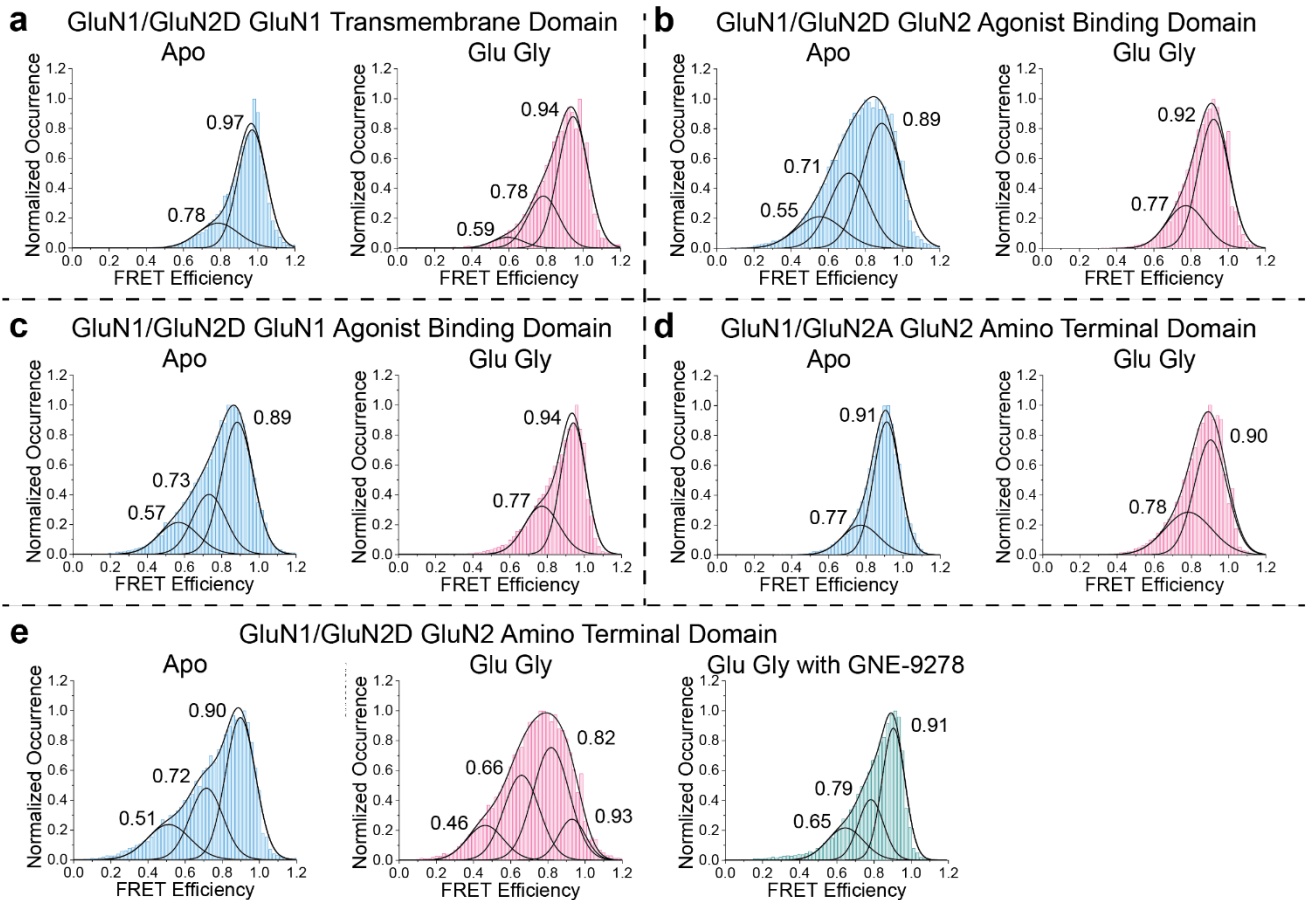
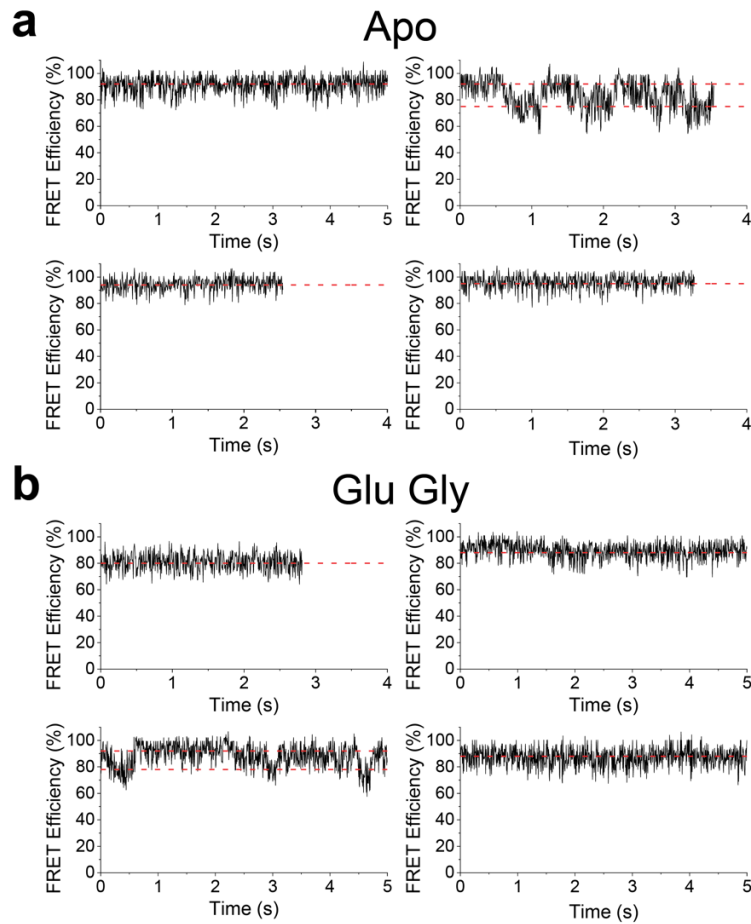


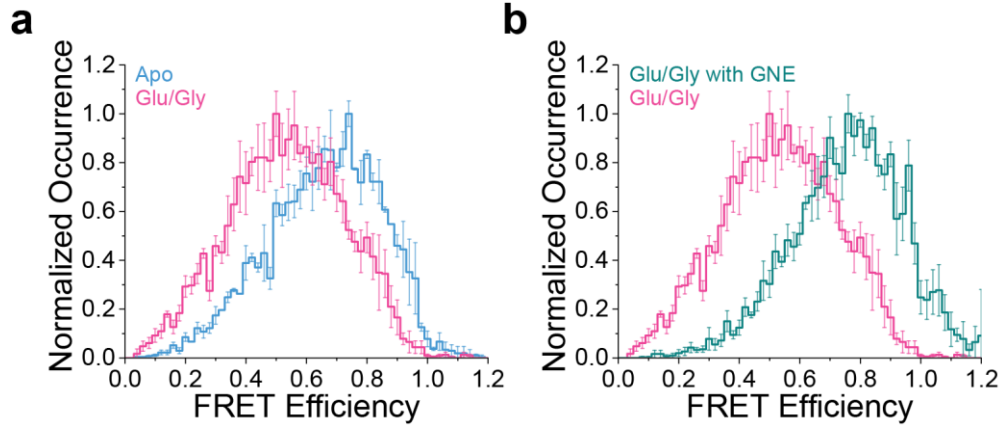
Supplementary Material



Supplementary Figure 1. Gaussian fits for the GluN1/GluN2D and GluN1/GluN2A smFRET constructs under various ligand conditions. Conformational states were predicted using hidden Markov model-based analysis of individual smFRET efficiency traces. The predicted states were used to inform Gaussian fittings of the cumulative FRET efficiency histograms in the apo (blue), glutamate and glycine bound (pink), and glutamate, glycine, and GNE-9278 bound (green) conditions. Gaussian fits are shown for the (a) GluN1/GluN2D GluN1 transmembrane domain, (b) GluN1/GluN2D GluN2 agonist binding domain, (c) GluN1/GluN2D GluN1 agonist binding domain, (d) GluN1/GluN2A amino-terminal domain, and (e) GluN1/GluN2D amino-terminal domain.



Supplementary Figure 2. Representative smFRET efficiency traces for the GluN2 amino-terminal domain in the GluN1/GluN2A receptor. Representative smFRET efficiency traces for the GluN2 amino-terminal domain in the GluN1/GluN2A receptor in the presence of **(a)** no ligand or **(b)** glutamate and glycine.



Supplementary Figure 3. smFRET measurements at the GluN2 amino-terminal domain in GluN1/GluN2D using Alexa 555 and Alexa 750. (a-b) Cumulative FRET efficiency histograms representing observed FRET efficiencies in the presence of no ligand (blue), glutamate and glycine (pink), and glutamate, glycine and GNE-9278 (green). Cumulative histogram values are mean $\pm$ SEM of data from at least two independent samples GluN1/GluN2D-ATD apo n=2 samples and 25 molecules, 5,526 data points; glu/gly =2 samples and 30 mol, 5,216 data points; glu/gly/GNE n=2 samples and 31 mol, 10,550 data points.

Constructs	Condition	Mean $\pm$ S.D.	<i>p</i> -value with respect to Apo
GluN1-TMD/GluN2D labeled with Alexa 555	Apo	0.234 $\pm$ 0.02	-
	Glu Gly	0.237 $\pm$ 0.004	0.8545
GluN1-TMD/GluN2D labeled with Alexa 647	Apo	0.071 $\pm$ 0.006	-
	Glu Gly	0.078 $\pm$ 0.007	0.3953
GluN1/GluN2D-ABD labeled with Alexa 555	Apo	0.240 $\pm$ 0.002	-
	Glu Gly	0.244 $\pm$ 0.003	0.2572
GluN1/GluN2D-ABD labeled with Alexa 647	Apo	0.071 $\pm$ 0.005	-
	Glu Gly	0.078 $\pm$ 0.007	0.3688
GluN1-ABD/GluN2D labeled with Alexa 555	Apo	0.259 $\pm$ 0.003	-
	Glu Gly	0.260 $\pm$ 0.002	0.392
GluN1-ABD/GluN2D labeled with Alexa 647	Apo	0.124 $\pm$ 0.005	-
	Glu Gly	0.119 $\pm$ 0.005	0.4608
GluN1/GluN2D-ATD labeled with Alexa 555	Apo	0.246 $\pm$ 0.004	-
	Glu Gly	0.248 $\pm$ 0.006	0.9668
	Glu Gly with GNE-9278	0.249 $\pm$ 0.014	0.7982
GluN1/GluN2D-ATD labeled with Alexa 647	Apo	0.121 $\pm$ 0.001	-
	Glu Gly	0.123 $\pm$ 0.003	0.4655
	Glu Gly with GNE-9278	0.126 $\pm$ 0.005	0.2999

Supplementary Table 1. Summary of statistical analysis for fluorescence polarization ratios obtained for the GluN1/GluN2D smFRET constructs. Polarization ratios were calculated for each GluN1/GluN2D smFRET construct with Alexa 555 or Alexa 647. Mean and standard deviation were calculated from at least two independent measurements and were compared using two-sided t-test.