPF-532.0; Kaiser Optical Systems), placed before the photodiodes. All experiments were performed in presence of a constant flow of a photostabilizer and oxygen scavenging solution buffer system (ROXS) consisting of 1mM methyl viologen, 1mM ascorbic acid, 1% w/w glucose oxidase, 0.1% v/v catalase and 33% w/w glucose (all from Sigma-Aldrich) in MB water. 1 mM glutamate was added to induce the desensitized state of the AMPA receptor.