Figure S1.

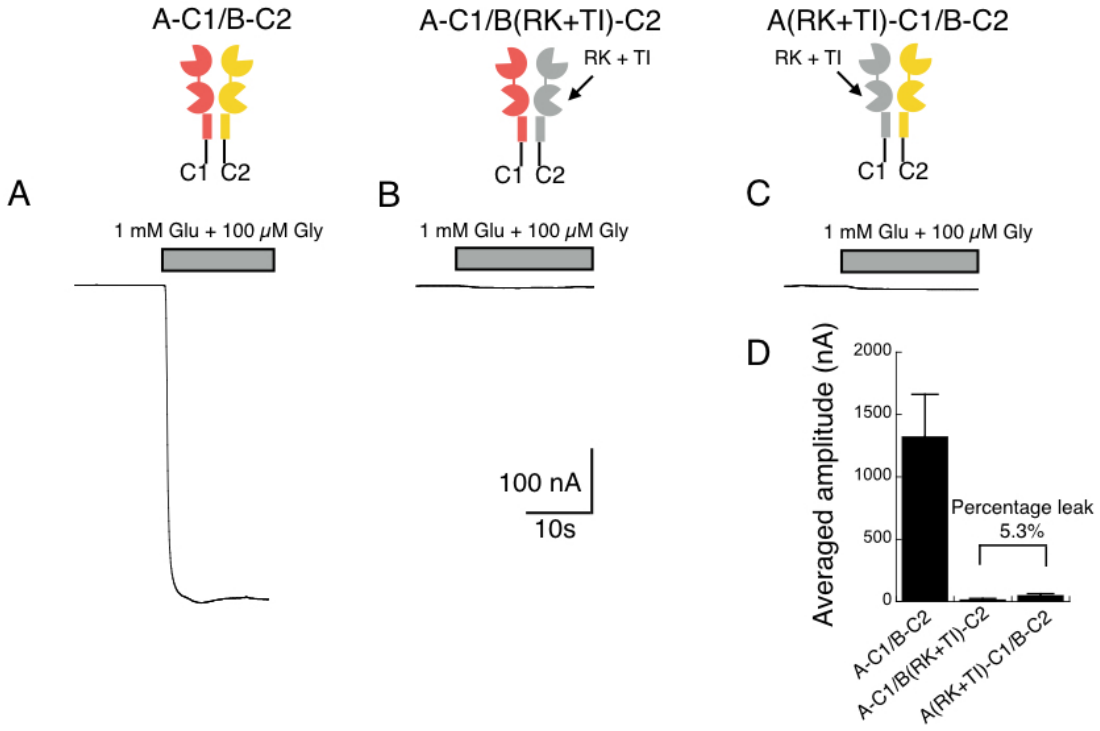


Fig. S1. (Related to Fig. 1, 2, 3) **Estimation of escape currents in the ER retention system for expression of triheteromeric NMDARs.** Co-expression of GluN1, GluN2A, and GluN2B subunits can produce three populations of functional NMDA receptors (i.e. A/B, A/A, and B/B), but expression of dihetomeric GluN1/2A (A/A) and GluN1/2B (B/B) receptors at the cell surface can be prevented by fusing the engineered C1 to the intracellular C-terminus of GluN2A (A-C1) and replacing the C-terminal domain of GluN2B with that of C2-tagged GluN2A (B-C2) (Hansen et al., 2014). Responses from A-C1/A-C1 and B-C2/B-C2 receptors that may have escaped ER retention (i.e. "leak" currents) can be assessed using RK+TI mutations, which abolish binding of glutamate and render NMDA receptors containing this subunit non-functional (Hansen et al., 2014). Currents activated by 1 mM glutamate plus 100 μM glycine from *Xenopus* oocytes injected with (**A**) GluN1, GluN2A-C1, and GluN2B-C2 cRNAs, (**B**) GluN1, GluN2A-C1, and mutated GluN2B-C2 (R519K and T691I) cRNAs, or (**C**) GluN1, mutated GluN2A-C1 (R518K and T690I), and GluN2B-C2 cRNAs. (**D**) Quantification of current amplitudes of each group. Data are mean ± SEM of current amplitudes from 6 oocytes for each group. The sum of the fractional "leak" currents assessed using the RK+TI mutations provides an estimate of the percent "leak" current in oocytes expressing triheteromeric A-C1/B-C2 receptors (Hansen et al., 2014).

Figure S2.

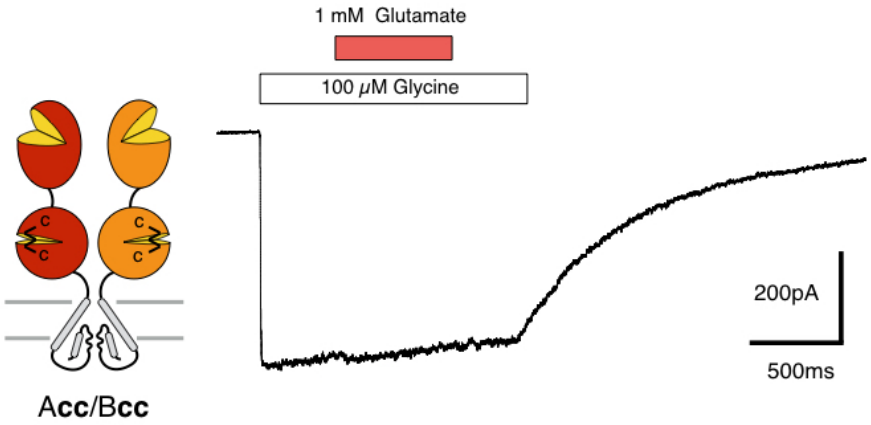
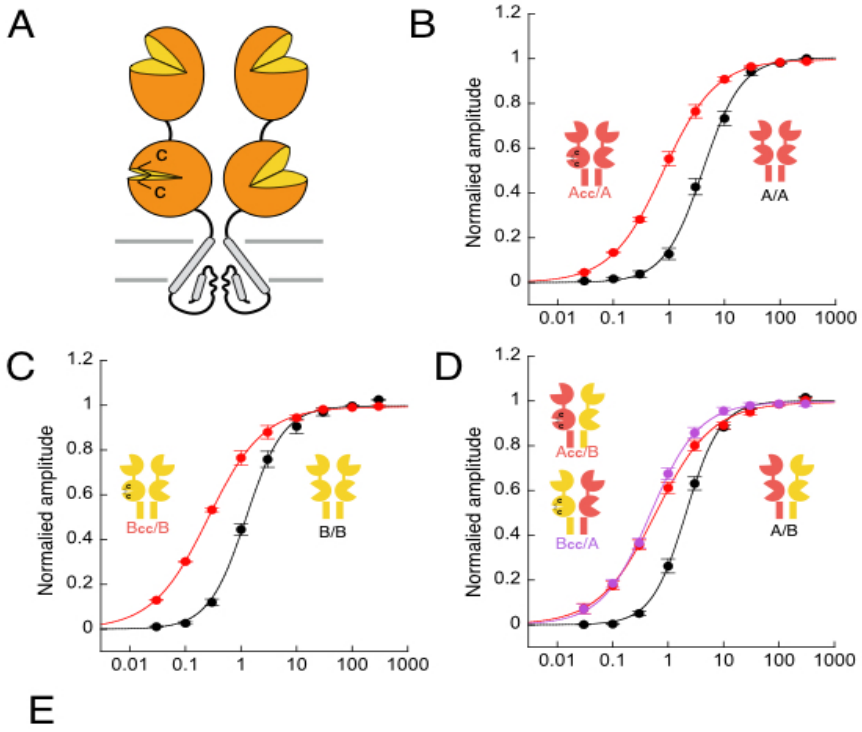


Fig. S2. (Related to Figure. 1) **Crosslinking of the GluN2 ligand binding domain is highly efficient.** Two cysteine mutations in GluN2A and GluN2B were designed to spontaneously form a disulfide bond that locks the ligand binding domain (LBD) in a conformation similar to the glutamate-bound state (GluN2A: K487C + N687C; GluN2B: K488C + N688C; hereafter Acc and Bcc) (Blanke and VanDongen, 2008, Kussius and Popescu, 2010, Dai and Zhou, 2016). Acc/Bcc receptors expressed on HEK-293 cells open fully when exposed to 100 μM glycine. Addition of 1 mM glutamate did not further increase the amplitude, indicating complete formation of the engineered disulfide bond. Current amplitude in glycine alone was 99.5 ± 1.0 % of the current amplitude in glutamate and glycine (n = 3).

Figure S3.



E

Group	EC_{50} (μM)	Hill Coefficient	n
A/A	4.2 ± 0.5	1.29 ± 0.04	8
A/B	2.1 ± 0.2	1.4 ± 0.01	3
B/B	1.3 ± 0.1	1.29 ± 0.06	6
Acc/A	0.9 ± 0.2	0.94 ± 0.05	6
Bcc/A	0.5 ± 0.1	1.00 ± 0.06	6
Bcc/B	0.3 ± 0.1	0.90 ± 0.06	7
Acc/B	0.6 ± 0.1	0.85 ± 0.05	9

Fig. S3. (Related to Figure. 1) **Crosslinking a single GluN2 within NMDARs increases glutamate potency and decreases Hill coefficient.** (**A**) Cartoon showing LBD crosslinking of a single GluN2 subunit. (**B-D**) Glutamate concentration-response relationships for wild type and single-GluN2 crosslinked NMDARs expressed in *Xenopus* oocytes measured using two-electrode voltage-clamp recordings. (**E**) Summary of EC_{50} values and Hill coefficients.

Figure S4.

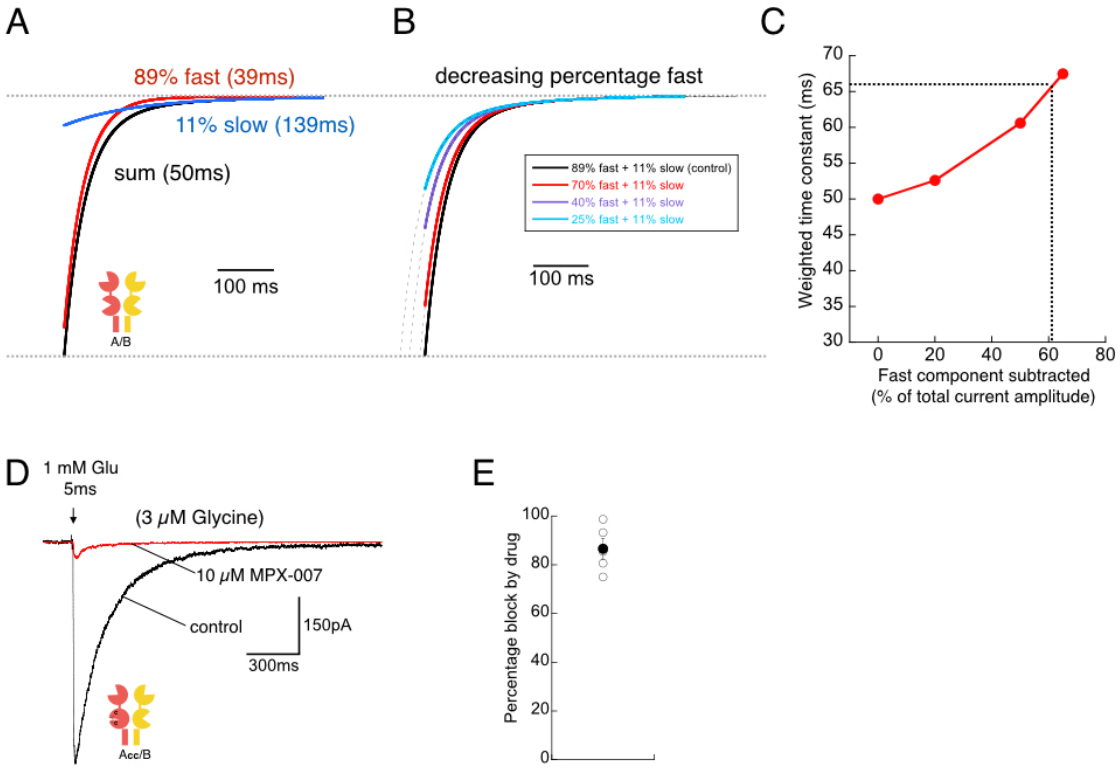


Fig. S4. (Related to Fig. 1 and STAR Methods) **A/B or Acc/B deactivation is not mediated by escaped A/A or B/B receptors, respectively.**
(**A**) The measured deactivation of A/B receptors can be fitted using a double exponential function, with 89% fast component (39 ms) and 11% slow component (139 ms). (**B**) Decreasing the amplitude of the fast component without changing the slow component results in a progressively slower weighted time constant, which is quantified in (**C**). This approach estimates that about 60% of the total current amplitude would have to be mediated by escaped A/A to cause the A/B deactivation time constant to be shifted from 66 ms to 50 ms. (**D**) The B/B escape current contributing to the measured Acc/B current was estimated by blocking the current using the GluN2A-selective NMDAR antagonist MPX-007 (kindly provided by Luc Therapeutics, Inc) (Volkmann et al., 2016). MPX-007 at 10 µM in the presence of 3 µM glycine blocks >95% of GluN2A-containing receptors (i.e. A/A and A/B), but does not affect B/B diheteromeric receptors (Yi et al., 2016). (E) MPX-007 blocked Acc/B responses by 87 ± 4 % (n=6), suggesting the majority of the expressed receptors contain at least one GluN2A subunit. The remaining <13% of the response, which is not inhibited by MPX-007, is mediated by unblocked A/B receptors and escaped B/B receptors.