Supplemental Experimental Procedures

Receptor Constructs. The constructs are derived from the previously reported *Xenopus laevis* NMDA 2 constructs (Lee et al., 2014). For cryo-EM experiments, the ATD non-crosslinked GluN2B construct was obtained by reverting the K216C mutation to the wild-type lysine residue (named $NMDA_{EM}$). For the DEER experiments, the cysteine substitution was introduced at the given positions based on the $NMDA_{EM}$ receptor. The four cysteines were introduced into the WT construct to have the LBD dimer interface disulfide crosslinked receptor (GluN1-N519C-L775C/GluN2B-E514C-L778C). For electrophysiology, the $NMDA_{EM}$-like construct was designed based on $NMDA_{EM}$ plus the R610G-R654E-R655E mutations as in the WT receptors.

Expression and Purification. The protein was expressed in HEK293S GnTI- cells grown in suspension and transduced using P2 BacMam virus at a multiplicity of infection (MOI) of 1:1 (GluN1:GluN2B) and incubated at 37 °C (Goehring et al., 2014). At 14 h post-transduction, 10 mM sodium butyrate was added to the cultures and the temperature was shifted to 30 °C. Cells were collected 60 h post-transduction and disrupted by sonication in 150 mM NaCl, and 20 mM Tris-HCl pH 8.0 (TBS buffer). The cell debris was removed by centrifugation for 20 mins at 8,000 r.p.m. Membranes were pelleted from the supernatant by centrifugation for 1 h at 40K r.p.m (Ti45 rotor), then homogenized and solubilized in TBS containing 1 mM PMSF, protease inhibitors (0.8 µM aprotinin, 2 µg/ml leupeptin, and 2 mM pepstatin A), 1% 2,2-didecylpropane-1,3-bis- -D-maltopyranoside (MNG-3) and 2 mM cholesterylhemisuccinate (CHS) for 1.5 h, 4 °C. The soluble fraction was bound to streptactin resin and eluted with buffer containing 5 mM desthiobiotin. The receptor was concentrated and digested with 3C protease overnight at 4 °C. The purified protein was further isolated by size-exclusion chromatography (SEC) in the buffer containing 400 mM NaCl, 20 mM MES pH 6.5, 1 mM *n*-dodecyl β-D-maltoside (DDM), 0.2 mM CHS. Peak fractions were pooled and concentrated.

Spin Labeling and DEER Experiments. For the DEER experiment, single cysteine mutations were introduced as labeling sites for spin label 1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethiosulfonate (MTSSL) based on the $NMDA_{EM}$. Receptor protein was expressed and purified as described above. Concentrated Strep-purified GFP-fused protein was digested with 3C protease at room temperature and dialyzed overnight against labeling buffer containing 150 mM NaCl, 20 mM Tris-HCl, 0.1% MNG-3 and 0.2 mM CHS, pH 7.2. The labeling reaction was initiated upon addition of a 10-fold molar excess of MTSSL (100 mM stock in dimethylformamide) at room temperature in the dark. After 1 hr, another 10-fold excess of MTSSL was added and incubated for an additional 1 hr. The labeled protein was purified by SEC in labeling buffer. Peak fractions containing labeled tetrameric protein were pooled and concentrated. The final sample molar concentration was determined using the molar extinction coefficient = 112,293 $M^{-1} \cdot cm^{-1}$, estimated for the GFP-free full-length construct (http://web.expasy.org/protparam/).

Intersubunit distances were measured using DEER methods (Jeschke et al., 2002; Zou and McHaourab, 2010). Dipolar time-evolution data were obtained at 83 K using a standard DEER four-pulse protocol, $(\pi/2)mw1 - \tau1 - (\pi)mw1 - \tau1 - (\pi)mw2 - \tau2 - (\pi)mw1 - \tau2 -$ echo (Pannier et al., 2000), on a 580 pulsed EPR spectrometer (Bruker) operating at Q-band frequency (33.9 GHz). The pulse lengths for $(\pi/2)mw1$ and $(\pi)mw1$ were 10 and 20 ns, respectively, and 40 ns for $(\pi)mw2$. DEER signals were analyzed assuming that the distance distributions, P(r), consists of a sum of Gaussians (Durr et al., 2014; Mishra et al., 2014; Stein et al., 2015).

Model Fitting and Structural Analysis. Fitting of coordinates into the ECD density maps were carried out using UCSF Chimera (Pettersen et al., 2004) fitting algorithm using maps simulated from atoms filtered to a resolution of 10 Å, and optimized using the correlation score. The separated ATD R1 (D23-P143 and K275-K347 for GluN1; P28-G142 and Y282-M353 for

GluN2B) or R2 (Y144-G274 and F348-S395 for GluN1; P143-D281 and H354-S391 for GluN2B) lobe, LBD D1 (T396-F532 and F748-D789 for GluN1; E392-I532 and Y745-K788 for GluN2B) or D2 (K533-K542 and E651-G747 for GluN1; E524-G536 and V646-G744 for GluN2B) lobe, were each fitted as independent rigid bodies. These rigid bodies are derived from the dimeric GluN1-GluN2B ATD crystal structure (PDB: 3QEL, (Karakas et al., 2011)), and dimeric GluN1-GluN2A LBD structure (PDB: 2A5T, (Furukawa et al., 2005)), respectively. The fittings were independent, there was no special consideration given to maintaining distances between split residues or preventing steric clashes. Nevertheless, the domain fittings all resulted in appropriate distances between the split residues, and non-existent to very minor steric clashes in the ATD domains of heterogenous antagonist-bound classes (class 2-6). The agreement between the independent fittings and preservation of these real constraints provided us with additional confidence in the overall correctness of these fittings, and the observed domain movement. After model fitting, the homology model of the *Xenopus laevis* GluN1-GluN2B receptor was generated based on the PDB coordinate files of separated domains using the SWISS-MODEL Automated Mode (Biasini et al., 2014). All structural measurements were carried out based on the PDB coordinates from ECD density maps. The figures were prepared with PyMOL or UCSF Chimera.

Two-Electrode Voltage-Clamp Electrophysiology. Recombinant NMDA receptors were expressed in *Xenopus laevis* oocytes after nuclear injection of a 36 nL mixture of RNA encoding GluN1 and GluN2 subunits (at 100 ng/µL concentration, ratio 1:1), and stored at 16°C in the presence of 50 µM competitive antagonist D-APV and 10 µg/ml gentamicin. Recordings were made using a bath solution containing 5 mM HEPES pH 7.3, 100 mM NaCl, 2.8 mM KCl, 0.3 mM $BaCl_2$ and 10 µM heavy-metal chelator ethylenediaminetetraacetic acid (EDTA). NMDA receptor-mediated currents were induced by simultaneous application of saturating concentrations of 100 µM glycine and 100 µM glutamate.

Ligand Binding Assays. The scintillation proximity assay (SPA) experiments were set up in TBS buffer plus 0.01% MNG and 0.02 mM CHS, except for the Ki of D-APV was performed in SEC buffer. Purified $NMDA_{EM}$ receptors (10-30 nM) were incubated with 0.5 mg ml^{-1} of Ysi-streptavidin (for Kd of ^{3}H-glycine and ^{3}H-glutamate, and Ki of DCKA) or Ysi-His (for Ki of D-APV) beads. Non-specific binding was determined by the addition of 1 mM DCKA (for ^{3}H-glycine) or 1 mM D-APV (for ^{3}H-glutamate). The inhibition constant of DCKA was determined in the presence of 1 μM glycine (10% ^{3}H-glycine), and of D-APV in the presence of 1 μM glutamate (10% ^{3}H-glutamate). All data were analyzed and fitted in GraphPad Prism using a one-site binding model.

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