Glutamate Binding to the GluN2B Subunit Controls Surface Trafficking of N-Methyl-D-aspartate (NMDA) Receptors**

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**This work was selected as a Paper of the Week.

Background: Ligand binding is essential for surface delivery of non-NMDA-type glutamate receptors.

Results: GluN2B ligand binding site mutants showed reduced surface and synaptic expression correlating with glutamate efficacy.

Conclusion: Glutamate binding controls surface delivery of NMDA receptors.

Significance: This work extends the receptor classes subject to glutamate regulation of surface delivery and reports parameters controlling synaptic delivery of NMDA receptors critical for neuron function.

Trafficking of NMDA receptors to the surface of neurons and to synapses is critical for proper brain function and activity-dependent plasticity. Recent evidence suggests that surface trafficking of other ionotropic glutamate receptors requires ligand binding for exit from the endoplasmic reticulum. Here, we show that glutamate binding to GluN2 is required for trafficking of NMDA receptors to the cell surface. We expressed a panel of GluN2B ligand binding mutants in heterologous cells with GluN1 or in rat cultured neurons and found that surface expression correlates with glutamate efficacy. Such a correlation was found even in the presence of dominant negative dynamin to inhibit endocytosis and surface expression correlated with Golgi localization, indicating differences in forward trafficking. Co-expression of wild type GluN2B did not enhance surface expression of the mutants, suggesting that glutamate must bind to both GluN2 subunits in a tetramer and that surface expression is limited by the least avid of the two glutamate binding sites. Surface trafficking of a constitutively closed cleft GluN2B was indistinguishable from that of wild type, suggesting that glutamate concentrations are typically not limiting for forward trafficking. YFP-GluN2B expressed in hippocampal neurons from GluN2B−/− mice rescued synaptic accumulation at similar levels to wild type. Under these conditions, surface synaptic accumulation of YFP-GluN2B mutants also correlated with apparent glutamate affinity. Altogether, these results indicate that glutamate controls forward trafficking of NMDA receptors to the cell surface and to synapses and raise the intriguing idea that NMDA receptors may be functional at intracellular sites.

N-Methyl-D-aspartate (NMDA) receptors are a subtype of ionotropic glutamate receptor present at excitatory synapses in the central nervous system. Composed of two obligate GluN1 subunits and two GluN2 or more rarely GluN3 subunits, channel opening requires L-glutamate binding to GluN2 and co-agonist glycine or D-serine binding to GluN1 (1, 2). In neurons, membrane depolarization is also required to clear Mg2+ from the pore to allow ion flux. Thus, NMDA receptors are exquisite coincidence detectors of pre- and postsynaptic activity. The residues in the receptor responsible for the Mg2+ block are also responsible for high permeability to Ca2+ (3), which triggers signal transduction cascades that mediate many forms of synaptic plasticity. NMDA receptors play key roles in brain development and in learning, memory, and cognitive functions (4, 5). NMDA receptors are also key pharmacological targets for therapeutic protection during stroke, epilepsy, and traumatic brain injury and for relief of neuropathic pain and multiple psychiatric disorders (2). Thus, it is important to understand mechanisms that control the subcellular trafficking of NMDA receptors.

Ionotropic glutamate receptors are multimeric protein complexes that require quality control mechanisms to ensure correct assembly before exit from the endoplasmic reticulum (ER). For example, ER retention signals in the cytoplasmic region C1 cassette of GluN1-1a and in the membrane domain M3 of both GluN1 and GluN2 are masked upon correct assembly into tetramers (6–8). Additional studies suggest that some ionotropic glutamate receptors undergo further quality control inspection for ligand binding prior to release from the ER. Mutations in GluK2 and GluK5 kainate receptors and GluA2 and GluA4 AMPA receptors that abolished agonist binding resulted in retention in the ER despite multimeric assembly (9–12). Studies of differentially spliced and edited isoforms of GluA2 and of mutant ligand binding domains of GluA4 and

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GluK2 suggest that ligand-induced clamshell closure is required for forward trafficking (12–14). Altogether, much evidence supports the idea that intracellular glutamate binding is required for surface delivery of AMPA and kainate receptors (15, 16).

When compared with AMPA and kainate receptors, little is known about the role of glutamate binding in surface delivery of NMDA receptors. A recent study of a co-agonist binding mutant suggested that glycine binding to GluN1 is essential for cell surface delivery of NMDA receptors (17). Here, we tested the role of glutamate binding to GluN2 in cell surface delivery of NMDA receptors in heterologous cells, in cultured rat neurons, and upon rescue in GluN2B−/− mouse neurons. Further, because it can be difficult to differentiate between a specific role for loss of ligand binding or more nonspecific conformational effects of mutants, we assessed a panel of GluN2B ligand binding mutants differing in glutamate efficacy. Our results support a critical role for glutamate binding in forward trafficking of NMDA receptors.

**EXPERIMENTAL PROCEDURES**

*DNA Constructs—*The dual promoter expression vector was derived from pVIVO (InvivoGen); the SV40 enhancer region promoter was replaced with a CMV enhancer from ECFP-N1 (Clontech). Enhanced cyan fluorescent protein (CFP; Clontech) followed by the PCR-generated linker LVPRGSRSR was developed from rat GluN2B (18) (a gift of M. Sheng, Genentech) by inserting enhanced yellow fluorescent protein (YFP; Clontech) followed by the PCR-generated linker LVPRGSRSR between amino acids 2 and 4 of the mature N terminus. This coding region was inserted into multiple cloning site 2 driven by the light chain human ferritin promoter, and the sequence of the coding region was confirmed. Single amino acid glutamate binding-deficient mutants R493K, E387A, S664G, F390S, and V660A as well as K462C/N662C were generated by site-directed mutagenesis, and the sequences of the coding region and mutations were confirmed. Wild type Myc-GluN2B was generated by replacing the YFP region of YFP-GluN2B by PCR. GW-GluN1-1a expresses rat GluN1-1a cDNA from the CMV promoter in vector GW1 (gift from M. Sheng). Dominant negative (DN) HA-dynamin-K44A and wild type HA-dynamin (19) expression vectors were gifts from Pietro DeCamilli (Yale University).

*Cell Culture—*Dissociated primary rat hippocampal neuron cultures were prepared from embryonic day 18 rats as described (20). GluN2B+/- mice (21) were maintained and genotyped as described (22); these heterozygous mice were timed-mated to obtain GluN2B−/− embryos. Hippocampi or cortices were dissected from individual 17–18 day embryos and stored overnight at 4 °C in Hibernate E (Brain Bits) supplemented with B-27 (Invitrogen or StemCell) pending parallel genotyping of brainstem and tail tissue. Mouse hippocampi were dissociated with papain (20 units/ml, 15 min, 37 °C), and the dissociation solution for cortices was supplemented with 85 units/ml deoxyribonuclease I (Invitrogen). 5 × 10^5 freshly dissociated neurons were pelleted at 80 × g for 4 min for nucleofection using an Amzaa Nucleofector II (Lonza) (program O-003 and program O-005 for rat and mouse neurons, respectively) with 6 μg of plasmid DNA. Nucleofected cells were plated onto poly-1-lysine-treated glass coverslips in 60-mm dishes for an effective estimated plating density of 3 × 10^5 cells/dish and inverted over a feeder layer of rat glia. Rat neurons were maintained in Neurobasal medium with B-27 and 1-glutamine (Invitrogen), and mouse neurons were additionally supplemented with 25 μg/ml bovine pancreatic insulin (Sigma-Aldrich). After 2 days in vitro (DIV), proliferation of glia was suppressed by the addition of 5 μM cytosine arabinoside (Calbiochem). Neurons were analyzed at DIV 6 and DIV 14.

COS-7 cells (ATCC CRL-1651) were maintained in DMEM (Sigma-Aldrich) with 10% fetal bovine serum (Invitrogen). 2.2 × 10^4 or 3.5 × 10^4 trypsin-dissociated cells were plated on 18-mm glass coverslips and transfected immediately with TransIT-LT1 transfection reagent (Mirus Bio) according to the manufacturer’s protocol. Cells were co-transfected with 0.5 μg each of GW-GluN1-1a and the YFP-GluN2B construct, and WT or DN HA-dynamin with 4.5 μl of LT1; and 0.5 μg each of GW-GluN1-1a, YFP-GluN2B construct, and vector control or Myc-GluN2B with 4.5 μl of LT1. All plasmids were premixed prior to addition to the transfection reagent. Cells were grown without (control) or with 11.25 μM (55,10R)-(-)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) (Enzo Life Science) and 150 μM (2R)-amino-5-phosphonovaleric acid (APV) as indicated. Cells were analyzed at 48 h after transfection.

**Live Antibody Labeling and Immunocytochemistry—**For surface YFP-GluN2B labeling, coverslips were incubated with anti-GFP antibody (rabbit polyclonal; 1:500; Invitrogen A11122) in conditioned media for 30 min at 37 °C (neurons) or in extracellular solution (COS-7 cells) prior to fixation for 15 min in prewarmed PBS with 4% paraformaldehyde and 4% sucrose, pH 7.4, followed by permeabilization with 0.25% Triton X-100 in PBS. For assessment of endocytosis, coverslips were incubated with anti-GFP antibody in conditioned media for 30 min at 37 °C and returned to their original media plus 300 μM APV (300 μM) and kynurenic acid (5 μM) for 1 h at 37 °C to promote endocytosis. Coverslips were then washed with cold PBS, acid-stripped in 0.5 M NaCl and 0.2 M acetic acid for 4 min on ice, and rewashed with cold PBS prior to fixation, permeabilization, blocking, and antibody staining. Neuronal cultures for immunodetection of GluN1 were fixed and permeabilized by a 2-min incubation in prewarmed PBS with 4% paraformaldehyde and 4% sucrose, pH 7.4, and then 10 min in cold 100% methanol and rehydrated in PBS for 30 s. Fixed and permeabilized cultures were blocked in PBS with 8% bovine serum albumin (BSA) and 1.2% normal goat serum for 30 min at 37 °C prior to incubation with primary antibodies in PBS with 3% BSA and 0.3% normal goat serum for 2 h at 37 °C and secondary
antibodies for 45 min at 37 °C. Coverslips were washed 6 × 2 min with PBS following each antibody incubation. For experiments with 4',6-diamido-2-phenylindole (DAPI) nuclear staining, coverslips were incubated with 300 nM DAPI in PBS for 10 min at room temperature and washed in parallel with the secondary antibody wash. Coverslips were mounted in Elvanol (Tris-HCl, glycerol, polyvinyl alcohol, and 2% 1,4-diazabicyclo[2,2,2]octane).

The following antibodies were used: anti-GluN2B (IgG2b; 1:200; University of California Davis/National Institutes of Health NeuroMab Facility N59/36), anti-GluN1 (IgG2a; 1:40,000; Invitrogen), and Thermo Fisher Scientific clone 6G6-1C9; recognizes PSD-95, PSD-93, SAP102, and SAP97), anti-VGlut1 (guinea pig polyclonal; 1:4000; Millipore AB5905), anti-GluN1 in oocytes. From the potential mutants, we chose a series exhibiting a range of glutamate efficacies that mapped well (Fig. 1A) to the more recent crystallographic data for the glutamate binding pocket of GluN2A (24) and thus likely reflect differences in glutamate affinity. Additionally, the selected point mutant amino acid residues are all conserved across GluN2A, GluN2B, GluN2C, and GluN2D (Fig. 1B). GluN2B mutants V660A, F390S, S664G, and E387A show reductions in apparent glutamate affinity of 18- to 237-fold when compared with wild type GluN2B, whereas mutant R493K was the most severe, giving no detectable glutamate response (23) (numbers correspond to the mature polypeptide after signal sequence cleavage). We generated the series of GluN2B mutants with an N-terminal YFP tag to facilitate measures of surface trafficking in a dual promoter mammalian expression vector co-expressing nuclear-targeted CFP (nCFP) to visualize transected cells.

Activation of expressed NMDA receptors by free l-glutamate and glycine in normal cell culture media has been shown to be toxic to transfected heterologous cell lines (25) primarily through the unregulated influx of calcium. We used this characteristic to functionally test the series of glutamate binding GluN2B mutants upon co-expression with GluN1-1a in COS-7 cells. Cells were transfected for GluN1-1a and nCFP vector with an empty multiple cloning site 2 or bearing each of the YFP-GluN2B variants, and cell survival 48 h later was assessed as the frequency of DAPI-positive cells expressing nCFP. In the absence of NMDA receptor inhibitors, COS-7 cell cultures co-transfected for GluN1-1a and nCFP vector bearing wild type GluN2B (YFP-GluN2B) had a dramatically decreased number of surviving transfected cells when compared with control cultures co-transfected for GluN1-1a and nCFP vector alone (Fig. 1C). Survival of YFP-GluN2B transfected cells was rescued to levels comparable with vector alone by chronic administration of NMDA receptor inhibitors MK-801 and APV (MK-801/ APV). In contrast to WT YFP-GluN2B, even in the absence of NMDA receptor inhibitors, survival of cells transfected for GluN1-1a and nCFP vector bearing YFP-GluN2B R493K, E387A, S664G, and F390S mutants was not significantly different from that of control cells (Fig. 1D). Survival of cells expressing the YFP-GluN2B V660A mutant with the highest glutamate efficacy was reduced by 21% relative to control, and this toxicity was rescued by MK-801/APV. Comparing all GluN2B constructs under control conditions, cell survival was significantly lower.
different (ANOVA, $p < 0.0001$), and all YFP-GluN2B mutants resulted in significantly enhanced survival when compared with WT (post hoc Bonferroni’s multiple comparison test, $p < 0.0001$). In contrast, when cells were grown with MK-801/APV, cell survival did not differ significantly among GluN2B constructs. Cell survival comparing control and MK-801/APV conditions was significantly different only for the YFP-GluN2B-V493K cells were grown without (control) or with inhibitors MK-801 and APV for 48 h and then fixed and stained (with) DAPI. Scale bar, 100 μm. D, in COS-7 cells transfected with the dual promoter vector, nCFP and YFP-GluN2B were always co-detected, and nCFP was generally brighter. There was a significant difference in overall expression (ANOVA, $p < 0.0001$) and post hoc Bonferroni’s multiple comparison test when compared with the wild type (control conditions, ANOVA, $p < 0.001$), and all YFP-GluN2B mutants result in significantly less toxicity when compared with WT under control conditions, but there was no difference in cultures grown in MK-801/APV conditions, ANOVA, $p < 0.0001$, and post hoc Bonferroni’s multiple comparison test when compared with WT, all $p < 0.001$; MK-801/APV conditions, ANOVA, $p > 0.05$; $n = 20–30$ fields for each condition from two independent experiments). These data suggest that glutamate binding-deficient GluN2 mutants and protection against NMDA receptor-mediated toxicity in heterologous cells.

Glutamate Binding Controls NMDA Receptor Surface Delivery—To determine the effect of reduced glutamate binding on surface expression, wild type or mutant YFP-GluN2B constructs were co-expressed with GluN1-1a in COS-7 cells and grown chronically with NMDA receptor inhibitors to prevent glutamate-induced excitotoxicity. After 48 h, transfected COS-7 cells were incubated live with membrane-impermeant anti-GFP antibody cross-reactive with the N-terminal YFP tag. After washing, cells were fixed, permeabilized, and stained for GluN1 (Fig. 2A) to confirm co-expression, and the ratio of labeled surface receptor integrated immunofluorescence to total YFP integrated fluorescence over the entire cell area was calculated to estimate what fraction of GluN2B is present on the cell surface. All of the mutants showed significantly decreased levels of surface expression when compared with the wild type GluN2B (Fig. 2B; ANOVA, $p < 0.0001$, and Bonferroni’s multiple comparison test, $p < 0.001$). Furthermore, the relative level of surface expression of the mutant GluN2B constructs correlated well with the reported glutamate efficacy (23) (Fig. 2B, right panel; Pearson’s coefficient of determination $r^2 = 0.95$, $p = 0.0044$ for WT, V660A, F390S, S664G, and E387A; R493K was excluded because it had no measurable glutamate response). There was no difference in total YFP intensity per COS-7 cell area among the YFP-GluN2B constructs, indicating no difference in overall expression (ANOVA $p > 0.1$, $n = 27$ per construct from two independent experiments). These data suggest that ligand binding is necessary for NMDA receptors to accumulate at steady state on the cell surface of heterologous cells, with apparent glutamate affinity directly regulating surface expression.
Glutamate Binding Regulates Forward Trafficking of NMDA Receptors

Surface expression of NMDA receptors is subject to a number of regulatory mechanisms including ER retention limiting forward trafficking and dynamin-dependent endocytosis via the AP-2 clathrin adaptor protein binding site present in the cytoplasmic tail of GluN2B (26, 27). To separate the relative contributions of forward trafficking from endocytosis, COS-7 cells were co-transfected with either DN HA-dynamin-K44A to inhibit clathrin-dependent endocytosis (28) or WT HA-dynamin along with the GluN2B and GluN1-1a constructs (Fig. 3A). Surprisingly, there were no differences in the ratio of cell surface to total NMDA receptors between cells co-expressing DN dynamin or WT dynamin (Fig. 3B), and results were similar to cells without dynamin co-expression (Fig. 2B). Relative surface levels were not significantly different for any of the YFP-GluN2B mutants with DN dynamin co-expression when compared with WT dynamin co-expression (Fig. 3B; Student’s t test, p > 0.1). Even in the presence of DN dynamin to inhibit endocytosis, all of the mutant GluN2B constructs exhibited reduced surface levels when compared with WT (Fig. 3B; ANOVA, p < 0.0001, and Bonferroni’s multiple comparison test, p < 0.001 for R493K, E387A, and S664G, p < 0.01 for F390S, and p < 0.05 for V660A). Efficacy of DN dynamin in inhibiting NMDA receptor endocytosis was confirmed by incubating transfected cells with anti-GFP antibody recognizing YFP-GluN2B, washing, incubating under conditions that promote receptor endocytosis, and then acid-stripping surface antibody and visualizing endocytosed antibody. DN dynamin but not WT dynamin inhibited antibody uptake in COS cells expressing YFP-GluN2B and GluN1 (Fig. 3, C and D).

As an independent method to assess potential differences in forward trafficking, we assessed colocalization of YFP-GluN2B wild type and mutants, co-expressed with GluN1, with the Golgi marker GM130 (29). Receptor that has successfully exited the ER en route to the surface will transiently reside in the Golgi; thus, we expect reduced forward trafficking ability to result in reduced colocalization with GM130. Indeed, YFP-GluN2B mutants appeared to exhibit less accumulation in GM130-positive Golgi than wild type (Fig. 4A). There was a significant difference among YFP-GluN2B constructs in Golgi localization measured as the average intensity of YFP-GluN2B colocalized with GM130 over the average intensity of the rest of the cellular YFP-GluN2B not colocalized with GM130 (Fig. 4B; ANOVA, p < 0.0001, and Bonferroni’s multiple comparison test, p < 0.001 for R493K and E387A when compared with WT). These data suggest that a difference in forward trafficking

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A

B

FIGURE 2. Glutamate binding to GluN2 regulates surface levels of NMDA receptors in heterologous cells. A, COS-7 cells co-transfected with untagged GluN1-1a and YFP-GluN2B-WT or the indicated glutamate binding mutants were surface-immunolabeled live for YFP-GluN2B and then fixed and permeabilized and immunolabeled to confirm co-expression of GluN1. Scale bar, 20 μm. B, quantitation of surface YFP-GluN2B to total YFP-GluN2B ratio normalized to WT revealed reduced surface levels of the mutants (ANOVA, p < 0.0001, and Bonferroni’s multiple comparison test when compared with WT, *, p < 0.001; n = 27–60 per construct from at least two independent experiments). Right panel, correlation of relative surface levels from the left panel with the log of the reported half-maximal effective concentration of each GluN2B construct for glutamate (23) shows a correlation between glutamate efficacy of GluN2B and surface levels of the expressed NMDA receptor (Pearson’s coefficient of determination r² = 0.95, p = 0.0044 excluding R493K, which had no detectable efficacy). Error bars indicate S.E.
to the cell surface is the primary contributor to differences in cell surface levels of the GluN2B mutants, implying that impaired glutamate binding reduces forward trafficking.

Co-expression of Wild Type GluN2B Cannot Rescue Surface Trafficking Deficits of GluN2B Glutamate Binding Mutants—Because NMDA receptors are tetramers composed of two GluN1 and two GluN2 subunits, we co-expressed WT Myc-GluN2B with each of the YFP-GluN2B mutants and GluN1-1a to attempt to rescue surface trafficking. In this situation, some of the YFP-GluN2B mutant subunits may be in GluN1/YFP-GluN2-mutant diheteromers, whereas some may be in GluN1/YFP-GluN2-mutant/Myc-GluN2-WT triheteromers. We wondered whether glutamate binding with normal affinity to one WT subunit in such a triheteromer might increase surface levels of associated mutant YFP-GluN2B. Thus, we co-transfected COS-7 cells with GluN1, YFP-GluN2B mutant, and either Myc-GluN2B-WT or empty vector for comparison and assayed for surface YFP staining relative to total YFP signal in cells immunopositive for Myc-GluN2B-WT (Fig. 5A). However, co-expression of Myc-GluN2B-WT did not rescue the surface...
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FIGURE 4. Glutamate binding-deficient GluN2 mutants show reduced Golgi localization. A, COS-7 cells co-transfected with untagged GluN1-1a and YFP-GluN2B WT or E387A mutant were fixed, permeabilized, and immunolabeled for the Golgi maker GM130 and GluN1. Scale bar, 10 μm. B, quantitation of the average intensity of YFP-GluN2B colocalized with GM130 over the average intensity of YFP-GluN2B in the rest of the cell not colocalized with GM130 revealed reduced Golgi localization of mutants (ANOVA, p < 0.0001, and Bonferroni’s multiple comparison test when compared with WT, *, p < 0.001; n = 20 per construct from two independent experiments). Error bars indicate S.E.

expression of the GluN2B mutants; they still exhibited poor surface expression when compared with WT (Fig. 5B; ANOVA, p < 0.0001). Comparison of Myc-GluN2B–WT co-expression with the empty vector condition for each YFP-GluN2B construct revealed no significant difference in surface level (Fig. 5B; Student’s t test, p > 0.1). Assuming the formation of GluN1/ YFP-GluN2-mutant/Myc-GluN2-WT triheteromers, these results suggest that occupancy of the glutamate binding sites on both GluN2B subunits is required for proper surface delivery and that the presence of one glutamate binding-deficient subunit is dominant in reducing surface expression. However, we cannot rule out the possibility that the mutations may affect selectivity of subunit assembly such that mutant and wild type GluN2B may form distinct diheteromers with GluN1.

Glutamate Binding Regulates Surface Expression of NMDA Receptors in Cultured Neurons—To assess the role of glutamate binding in surface expression of NMDA receptors in their native cell type, we transfected YFP-GluN2B and the series of mutants with reduced glutamate efficacy into cultured rat hippocampal neurons. Neurons were transfected at plating, grown under normal conditions in the absence of inhibitors, and analyzed at 6 DIV, allowing time for the YFP-GluN2B to associate with endogenous GluN1 and traffic to the cell surface. We chose 6 DIV for initial analysis as a developmental stage when receptors are present on the neuron surface but not yet well clustered at synapses in cultured hippocampal neurons (30–32) to assess general mechanisms of surface expression relatively independent of synapse-specific mechanisms regulating trafficking. Transfected neurons were incubated live with membrane-impermeant anti-GFP antibody to detect surface YFP-NR2B and then fixed, permeabilized, and immunolabeled for the somatodendritic marker MAP2.

In DIV 6 rat hippocampal neurons, YFP fluorescence corresponding to YFP-GluN2B–WT was detected in the soma and dendrites, and surface-labeled YFP-GluN2B receptors were also detected throughout the somatodendritic domain (Fig. 6A). We observed similar somatodendritic expression patterns of total YFP fluorescence for all YFP-GluN2B glutamate binding-deficient mutants. However, the mutants exhibited varying degrees of diminished surface immunofluorescence relative to the wild type (Fig. 6A shows examples for R493K, S664G, and V660A). Some nonuniformity was seen in the surface label for all receptors, presumably due to surface patching of the diffusible receptors during live cell antibody incubation, as reported previously for GluA1 in immature neurons (33). Thus, for quantitation, we measured the ratio of surface YFP-GluN2B to total YFP-GluN2B over the entire MAP2-positive somatodendritic domain per field for transfected cells chosen by the CFP and YFP channels. All mutants analyzed showed significant surface expression deficits when compared with the YFP-GluN2B–WT (Fig. 6B; ANOVA, p < 0.0001, and Bonferroni’s multiple comparison test, p < 0.001, each mutant when compared with wild type). Furthermore, the relative surface expression of these mutants in neurons correlated well with their reported glutamate efficacies (Fig. 6C; Pearson’s coefficient of determination r² = 0.93, p = 0.0088 for WT, V660A, F390S, S664G, and E387A).

Because the presence of endogenous GluN2B in the rat hippocampal neurons may affect the surface expression of exogenously expressed mutant GluN2B either negatively through a competitive mechanism or positively through an associative mechanism, we also investigated the surface expression of our panel of YFP-GluN2B constructs in GluN2B−/− mouse cortical neurons at DIV 6. Similar to the results in rat hippocampal neurons, in cultured mouse cortical neurons lacking native GluN2B, the YFP-GluN2B glutamate binding-deficient mutants exhibited reduced surface expression relative to wild type. The fraction of YFP-GluN2B found on the cell surface was significantly reduced for all the mutants when compared with YFP-GluN2B–WT (Fig. 6D; ANOVA, p < 0.0001, and Bonferroni’s multiple comparison test of each mutant when compared with wild type, p < 0.001). Furthermore, consistent with results in rat hippocampal cells and heterologous COS-7 cells, relative surface expression in GluN2B−/− neurons correlated well with
the reported glutamate efficacies (Fig. 6E; Pearson’s coefficient of determination $r^2 = 0.95$, $p = 0.0056$ for WT, V660A, F390S, S664G, and E387A).

**A Constitutively Closed Cleft GluN2B Mutant Is Similar to Wild Type in Surface Expression**—It has been suggested that reversible gating motions induced by ligand binding are required for GluA2 surface trafficking and that transition through multiple states is essential (13). This idea was based in part on the finding that a constitutively closed cleft GluA2 mutant was retained in the ER (13). On the other hand, if a simple closed cleft conformation promotes surface trafficking, then one might expect a mutant with a constitutively closed cleft to traffic to the surface to the same extent or more efficiently than wild type. To test these ideas, we generated YFP-GluN2B K462C/N662C (KNCC). These two residues are close enough to form a disulfide bond between the S1 and S2 lobes, stabilizing the closed cleft conformation, and indeed, the homologous GluN2A KNCC mutant when expressed with GluN1 generates a constitutively active receptor with 90% maximal current in the absence of glutamate (34). Glutamate could still activate the GluN2A KNCC mutant receptor to 100% with an EC$_{50}$ of 0.76 μM (34). In the cell surface expression assays in COS-7 cells, the GluN2B KNCC mutant behaved indistinguishably from wild type GluN2B. Surface expression of YFP-GluN2B KNCC was essentially identical to that of wild type GluN2B and was not affected by co-expression of either dominant negative dynamin or wild type Myc-GluN2B (Fig. 7, A and B; ANOVA $p > 0.1$). Thus, forward trafficking of GluN2B was neither enhanced nor reduced by the KNCC mutation generating a constitutive closed cleft conformation. Furthermore, surface expression of YFP-GluN2B KNCC was also indistinguishable from that of wild type YFP-GluN2B upon expression in mouse GluN2B$^{-/-}$ cortical neurons at 6 DIV (Fig. 7, C and D; Student’s $t$ test, $p > 0.1$).

**Glutamate Binding of GluN2B Regulates Synaptic Expression upon Rescue in GluN2B$^{-/-}$ Hippocampal Neurons**—The pool of NMDA receptors in neurons that mediate most forms of synaptic plasticity are those that are localized to synapses. Thus, we assessed the role of glutamate binding to GluN2B in trafficking to synapses in mature cultured neurons. We first established conditions to essentially replace the endogenous synaptic GluN2B with recombinant YFP-GluN2B. Hippocampal neurons from GluN2B$^{-/-}$ mice were transfected at plating with YFP-GluN2B under control of the human ferritin promoter and analyzed at 14 DIV. In this rescue system, the recombinant YFP-GluN2B localized appropriately to synapses and colocalized with GluN1 (Fig. 8A). Furthermore, we were fortunate to obtain just the right expression level of YFP-GluN2B to rescue anti-GluN2B immunoreactive puncta number and intensity in the GluN2B$^{-/-}$ neurons to levels indistinguishable from that of wild type sister neurons (Fig. 8B; ANOVA, $p < 0.0001$, and Bonferroni’s multiple comparison, test, $p < 0.001$ for GluN2B$^{-/-}$ when compared with WT, $p > 0.05$ for GluN2B$^{-/-}$ plus YFP-GluN2B when compared with WT).

We next assessed synaptic surface accumulation of the glutamate binding-deficient GluN2B mutants when compared with wild type in this rescue system. Mouse GluN2B$^{-/-}$ hippocampal neurons were transfected with the YFP-GluN2B constructs at plating and analyzed at DIV 14. Neurons were immunostained live for surface YFP-GluN2B and then fixed, permeabilized, and immunostained for excitatory postsynaptic scaffolding PSD-95 protein family and excitatory presynaptic vesicular glutamate transporter VGlut1. The glutamate bind-
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A

|        | WT    | R493K | S664G | V660A |
|--------|-------|-------|-------|-------|
| YFP-GluN2B |       |       |       |       |
| Surface/Total YFP-GluN2B |       |       |       |       |

FIGURE 6. Glutamate binding of GluN2 regulates surface levels in neurons. A, rat hippocampal neurons were transfected at plating with the indicated YFP-GluN2B construct and labeled live at 6 DIV for surface YFP-GluN2B and then fixed, permeabilized, and immunolabelled for the somatodendritic marker MAP2. Scale bar, 10 μm. B, quantitation of somatodendritic surface YFP-GluN2B to total YFP-GluN2B ratio normalized to WT revealed reduced surface levels of all mutants (ANOVA, p < 0.0001, and Bonferroni’s multiple comparison test when compared with WT, *p < 0.001; n = 45 per construct from three independent experiments). C, the relative surface levels correlated well with the log of the reported EC50 for glutamate (Laube et al. (23)) for the series of YFP-GluN2B constructs (Pearson’s coefficient of determination $r^2 = 0.93, p = 0.0088$ excluding R493K, which had no measured response). D and E, similar experiments in GluN2B−/− mouse cortical neurons revealed similarly reduced surface levels of all mutant YFP-GluN2B receptors (ANOVA, $p < 0.0001$, and Bonferroni’s multiple comparison test when compared with WT, *$p < 0.001$; n = 30 per construct from two independent experiments) and correlation between glutamate efficacy of GluN2B and surface levels (Pearson’s coefficient of determination $r^2 = 0.95, p = 0.0056$ excluding R493K). Error bars in B–E indicate S.E.

B

C

D

E

DISCUSSION

Here, we used a range of GluN2B ligand binding point mutants with reduced glutamate efficacy to assess the role of glutamate binding in surface trafficking of NMDA receptors. Both in heterologous cells co-expressing GluN1 and in primary neuron cultures, surface expression of the GluN2B mutants correlated with apparent affinity for glutamate. Similar results were found in the presence of dominant negative dynamin to inhibit endocytosis, and surface expression also correlated with Golgi localization, suggesting that glutamate binding is required for forward trafficking of NMDA receptors to the cell surface. Co-expressed wild type GluN2B did not enhance surface expression of mutant GluN2B, suggesting that the ligand binding sites of both GluN2 subunits in a tetramer need to be occupied for surface expression. Further, in a molecular replacement strategy, we expressed YFP-GluN2B and the mutants in cultured neurons from GluN2B−/− mice and found that apparent glutamate affinity controlled surface
expression in young neurons and surface synaptic levels in mature neurons. Altogether, these data strengthen the accumulating evidence that glutamate is required for forward trafficking of multiple ionotropic glutamate receptors (15, 16) and indicate that NMDA receptors share this requirement.

Prior studies on AMPA and kainate receptors were reported in a more binary fashion, in the sense that severe ligand binding mutants were studied and found to be strongly and similarly impaired in surface expression (e.g. Refs. 9–12). For example, when compared with GluK2 wild type glutamate EC_{50} 191 \mu M or GluA4 wild type EC_{50} 2 \mu M, mutants impaired in surface trafficking had undetectable response (9, 12). Here, using a panel of GluN2B ligand binding mutants covering a spectrum of glutamate efficacies, we observed a correlation between glutamate efficacy and surface expression, both in heterologous cells and in neurons. When compared with glutamate EC_{50} 1.5 \mu M for wild type GluN2B, mutants V660A, F390S, S664G, and E387A with EC_{50} in the range of 27–355 \mu M and R493K with undetectable response (Laube

**FIGURE 7. A constitutively closed cleft GluN2B mutant is similar to wild type in surface expression.** A, COS-7 cells were transfected with GluN1-1a and YFP-GluN2B WT or KNCC plus either wild type dynamin or DN HA-dynamin-K44A and processed as in Fig. 3. Quantitation of surface YFP-GluN2B to total YFP-GluN2B ratio normalized to WT revealed no effect of the KNCC mutation on surface expression, with similar results upon co-expression of WT HA-dynamin or DN HA-dynamin-K44A (ANOVA p > 0.1; n = 24 per construct from two independent experiments). Error bars indicate S.E. B, COS-7 cells were co-transfected with Myc-GluN2B WT, GluN1-1a, and YFP-GluN2B WT or KNCC and processed as in Fig. 5. Quantitation of surface YFP-GluN2B to total YFP-GluN2B ratio normalized to WT revealed no effect of the KNCC mutant on surface expression, with similar results upon co-expression of Myc-GluN2B-WT or the empty vector control (ANOVA p > 0.1; n = 24 per construct from two independent experiments). Error bars indicate S.E. C, GluN2B^{-/-} mouse cortical neurons were transfected at plating with YFP-GluN2B WT or KNCC and processed as in Fig. 6. Quantitation of somatodendritic surface YFP-GluN2B to total YFP-GluN2B ratio normalized to WT revealed no effect of the KNCC mutant on surface expression (Student’s t test, p > 0.1; n = 30 per construct from two independent experiments). Error bars indicate S.E. D, representative images of GluN2B^{-/-} mouse cortical neurons expressing YFP-GluN2B WT or KNCC and labeled live at 6 DIV for surface YFP-GluN2B and then fixed, permeabilized, and immunolabeled for the somatodendritic marker MAP2. Scale bar, 10 \mu m.
et al. (23)) showed a corresponding range of surface expression in heterologous cells (Fig. 2) and in neurons (Fig. 6). Combined with the evidence of a role for glutamate binding at the stage of forward trafficking (Figs. 3 and 4), these results suggest that the rate of receptor release for forward trafficking is dependent on the rate of ligand binding.
Glutamate Binding Controls NMDA Receptor Surface Delivery

ER retention of mutant AMPA and kainate receptors has been demonstrated by sensitivity to endoglycosidase H (EndoH) (9, 11, 12). EndoH cleaves N-linked high mannose-containing sugars on immature receptors found in the ER, whereas maturation through the Golgi renders receptors EndoH-resistant. We were not able to detect pools of GluN2B with differential EndoH sensitivity (data not shown), and previous studies could not detect EndoH-resistant GluN1; all GluN1 was EndoH-sensitive despite evidence for a large cytoplasmic pool (36), suggesting that ER-localized NMDA receptors may be largely EndoH-sensitive. Nonetheless, the reduced forward trafficking of GluN2B mutants when compared with wild type even during inhibition of endocytosis (Fig. 3) and reduced colocalization of GluN2B mutants with Golgi marker GM130 (Fig. 4) support the idea that the mutant receptors accumulate in the ER. Although accurate estimates of glutamate concentration in the ER lumen are lacking, glutamate is readily detected in the ER, and average intracellular concentrations are thought to be in the millimolar range (e.g. 4–16 mM in various brain regions (15, 37, 38)). The finding here that the constitutively closed cleft GluN2B KNCC traffics to the surface like wild type GluN2B and does not exhibit enhanced surface expression (Fig. 7) suggests that glutamate concentrations are not normally limiting for forward trafficking of GluN2B. Furthermore, the conserved glutamate binding pocket and similar affinities of GluN2A, GluN2B, GluN2C, and GluN2D subunits for glutamate, ranging from EC_{50} 0.51 to 3.3 μM (2, 39), suggest that glutamate is not typically limiting for forward trafficking of native NMDA receptors. The range of glutamate concentration that mediates maximal activation of wild type GluN2B while detecting differences in activation of the GluN2B mutants is roughly from 50 μM to 1 mM (23). Assuming that the glutamate pool that controls forward trafficking is the same for AMPA, kainate, and NMDA receptors, thus also taking into account surface trafficking of GluA2 and GluK2 and one mutant of each with reduced glutamate efficacy (9, 12), would put the relevant glutamate concentration at the high end of our range.

Interestingly, co-expression of WT GluK2(Q) partially rescued surface expression of a glutamate binding-deficient mutant GluK2(R) E738G, assayed by rectification ratio in patch clamp recordings (9). In contrast, surface expression of GluN2B glutamate binding-deficient mutants was not enhanced by co-expression of WT GluN2B (Fig. 5). The apparent difference between these results may lie partly in the nature of the assay, as electrophysiological tagging is very sensitive to detect low surface levels, or may reflect a difference between ionotropic receptor subtypes. Our results suggest that ligand must interact at both GluN2 subunits for proper trafficking and the least avid binding site may be limiting, consistent with the requirement for two molecules of glutamate (and two of glycine) for activation of NMDA receptors (40, 41) and with recent structural studies (24). However, we cannot rule out the alternate possibility that the GluN2B mutations may alter selectivity of subunit assembly such that mutant GluN2B preferentially assembles with mutant and wild type with wild type. Partial rescue of surface trafficking was also achieved for ligand binding-deficient mutants of GluA4 by co-expression of stargazin (12) and of GluN1-D732A by treatment with a cell-permeable competitive glycine site antagonist (17). However, stargazin does not interact with NMDA receptors, and although the idea that binding of a competitive antagonist might mimic ligand binding to rescue surface trafficking is attractive, the low cell permeability of competitive antagonists for GluN2 precluded the ability to test this idea.

Previous studies, as discussed above, assayed the role of ligand binding to ionotropic glutamate receptors in surface trafficking in heterologous cells and by overexpression in neurons. Here, we extend these assays to include rescue of synaptic GluN2B at an appropriate expression level in GluN2B−/− neurons (Fig. 8). These results indicate that differences in ligand binding correlate with differences in surface synaptic accumulation in mature neurons. Thus, ligand binding controls delivery of this functionally important pool of NMDA receptors. NMDA receptor accumulation at synapses is also controlled by other features of GluN1 and GluN2 subunits (42, 43) and multiple interacting proteins regulated by activity and kinases (27, 44, 45).

The precise mechanism by which ligand binding promotes forward trafficking of ionotropic glutamate receptors is not well understood. The mechanism is not likely to require ion flux because mutations in the isolated S1−S2 ligand binding domain of GluA4 or GluK2 affected their secretion in a parallel manner to effects on surface expression of the full-length receptors (12, 14). One idea is that reversible gating motions associated with reversible cleft closure induced by ligand binding are sensed in the ER to promote export (13). In support of this idea, a constitutively closed cleft GluA2 ETCC mutant was retained in the ER (13), and a constitutively closed cleft GluK2 ENCC mutant showed reduced surface expression when compared with WT GluK2 (14). However, in our study (Fig. 7), the constitutively closed cleft GluN2B KNCC mutant trafficked to the surface indistinguishably from WT GluN2B. These results suggest that GluN2 does not have to undergo reversible gating motions or

FIGURE 8. Glutamate binding of GluN2B regulates rescue of synaptic surface GluN2B in GluN2B−/− neurons. A, mouse hippocampal GluN2B−/− neurons were transfected at plating with YFP-GluN2B-WT and immunolabeled at 14 DIV for GluN2B and either synapsin (left) or GluN1 (right). The recombinant YFP-GluN2B localized appropriately opposite synapsin-labeled terminals (arrows) and colocalized with endogenous GluN1. Arrowheads indicate dendrites from a neighbor nontransfected neuron lacking GluN2B. Scale bars, 5 μm. B, quantitation of GluN2B immunofluorescent puncta confirmed a complete absence in GluN2B−/− cultures and showed rescue to levels indistinguishable from WT neurons in GluN2B−/− neurons transfected with YFP-GluN2B (ANOVA, p < 0.0001, and Bonferroni’s multiple comparison test, *p < 0.001 for GluN2B−/− when compared with WT neurons, p > 0.05 for GluN2B−/− plus YFP-GluN2B when compared with WT neurons; n = 12–15). Error bars indicate S.E. C, mouse hippocampal GluN2B−/− hippocampal neurons were transfected at plating with the indicated YFP-GluN2B constructs and analyzed at 14 DIV. Neurons were immunolabeled live for surface YFP-GluN2B and then fixed, permeabilized and immunolabeled for PSD-95 and VGlut1. Scale bar, 5 μm. D, quantitation of the number of surface YFP-GluN2B puncta colocalized with PSD-95 per dendrite length showed reduced surface synaptic levels for all GluN2B mutants (ANOVA, p < 0.0001, and Bonferroni’s multiple comparison test when compared with WT, *p < 0.001). E, the density of surface synaptic puncta correlated well with the log of the reported EC_{50} for glutamate (Laube et al. (23)) for the series of YFP-GluN2B constructs (Pearson’s coefficient of determination r^2 = 0.99, p = 0.0003 excluding R493K). F, there was no difference in the density of PSD-95 puncta among groups (ANOVA p > 0.05). Error bars in D–F indicate S.E.
reversible cleft closure and that other aspects of the ligand-induced conformation promote forward trafficking to the cell surface. Other recent studies on AMPA receptors also support a mechanism of ligand-induced conformation changes promoting forward trafficking independent of activity and suggest that the ligand binding domain is the primary site for ER quality control of AMPA receptors (16).

Ligand binding appears to control forward trafficking for all ionotropic glutamate classes, glutamate binding to AMPA and kainate receptors (15), glycine binding to GluN1 (17), and glutamate binding to GluN2 (this work). A primary reason may be for quality control, so that only functional assembled ligand-gated ion channels traffic to the surface. An intriguing corollary is that NMDA receptors may be functional in the ER. Indeed, the proposed requirement for glutamate and glycine binding for ER exit suggests that NMDA receptors would be at least transiently open at intracellular sites. Interestingly, activation of the metabotropic receptor mGluR5 on intracellular membranes triggers different signaling cascades from activation of cell surface mGluR5, resulting in differential gene transcription (46). Thus, it is possible that ligand binding to intracellular receptors may have a function in addition to quality control, that sodium and calcium efflux from the ER or transport intermediates to the cytosol mediated by NMDA receptors may trigger local signaling in neurons.

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REFERENCES

1. Mori, H., and Mishina, M. (1995) Structure and function of the NMDA receptor channel. Neuropharmacology 34, 1219–1237
2. Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., Hansen, K. B., Yuan, H., Myers, S. J., and Dingledine, R. (2010) Glutamate receptor ion channels: structure, regulation, and function. Pharmacol. Rev. 62, 405–496
3. Burnashev, N., Schoepfer, R., Monyer, H., Ruppersberg, J. P., Günther, W., Seeburg, P. H., and Sakmann, B. (1992) Control by asparagine residues of calcium permeability and magnesium blockade in the NMDA receptor. Science 257, 1415–1419
4. Lee, Y. S., and Silva, A. J. (2009) The molecular and cellular biology of enhanced cognition. Nat. Rev. Neurosci. 10, 126–140
5. Wang, H., Hu, Y., and Tsien, J. Z. (2006) Molecular and systems mechanisms of memory consolidation and storage. Prog. Neurobiol. 79, 123–135
6. Horak, M., Chang, K., and Wenthold, R. J. (2008) Masking of the endoplasmic reticulum retention signals during assembly of the NMDA receptor. J. Neurosci. 28, 3500–3509
7. Hawkins, L. M., Przybyslawski, K., Chang, K., Mousann, C., Stephenson, F. A., and Wenthold, R. J. (2004) Export from the endoplasmic reticulum of assembeled N-methyl-D-aspartic acid receptors is controlled by a motif in the C-terminus of the NR2 subunit. J. Biol. Chem. 279, 28903–28910
8. Yang, W., Zheng, C., Song, Q., Yang, X., Qiu, S., Liu, C., Chen, Z., Duan, S., and Luo, J. (2007) A three-amino acid tail following the TM4 region of the N-methyl-D-aspartate receptor (NR) 2 subunits is sufficient to overcome endoplasmic reticulum retention of NR1–1a subunit. J. Biol. Chem. 282, 9269–9278
9. Mah, S. J., Cornell, E., Mitchell, N. A., and Fleck, M. W. (2005) Glutamate receptor trafficking: endoplasmic reticulum quality control involves ligand binding and receptor function. J. Neurosci. 25, 2215–2225
10. Valluru, L., Xu, J., Zhu, Y., Yan, S., Contractor, A., and Swanson, G. T. (2005) Ligand binding is a critical requirement for plasma membrane expression of heteromeric kainate receptors. J. Biol. Chem. 280, 6085–6093
11. Greger, I. H., Akamine, P., Khatri, L., and Ziff, E. B. (2006) Developmentally regulated, combinatorial RNA processing modulates AMPA receptor biogenesis. Neuron 51, 85–97
12. Colemen, S. K., Møystky, T., Joupplia, A., Koskelainen, S., Rivera, C., Korpi, E. R., and Keinänen, K. (2009) Agonist occupancy is essential for forward trafficking of AMPA receptors. J. Neurosci. 29, 303–312
13. Penn, A. C., Williams, S. R., and Greger, I. H. (2008) Gating motions underlie AMPA receptor secretion from the endoplasmic reticulum. EMBO J. 27, 3056–3068
14. Gill, M. B., Vivithanaporn, P., and Swanson, G. T. (2009) Glutamate binding and conformational flexibility of ligand binding domains are critical early determinants of efficient kainate receptor biogenesis. J. Biol. Chem. 284, 14503–14512
15. Fleck, M. W. (2006) Glutamate receptors and endoplasmic reticulum quality control: looking beneath the surface. Neuroscientist 12, 232–244
16. Colemen, S. K., Møystky, T., Hinkkari, S., Vahtera, L., Korpi, E. R., Pentikäinen, O. T., and Keinänen, K. (2010) Ligand binding domain determines endoplasmic reticulum exit of AMPA receptors. J. Biol. Chem. 285, 36302–36309
17. Kenny, A. V., Cousins, S. L., Pinho, L., and Stephenson, F. A. (2009) The integrity of the glycine co-agonist binding site of N-methyl-D-aspartate receptors is a functional quality control checkpoint for cell surface delivery. J. Biol. Chem. 284, 324–333
18. Kim, M. J., Dunah, A. W., Wang, Y. T., and Sheng, M. (2005) Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking. Neuron 46, 745–760
19. Ochoa, G. C., Slepnev, V. I., Neff, L., Ringstad, N., Takei, K., Daniell, L., Kim, W., Cao, H., McNiven, M., Baron, R., and De Camilli, P. (2000) A functional link between dynamin and the actin cytoskeleton at podosomes. J. Cell Biol. 150, 377–389
20. Kaeche, S., and Banker, G. (2006) Culticping hippocampal neurons. Nat. Prost. 1, 2406–2415
21. Kutsuwada, T., Sakimura, K., Manabe, T., Takayama, C., Katakur, N., Kushiya, E., Natsume, R., Watanabe, M., Inoue, Y., Yagi, T., Aizawa, S., Arakawa, M., Takahashi, T., Nakamura, Y., Mori, H., and Mishina, M. (1996) Impairment of suckling response, trigeminal neuronal pattern formation, and hippocampal LTD in NMDA receptor ε 2 subunit mutant mice. Neuron 16, 333–344
22. Liu, Y., Wong, T. P., Aarts, M., Rooyackers, A., Liu, L., Lai, T. W., Wu, D. C., Lu, J., Tymianski, M., Craig, A. M., and Wang, Y. T. (2007) NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. J. Neurosci. 27, 2846–2857
23. Laube, B., Hirai, H., Sturgess, M., Betz, H., and Kuhse, J. (1997) Molecular determinants of agonist discrimination by NMDA receptor subunits: analysis of the glutamate binding site on the NR2B subunit. Neuron 18, 493–503
24. Furukawa, H., Singh, S. K., Mancusso, R., and Gouaux, E. (2005) Subunit arrangement and function in NMDA receptors. Nature 438, 185–192
25. Cik, M., Chazot, P. L., and Stephenson, F. A. (1993) Optimal expression of cloned NMDAR1/NMDAR2A heteromeric glutamate receptors: a biochemical characterization. Biochem. J. 296, 877–883
26. Przybyslawski, K., Chang, K., Ennis, N., Kan, L., Vicini, S., and Wenthold, R. J. (2005) The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PKD proteins and AP-2. Neuron 47, 845–857
27. Lau, C. G., and Zukan, B. S. (2007) NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. Nat. Rev. Neurosci. 8, 413–426
28. Herskovits, J. S., Burgess, C. C., Obar, R. A., and Valley, R. B. (1993) Effects of mutant rat dynamin on endocytosis. J. Cell Biol. 122, 565–578
29. Nakamura, N., Rabouille, C., Watson, R., Nilsson, T., Hui, N., Slusarewicz, P., Kreis, T. E., and Warren, G. (1995) Characterization of a cís-Golgi matrix protein, GM130. J. Cell Biol. 131, 1715–1726
30. Cottrell, I. R., Dubé, G. R., Egle, C., and Liu, G. (2000) Distribution, density, and clustering of functional glutamate receptors before and after synaptogenesis in hippocampal neurons. J. Neurophysiol. 84, 1573–1587
31. Crump, F. T., Dillman, K., and Craig, A. M. (2001) CaM-dependent protein kinase mediates activity-regulated synaptic targeting of NMDA
32. Rao, A., Kim, E., Sheng, M., and Craig, A. M. (1998) Heterogeneity in the molecular composition of excitatory postsynaptic sites during development of hippocampal neurons in culture. J. Neurosci. 18, 1217–1229.

33. Mammen, A. L., Huganir, R. L., and O’Brien, R. J. (1997) Redistribution and stabilization of cell surface glutamate receptors during synapse formation. J. Neurosci. 17, 7351–7358.

34. Blanke, M. L., and VanDongen, A. M. (2008) Constitutive activation of the N-methyl-D-aspartate receptor via cleft-spanning disulfide bonds. J. Biol. Chem. 283, 21519–21529.

35. Ramírez, O. A., and Couve, A. (2011) The endoplasmic reticulum and protein trafficking in dendrites and axons. Trends Cell Biol. 21, 219–227.

36. Huh, K. H., and Wenthold, R. J. (1999) Turnover analysis of glutamate receptors identifies a rapidly degraded pool of the N-methyl-D-aspartate receptor subunit, NR1, in cultured cerebellar granule cells. J. Biol. Chem. 274, 151–157.

37. Berger, S. J., Carter, J. C., and Lowry, O. H. (1977) The distribution of glycine, GABA, glutamate, and aspartate in rabbit spinal cord, cerebellum, and hippocampus. J. Neurochem. 28, 149–158.

38. Meeker, R. B., Swanson, D. J., and Hayward, J. N. (1989) Light and electron microscopic localization of glutamate immunoreactivity in the supraoptic nucleus of the rat hypothalamus. Neuroscience 33, 157–167.

39. Erreger, K., Geballe, M. T., Kristensen, A., Chen, P. E., Hansen, K. B., Lee, C. J., Yuan, H., Le, P., Lyuboslavsky, P. N., Micale, N., Jørgensen, L., Clausen, R. P., Wylie, D. J., Snyder, J. P., and Traynelis, S. F. (2007) Subunit-specific agonist activity at NR2A-, NR2B-, NR2C-, and NR2D-containing N-methyl-D-aspartate glutamate receptors. Mol. Pharmacol. 72, 907–920.

40. Clements, J. D., and Westbrook, G. L. (1991) Activation kinetics reveal the number of glutamate and glycine binding sites on the N-methyl-D-aspartate receptor. Neuron 7, 605–613.

41. Benveniste, M., and Mayer, M. L. (1991) Kinetic analysis of antagonist action at N-methyl-D-aspartic acid receptors: two binding sites each for glutamate and glycine. Biophys. J. 59, 560–573.

42. Ferreira, J. S., Rooyakkers, A., She, K., Ribeiro, L., Carvalho, A. L., and Craig, A. M. (2011) Activity and protein kinase C regulate synaptic accumulation of N-methyl-D-aspartate (NMDA) receptors independently of GluN1 splice variant. J. Biol. Chem. 286, 28331–28342.

43. Storey, G. P., Opitz-Araya, X., and Barria, A. (2011) Molecular determinants controlling NMDA receptor synaptic incorporation. J. Neurosci. 31, 6311–6316.

44. Sanz-Clemente, A., Matta, J. A., Isaac, J. T., and Roche, K. W. (2010) Casein kinase 2 regulates the NR2 subunit composition of synaptic NMDA receptors. Neuron 67, 984–996.

45. Nolt, M. J., Lin, Y., Hruska, M., Murphy, J., Sheffler-Colins, S. I., Kayser, M. S., Passer, J., Bennett, M. V., Zukin, R. S., and Dalva, M. B. (2011) EphB controls NMDA receptor function and synaptic targeting in a subunit-specific manner. J. Neurosci. 31, 5353–5364.

46. Jong, Y. J., Kumar, V., and O’Malley, K. L. (2009) Intracellular metabotropic glutamate receptor 5 (mGluR5) activates signaling cascades distinct from cell surface counterparts. J. Biol. Chem. 284, 35827–35838.