Transmembrane Region of N-Methyl-D-aspartate Receptor (NMDAR) Subunit Is Required for Receptor Subunit Assembly*5

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N-Methyl-D-aspartate receptors (NMDARs), one of three main classes of ionotropic glutamate receptors, play major roles in synaptic plasticity, synaptogenesis, and excitotoxicity. Unlike non-NMDA receptors, NMDARs are thought to comprise obligatory heterotetrameric complexes mainly composed of GluN1 and GluN2 subunits. When expressed alone in heterogenous cells, such as HEK293 cells, most of the NMDAR subunits can neither leave the endoplasmic reticulum (ER) nor be expressed in the cell membrane because of the ER retention signals. Only when NMDARs are heteromerically assembled can the ER retention signals be masked and NMDARs be expressed in the surface membrane. However, the mechanisms underlying NMDAR assembly remain poorly understood. To identify regions in subunits that mediate this assembly, we made a series of truncated or chimeric cDNA constructs. Using FRET measurement in living cells combined with immunostaining and coimmunoprecipitation analysis, we examined the assembly-determining domains of NMDAR subunits. Our results indicate that the transmembrane region of subunits is necessary for the assembly of NMDAR subunits, both for the homodimer and the heteromer.

N-Methyl-D-aspartate receptors (NMDARs)3, a subtype of the ionotropic glutamate receptors, play critical roles in excitatory synaptic transmission, plasticity, and excitotoxicity in the central nervous system. Over the past two decades, a variety of NMDAR subunits have been identified: the ubiquitously expressed GluN1 subunit, which occurs in eight variants, a family of four distinct GluN2 subunits (GluN2A–D), and two GluN3 subunits (GluN3A and GluN3B). All of these subunits have a similar membrane topology, which is shared by all ionotropic glutamate receptor subunits, with a large extracellular N terminus (NT), three transmembrane segments (M1, M3, and M4), a reentrant membrane loop (M2), and a cytoplasmic C terminus (1, 2) (Fig. 1A). On the basis of the sequence homology to bacterial periplasmic binding proteins, the NT of iGluRs can be divided into two domains in tandem: the amino-terminal domain (ATD) or the LIVBP-like domain (leucine, isoleucine, valine binding protein), which includes the first 400 or so amino acids (3), and the following S1 domain preceding M1, which forms the ligand-binding domain or LAOBP-like domain (lysine, arginine, ornithine binding protein) together with the extracellular loop between M3 and M4 (S2 domain) (4, 5).

Among the ionotropic glutamate receptors, NMDARs are special in that conventional NMDARs are obligatory heterotetrameric membrane proteins composed of two GluN1 and two GluN2 subunits (6, 7). When expressed alone in heterogeneous cells, the major GluN1 isoform (GluN1-1) and GluN2 are both retained in the ER because of multiple ER retention signals existing in the ATD, transmembrane regions, and C termini of subunits. When GluN1 and GluN2 subunits assemble together, these ER retention signals are masked, and the assembled receptors are trafficked to the surface membrane and expressed as functional channels (8–10). Consequently, correct assembly of subunits is a prerequisite for NMDARs to be exported from the ER and perform normal functions in the cell membrane. However, the molecular mechanisms governing the selective assembly of NMDAR subunits are still far from being well understood.

Studies on AMPA receptors indicate that they assemble as tetramers in a pattern of dimer-to-dimer association. The initial dimer formation is mediated by ATD interactions, whereas subsequent tetramerization occurs through interactions of the ligand-binding domains and the transmembrane domains (11–14). Similarly, NMDARs have also been shown to assemble as a dimer of dimers, but it is unclear which region is necessary for receptor subunit assembly. Studies using biochemical methods and immunostaining indicate that the C terminus and/or M4 are not involved in the heteromer formation between GluN1 and GluN2 subunits (9, 15), whereas the ATD or even the whole N terminus is necessary for NMDAR subunit assembly (15, 16). However, still other research indicates that the ATD mainly plays modulatory roles in NMDAR trafficking and function and is not directly involved in receptor assembly (10, 17, 18).

In this study, we constructed a series of truncated or chimeric NMDAR subunit mutants tagged with CFP or YFP and analyzed their homomeric and heteromeric assembly using a FRET
assay. We found that the transmembrane region rather than the N terminus of NMDAR subunits was required for the assembly of NMDAR complexes.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The expression vectors for CFP/YFP-GluN1, CFP/YFP-GluN2A, CFP/YFP-GluN2B, and GABA<sub>α</sub>1-YFP have been described previously (19). All constructs of GluN1 were generated from rat GluN1-1a cDNA. GluN1-CFP/YFP was constructed by first deleting the stop codon of the GluN1-1a subunit and then fusing the CFP or YFP to the C terminus of the subunit. The N-terminally truncated versions of the GluN1, GluN2A, or GluN2B subunits used in this study were constructed using conventional DNA mutagenesis techniques and named XFP-GluN<sub>Ant</sub> or GluN<sub>Ant</sub>-XFP with XFP tagged to the N terminus or C terminus of GluN, respectively (Fig. 1A). XFP represents CFP or YFP. Chimeras of YFP-GluN<sub>M1–4(GluA1)</sub>, YFP-GluN<sub>M1–3(GluN1)</sub> and YFP-GluN<sub>S2M4(GluA1)</sub> were constructed by replacing the M1–4, M1–3 or S2M4 region of YFP-GluN1 by the corresponding parts of GluA1. YFP-GluA1<sub>M(GluN1)</sub> was constructed by replacing the N and C termini of YFP-GluN1 by the corresponding part of GluA1 (Figs. 3A and 4A). A series of constructs of the GluN1 membrane domain tagged with CFP or YFP were made (Fig. 5A) and named XFP-M<sub>GluN1</sub>. For example, XFP-M<sub>1</sub>GluN1 represents the construct of M1 of GluN1 tagged with CFP or YFP in the N terminus. For each construct, the amino acid number (relative to the first methionine in the open reading frame) at the appropriate junction is indicated. All constructs were verified using DNA sequencing.

**Cell Culture and Transfection**—HEK293 cells and COS-7 cells were grown in DMEM, supplemented with 10% fetal bovine serum and antibiotics (all from Invitrogen), and transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The transfection mixture was replaced 3–5 h after transfection with fresh culture medium. Cells were examined within 2 days after transfection in an extracellular solution composed of 145 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 5 mM glucose, 0.01 mM glycine, and 5 mM HEPES (pH 7.4) with NaOH.

**Surface Immunostaining and Quantitative Analysis**—These methods have been described previously (20, 21). Cells were examined through a 60 × 1.4 (numerical aperture) oil immersion objective on a FV1000 confocal laser scanning microscope equipped with imaging analysis software (Olympus, Tokyo, Japan). All processes were performed at room temperature. Surface expression of YFP-tagged NMDARs in HEK293 cells was measured as the percentage of surface-stained cells (red) in the population of total cells transfected with YFP fusion protein (green). For each sample, more than 200 YFP-positive cells were counted, and the means were obtained from three to five different cultures.

**Immunocytochemistry**—Cultured COS-7 cells were fixed in 4% paraformaldehyde in PBS for 10 min and were permeabilized in PBS containing 0.2% Triton X-100 and 5% BSA for 30 min at room temperature. Cells were then incubated in primary rabbit polyclonal BiP antibody (a marker for ER, Abcam) with primary mouse monoclonal GFP antibody (Abcam) in PBS containing 5% BSA for 1 h. After washing three times with PBS, cells were incubated in anti-rabbit Alexa Fluor 546-conjugated secondary antibody (Molecular Probes, Eugene, OR) with or without anti-mouse Alexa Fluor 488-conjugated secondary antibody (Molecular Probes) in PBS containing 5% BSA for another 1 h. After washing three times with PBS, cells were observed on a Fluoview FV1000 confocal microscope (Olympus).

**Immunoblotting and Immunoprecipitation**—Wild-type GluN1 was cotransfected with YFP-GluN1 or with YFP-GluN<sub>M1–4(GluA1)</sub> in HEK293 cells. Thirty-six hours after transfection, the transfected cells were lysed by radioligand precipitation assay buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, proteinase inhibitor) and then incubated with the GFP antibody/protein A complex at 4 °C overnight. The complex was washed four times with radioligand precipitation assay buffer. Finally, the immunoprecipitated proteins were eluted with 1 × SDS sample buffer and analyzed by Western blot analysis using GluN1 (C-20) (SC-1467) antibody.

**Detection of FRET Using Three-cube FRET Measurement**—The fluorescence imaging workstation for FRET and the quantification method have been described previously (19, 22, 23). Briefly, the fluorescence imaging workstation consisted of a TE2000 inverted microscope (Nikon), Dual-ViewTM (Optical Insights), and a SNAP-HQ-cooled CCD (Roper Scientific). MetaMorph version 5.0 software was used for analysis of the cell image data. The FRET ratio (FR) was calculated according to Erickson et al. (22) as Equation 1:

\[
FR = \frac{S_{\text{FRET}}(DA) - R_{\text{D1}} \cdot S_{\text{CFP}}(DA)}{R_{\text{A1}} \cdot S_{\text{YFP}}(DA)}
\]

\(S_{\text{SPECIMEN}}\) indicates whether the cell is expressing donor (D), CFP), acceptor (A, YFP), or both (DA). \(R_{\text{D1}}\) equals \(S_{\text{FRET}}(D)/ S_{\text{CFP}}(D)\), and \(R_{\text{A1}}\) equals \(S_{\text{FRET}}(A)/S_{\text{YFP}}(A)\). \(R_{\text{D1}}\) was calculated when the HEK293 cells expressed CFP-KDEL, and \(R_{\text{A1}}\) was calculated when the cells expressed YFP-KDEL. FRET signals were acquired from intact cells expressing receptor subunits tagged with fluorescent protein.

**RESULTS**

**N Terminus of NMDA Receptor Subunit Is Not Necessary for Subunit Assembly but Is Essential for Trafficking to the Cell Membrane**—To determine the role of the NT in the assembly of NMDAR subunits, we generated a series of NT deletions in the CFP- or YFP-tagged NMDAR subunit (Fig. 1B). In previous studies, we analyzed the assembly of native NMDAR subunits using a three-cube FRET measurement (19). With the same approach, we tested the ability of these truncated subunits to form homodimers or heteromers. To exclude the possibility that the FRET signals may have come from unordered aggregates in intracellular compartments or random encounters between overexpressed subunits, we cotransfected CFP-GluN1 and GABA<sub>α</sub>1-YFP in HEK293 cells and measured the FRET signals. As a result, no significant FRET signal was produced, the FR being 1.01 ± 0.04, \(n = 31\) (Fig. 1C).
NMDAR Subunit Assembly

Next, we found that when CFP-GluN1\(_{\Delta NT}\) and YFP-GluN1\(_{\Delta NT}\) were cotransfected in HEK293 cells, FRET signals were produced. The FR value was calculated to be 4.69 ± 0.15 (\(n = 46\)) (Fig. 1C). This value was significantly different from that obtained from coexpression of CFP-GluN1 and GABA\(_A\) \(\alpha1\) constructs. These results indicated that the deletion of NT did not affect the formation of homomers between these truncated GluN1 subunits. To further confirm that NT-deleted GluN1 can form a homodimer, we tagged CFP or YFP to the intracellular C terminus of this subunit mutant and analyzed the interaction between these deletion constructs. The results showed that coexpression of the NT-deleted GluN1 subunits tagged with CFP or YFP at the C terminus also yielded positive FRET signals. The FR value was 2.56 ± 0.07 (\(n = 37\)) (Fig. 1C). In addition, coexpression of NT-deleted GluN1 subunits with a C-terminal CFP tag and the wild-type GluN1 subunit with a C-terminal YFP tag also produced FRET signals with an FR value of 2.44 ± 0.06 (\(n = 42\)) (Fig. 1C). These results further indicated that FRET reflected genuine subunit interaction and that the NT was not required for subunit assembly. Similar results were obtained from the GluN2A and GluN2B subunits. For the GluN2A or GluN2B subunits with NT deleted (Fig. 1B), the FR values were 4.29 ± 0.17 (\(n = 40\)) or 4.30 ± 0.10 (\(n = 49\)), respectively (C). These results showed that GluN2 subunits could still assemble into homomers even when the whole NT was removed.

To determine whether the formation of heteromers between the GluN1 and GluN2 subunits was affected when the NT was deleted, we cotransfected CFP-GluN1\(_{\Delta NT}\) with YFP-GluN2A\(_{\Delta NT}\) or CFP-GluN1\(_{\Delta NT}\) with YFP-GluN2B\(_{\Delta NT}\) into HEK293 cells and implemented the FRET experiments. Consequently, positive FRET signals were observed, the FR values being 4.52 ± 0.16 (\(n = 38\)) or 3.02 ± 0.12 (\(n = 34\)), respectively (Fig. 1D). In the previous study, we have shown in living cells that in the absence of the GluN1 subunit, the GluN2A and GluN2B subunits can form heteromers (19). When truncated GluN2A and GluN2B subunits were cotransfected into HEK293 cells, FRET signals were detected as 3.21 ± 0.14 (\(n = 25\)) for GluN2A\(_{\Delta NT}\) with GluN2B\(_{\Delta NT}\) (Fig. 1D). Taken together, these results suggested that deletion of the whole NT did not eliminate the formation of homomers among the GluN1, GluN2A, or GluN2B subunits, and the formation of heteromers between the GluN1 and GluN2 subunits or between the GluN2A and GluN2B subunits.

Because deletion of the whole NT did not affect NMDAR subunit assembly, we used surface immunostaining to determine whether these assembled complexes could be trafficked to the cell membrane. Because the fluorescent protein was tagged to the extracellular side of each subunit, the expression of GluN1 and GluN2A or GluN2B in the plasma membrane was detectable by immunostaining in live cells. We used a polyclonal anti-GFP antibody followed by a Cy3-conjugated secondary antibody to label the receptors (Fig. 2, A–C). Expression in the plasma membrane was indicated by the presence of red puncta scattered on the cell surface (Fig. 2A). When we coexpressed CFP-GluN1 with YFP-GluN2A or CFP-GluN1 with YFP-GluN2B, 66.0 ± 3.6% or 77.6 ± 1.8% of cells had positive surface labeling (Fig. 2, A and D). However, when cells were cotransfected with CFP-GluN1\(_{\Delta NT}\) and YFP-GluN2A or with CFP-GluN1\(_{\Delta NT}\) and YFP-GluN2B, we did not find significant
surface expression of these subunits (11.3 ± 1.2% or 4.0 ± 3.6%, respectively) (Fig. 2, B and D). Similarly, when cells were cotransfected with CFP-GluN1 and YFP-GluN2A<sub>ΔNT</sub> or CFP-GluN1 and YFP-GluN2B<sub>ΔNT</sub>, no significant surface expression of these subunits was observed (9.7 ± 0.5% or 4.7 ± 4.7%, respectively) (Fig. 2, C and D). Consistent with the immunostaining results, no glutamate-evoked current was detected from cells expressing these subunit combinations (data not shown). We checked the surface expression of all the intact or NT-deleted subunits alone and did not find significant surface staining (supplemental Fig. 2). Taken together, these results suggested that deletion of the NT blocked the trafficking of the assembled receptor mutants from the ER to the cell membrane.

**Transmembrane Region of NMDA Receptor Subunit Is Required for Receptor Subunit Assembly**—Because we demonstrated that the N terminus was not necessary for the assembly of NMDAR subunits and previous studies have indicated that C terminus-deleted GluN1 and GluN2 can assemble and be expressed in the cell membrane (9, 15), the transmembrane regions may be required for subunit assembly. To investigate the role of the transmembrane region of GluN1 in subunit assembly, we generated a chimeric construct of YFP-GluN1M1–4(GluA1) (Fig. 3A) by replacing the region between the M1 and M4 of YFP-GluN1 by the counterpart of the GluA1 subunit, which does not physically associate with the NMDAR subunit (19, 24). In this study, we found that coexpression of YFP-GluN1M1–4(GluA1) and CFP-GluN1 or YFP-GluN1M1–4(GluA1) and CFP-GluN2B did not produce any significant FRET signal, the FR being 1.056 ± 0.020 (n = 30) or 1.025 ± 0.052 (n = 15), respectively (Fig. 3B). These results showed that YFP-GluN1M1–4(GluA1) was neither able to assemble with CFP-GluN1, nor with CFP-GluN2B.

We then assessed the assembly of YFP-GluN1M1–4(GluA1) with CFP-GluN1 by coimmunoprecipitation in HEK293 cells.
When YFP-GluN1 was cotransfected with wild-type GluN1, we found two bands with an antibody specifically recognizing the C terminus of GluN1 after immunoprecipitation with an anti-GFP antibody. The lower band was the wild-type GluN1 subunit and the upper was the YFP-tagged GluN1 subunit. However, when YFP-GluN1M1–4(GluA1) was cotransfected with wild-type GluN1, we only detected the band of YFP-GluN1M1–4(GluA1) but not the wild-type GluN1 subunit (Fig. 3C). In accordance with the FRET results, this coimmunoprecipitation result suggested that GluN1 could not assemble with YFP-GluN1M1–4(GluA1). Taken together, these results suggested that the M1–4 region of GluN1 was necessary for GluN1 homoassembly and heteroassembly with GluN2B.

We further constructed a chimera named YFP-GluA1M(GluN1) in which we replaced both the N terminus and C terminus of YFP-GluN1 with the counterparts of GluA1 (Fig. 3A). Interestingly, when this chimera was coexpressed with CFP-GluN1 or with CFP-GluN2B in HEK293 cells, the resulting FRET signals were positive, the FR values being 1.30 ± 0.05 (n = 18) and 1.34 ± 0.04 (n = 20), respectively (Fig. 3B). This result indicated that the M1–4 region of GluN1 could mediate GluN1 homoassembly and GluN1/GluN2B heteroassembly. We also assessed the surface expression when HEK293 cells were cotransfected with YFP-GluA1M(GluN1) and CFP-GluN2B, but only 3.52% of cells showed positive surface labeling (data not shown), which indicated that these assembled chimeric subunits could not traffic into the cell membrane.

**M1–3 Region Is Required for GluN1 Homodimer Assembly**—To further determine the domain in M1–4 required for NMDAR subunit assembly, we divided M1–4 into two parts, M1–3 and S2M4, and replaced them with the corresponding regions of GluA1 to get YFP-GluN1M1–3(GluA1) and YFP-GluN1S2M4(GluA1) (Fig. 4A). We cotransfected YFP-GluN1M1–3(GluA1) with CFP-GluN1 or YFP-GluN1S2M4(GluA1) with CFP-GluN1 in HEK293 cells and detected the FRET signals. The results showed that when YFP-GluN1M1–3(GluA1) was coexpressed with CFP-GluN1, no significant FRET signals were observed; FR = 1.03 ± 0.04 (n = 26). In contrast, coexpression of YFP-GluN1S2M4(GluA1) and CFP-GluN1 produced significant FRET signals, with FR = 1.33 ± 0.05 (n = 35) (Fig. 4B). These results indicated that M1–3 but not S2M4 was necessary for GluN1 homodimer formation.

We also made FRET measurements between CFP-GluN2B and YFP-GluN1M1–3(GluA1) or between CFP-GluN2B and YFP-
YFP.

FR being 1.37

significant FRET signals were produced in both combinations, the

cells measured were between 22 and 28.

merization of different GluN1 membrane domain constructs. The numbers of

AUGUST 5, 2011 •

expressed in the cell membrane.

positive surface labeling, respectively (data not shown). These

We further assessed the surface expression of these two assem-

FIGURE 5.

Membrane segments of GluN1 can assemble as homooligomers. A, schematic representation of different GluN1 transmembrane domain

GluN1, in HEK293 cells. The results showed that sig-

FR being 1.04

molecular structure, which indicates that each putative membrane seg-

GluN1 can assemble as homooligomers, a series of con-

A

GluN1 can assemble with its cognate one selectively.

B

Membrane Segments of GluN1 Can Form Homooligomers—

Our data from chimeras indicated that the transmembrane

region should have the ability to mediate GluN1 homooligomer

assembly (Fig. 3). To further test this, we made constructs of

M1–4 or M1–3 of GluN1 and tagged CFP or YFP on their N

termi, as XFP-M1–4GluN1 or XFP-M1–3GluN1 (Fig. 5A).

When CFP-M1–4GluN1 and YFP-M1–4GluN1 or CFP-M1–

3GluN1 and YFP-M1–3GluN1 were coexpressed in HEK293 cells, the

resulting FRET signals were both positive, the FR values

being 2.20 ± 0.10 (n = 28) or 1.84 ± 0.07 (n = 28), respectively

(Fig. 5B). These data implied that the transmembrane region of

GluN1 could form homooligomers.

To further investigate whether each putative membrane seg-

ment in GluN1 could form homooligomers, a series of con-

structs containing different membrane segments tagged with

CFP or YFP were made, named CFP- or YFP-M1GluN1, CFP- or

YFP-M23GluN1, and CFP- or YFP-M4GluN1 for the GluN1 mem-

brane segments and CFP-M1GluA1 for the GluA1 membrane

segment (Fig. 5A). Because M2 is a reentrant loop, we con-

structed M2 and M3 as an integral part. As the data showed,

FRET signals from the cells coexpressing CFP-M1GluN1 and

YFP-M1GluN1, CFP-M23GluN1 and YFP-M23GluN1, or CFP-

M4GluN1 and YFP-M4GluN1 were all positive, the FR being

2.03 ± 0.07 (n = 22), 1.41 ± 0.04 (n = 28), and 1.47 ± 0.05 (n =

28), respectively (Fig. 5B). In contrast, when YFP-M1GluN1 and

CFP-M1GluA1 were coexpressed in HEK293 cells, no FRET sig-

nal was detected, the FR being 1.07 ± 0.04 (n = 28). Further-

more, we wondered whether there was a heteroassembly

between M1 and M4 of GluN1 and checked the FRET signal in

cells coexpressing these two segments. As a result, we did not

find any FRET signal between them, as the FR was 1.04 ± 0.04

(n = 19). These results indicated that hetero-oligomers could

not form either between M1 of GluN1 and the corresponding

segment of GluA1 or between distinct membrane segments of

GluN1. It implies that each putative membrane segment of

GluN1 can assemble with its cognate one selectively.

DISCUSSION

In this study, we investigated the molecular mechanism of

NMDA receptor assembly by means of FRET measurement,

immunostaining, and biochemical analysis. We made a series of

trunced or chimeric NMDAR subunit constructs tagged with

CFP or YFP for FRET detection and found that the transmem-

brane region rather than the N terminus of NMDAR subunits

was required for the assembly of NMDAR complexes.

The NMDAR is special in its obligatory heterotetrameric

structure, which indicates that assembly of receptor subunits is

under critical control. However, there was still controversy over

the domain determining the subunit assembly. Here, we found

that the transmembrane domain is very important for subunit

assembly. First, we showed that NMDAR subunits with the NT

deleted can still assemble, which indicates that the N terminus

is not the crucial domain determining subunit assembly. Previ-

ous studies using coimmunoprecipitation indicated that a com-

plete NT is critical but not sufficient for oligomerization of

GluN1 with GluN2A (15). We attribute this discrepancy to

the different methods our two groups used. FRET is measured in

intact cells and is a more sensitive method (25). Weak interac-

tions can be preserved and reliably detected. Another group

using FRET measurement found that the full-length GluN1

subunit assembles in a dimeric form, whereas neither GluN2A

nor GluN2B show defined aggregation (18). Their results are

inconsistent with our result showing that the GluN2 subunit

can also form homodimers when expressed alone (Fig. 1). This
may be due to the different tagging system we used. The fluorescent protein was tagged to the C terminus of the GluN2 subunit in their experiment, whereas it was tagged to the N terminus of the GluN2 subunit in our experiment. As we know, the GluN2 subunit has a relatively longer and more flexible C terminus, which may make the C terminus tagging inappropriate for FRET analysis, a method requiring a distance of less than 10 nm between the donor and acceptor. To exclude the possibility that misfolded subunits can cause FRET signals in our system, we also checked the FRET between N-terminally labeled GluN1 subunits with C-terminally labeled ones, where FR of YFP-GluN1/GluN1-CFP was 1.01 ± 0.056 (n = 24) as a negative value for FRET.

Second, the results from the subunit chimeras of NMDAR with AMPA receptors showed that the transmembrane region was necessary for subunit assembly, because YFP-GluN1M1–4(GluA1) was neither able to assemble with CFP-GluN1 nor with CFP-GluN2B (Fig. 3).

Third, the transmembrane region was indeed able to assemble as a homodimer (Figs. 4 and 5). These results provided strong evidence that the transmembrane region was required for NMDAR subunit assembly. So, for NMDARs, the transmembrane domain is not only the region determining the formation of the ion channel but also the crucial region for receptor assembly. In fact, the transmembrane region is the basis for the assembly of many receptors and ion channels, including immune receptors, proton channels, and nicotinic receptors (26–28). The study of AMPA receptor assembly also suggested that although the ATD region interactions mediate dimer formation, interaction of the transmembrane regions is critical for subsequent tetramerization (11).

More interestingly, we found that the regions responsible for homodimer formation and heteromer formation were a little different. One interacting site in M1–3 is responsible for GluN1 homodimer formation, whereas two independent interacting sites are responsible for GluN1/GluN2 heteromer formation in M1–3 and S2M4, respectively. Previous crystallographic studies showed that the ligand-binding domain of GluN1 cannot form a homodimer (29), whereas the ligand-binding domains (including the S1 and S2 domains) can form heterodimers between GluN1 and GluN2A (30). Because S2 belongs to the ligand domain, these data together with our results indicate that the S2 region may be important for GluN1 and GluN2 subunit heteromer assembly but not for GluN1 homodimer assembly. Studies on AMPA receptors also suggest that compatibility in the membrane domains (M1–3 and M4) as well as part of S2 is important for functional heteromeric assembly of AMPA receptors. As to homodimer formation, only M1–3 is necessary. Structure and function studies of GluR0 have shown that this prokaryotic precursor to eukaryotic GluRs can assemble and function without M4 and the C terminus in the structure (31, 32), which also indicates that M4 is not important for homodimer formation.

In addition to its role in receptor assembly, the membrane domain of the NMDAR subunit is also involved in ER retention. Recently, there is evidence that the putative membrane domains of the NMDAR subunits also contain ER retention signals. In both the GluN1 and GluN2B subunits, two distinct ER retention signals have been identified, present in M3 and in the C termini, and these signals help to retain individual subunits and incomplete oligomers in the ER until they are masked by the correct oligomeric assembly (8, 9, 33, 34). In particular, M3 and M4 of GluN1 are both necessary for masking the ER retention signal existing in M3 of GluN2B (9). Our results show that the transmembrane region is critical for assembly between GluN1 and GluN2B, and it is likely that membrane region-mediated assembly is the mechanism underlying the masking of the ER retention signals existing in GluN1 and GluN2B. However, we also found that even if the NT-deleted subunits assemble, they still cannot traffic to the cell membrane but instead are retained in the ER. This result means that these assembled receptor mutants are not sufficient for trafficking to the cell membrane and that the N terminus of the GluN1/2 subunits may contain certain structures necessary for delivery of the receptors to cell membrane.

More and more evidence indicates that ER quality control of many receptors involves a process of ligand binding and receptor function, in which pharmacological ligands act as functional
chaperones to facilitate receptor trafficking through the ER-Golgi secretory pathway, including glutamate receptors (35–37), δ-opioid receptors (38) and vasopressin V2 receptors (39). It is known that the ligand-binding domain (including S1 and S2) is critical for agonist binding and activation of glutamate receptors. Accordingly, we found that when the ligand-binding domain is deleted or replaced, these NMDAR subunit mutants are retained in the ER rather than be expressed in the surface membrane or form functional channels, even though they still assemble as heteromers.

Taken together, our results are compatible with a model (Fig. 6), in which the determinants of NMDAR subunit assembly are in the transmembrane region (Fig. 6A), rather than the N termini or C termini (Fig. 6A). Furthermore, it is likely that homomeric and heteromeric assembly of NMDAR subunits is mediated by different molecular modules in the GluN1 transmembrane region. The M1–3 domain of GluN1 is responsible for GluN1 homodimer assembly, and two independent interacting sites are involved in GluN1/GluN2 heteromer assembly (Fig. 6C).

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