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*

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The cytoplasmic C-terminal domains of NR2 subunits have been proposed to modulate the assembly and trafficking of NMDA receptors. However, questions remain concerning which domains in the C terminus of NR2 subunits control the assembly of receptor complexes and how the assembled complexes are selectively trafficked through the various cellular compartments such as endoplasmic reticulum (ER) to the cell surface. In the present study, we found that the three amino acid tail after the TM4 region of NR2 subunits is necessary for surface expression of functional NMDA receptors, while truncations with only two amino acids following the TM4 region (NR2Δ2) completely eliminated surface expression of the NMDA receptor on co-expression with NR1-1a in HEK293 cells. FRET (fluorescence resonance energy transfer) analysis showed that these NR2Δ2 truncations are able to form homomers and heteromers on co-expression with NR1-1a. Furthermore, when NR2Δ2 subunits were cotransfected with either the NR1-4a or NR1-1aAAA mutant, lacking the ER retention motif (RRR), functional NMDA receptors were detected in the transfected HEK293 cells. Unexpectedly, we found that the replacement of five residues after TM4 with alanines gave results indistinguishable from those of found that the replacement of five residues after TM4

N-methyl-d-aspartate (NMDA)3 receptors are heteromeric complexes primarily assembled from two subunit classes: NR1 and NR2. Co-assembly of NR1 and NR2 subunits is essential for formation of a functional channel, presumed to be a tetramer containing two NR1 and two NR2 subunits (1–2). NR1 is a single subunit with eight splicing variants, which have distinct trafficking and functional properties (3). NR2 subunits are coded by four separate genes, NR2A-D, each of which can endow the receptor channel with different properties (4). Thus, the subunit composition of NMDA receptors is a major determinant of NMDA receptor-mediated activity in the central nervous system. Although much is known about the physiological roles that NMDARs play in long term potentiation (LTP), learning and memory (5–7), much remains to be learned about the mechanisms by which these receptors are assembled, sorted, targeted, and anchored to the appropriate location.

The C-terminal domains of the receptor subunits contain critical determinants of subcellular receptor localization. Regulation of receptor trafficking by these determinants ensures that only fully assembled multimeric receptors are expressed on the plasma membrane. When expressed alone in heterogeneous cells, the major NR1 isoform (NR1-1) is retained in the ER because of an RRR motif in its C1 cassette, but NR1-2, NR1-3, and NR1-4 are not retained in the ER because of their lack of the RRR motif and/or their possession of the C2’ cassette (3, 8–9). The presence of NR2 subunits can overcome the ER retention mediated by the RRR motif in NR1-1 and deliver the NR1-1/NR2 receptor complexes to the membrane surface. The NR2 subunit is also retained in the ER when expressed alone in heterogeneous cells (10) and in neurons lacking the NR1 subunit (11). That the intracellular C terminus of the NR2B subunit plays a role in its ER retention is suggested by a study using Tac chimeras of its C terminus (10), but to date there is no particular domain or motif identified as being responsible for the ER retention. Partial truncation of the C terminus of NR2B subunits does not abolish the assembly and surface expression of func-

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3 The abbreviations used are: NMDA, N-methyl-d-aspartate; FRET, fluorescence resonance energy transfer; HEK293 cell, human embryonic kidney 293 cell; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; NR subunit, NMDA receptor subunit; TM4, the fourth transmembrane domain; DIV, days in vitro; FR, FRET ratio; E_{EEFF}, effective FRET efficiency; ER, endoplasmic reticulum; GFP, green fluorescent protein.
tional NMDA receptor channels in neurons from genetically modified mice, although the synaptic localization of the receptors is impaired as a result of loss of the PDZ binding domain (12–16). Mutation of the four amino acids (HLFY) following the TM4 region of the NR2B subunit markedly reduces the level of surface expression of NR1/NR2B complexes in heterologous cells (10). Taken together, these studies strongly suggest that the several amino acids following the TM4 domain of the NR2 subunit play a crucial role in releasing the assembled functional NMDA receptor complex from the ER. However, the interpretation of these studies is complicated by the fact that no clear motif in the C terminus of NR2 subunits or related exporting signal have been found to control the assembly and surface expression of the NMDA receptor complex.

In this study, we addressed these questions further, especially in regard to the role of the C terminus of both NR2A and NR2B in the assembly and surface expression of NMDA receptors. Although the C terminus of NR2A and NR2B shares high sequence identity, several elegant studies have suggested that different mechanisms may apply to regulate the trafficking and synaptic localization of NR2A containing NMDARs versus NR2B-containing ones (17–19). To this end, a series of C-terminally truncated NR2A or NR2B mutants tagged with green fluorescent protein (GFP) were made for detecting surface expression by whole cell patch clamp recordings and live cell surface immunocytochemical staining. We found that a short tail of at least three amino acids, which required no sequence specificity, following the TM4 of NR2 subunits was required for overcoming the ER retention motif of NR1 subunits, but was not necessary for the assembly of NMDAR complexes.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The expression vectors for GFP-NR2A and GFP-NR2B were generated as described previously (20). A variety of mutants for NR2A and NR2B with C-terminal truncation were constructed from GFP-NR2A and GFP-NR2B, respectively, using conventional DNA cloning techniques. These mutants were named 2AΔ or 2BΔ followed by a number indicating the number of residues left following the TM4 region and/or by a subscript “A” indicating an alanine substitute (Figs. 1A and 2A). For example, 2BΔ5AAAAA represents a GFP-NR2B mutant with five residues (AAAAA) left immediately following TM4. All constructs were verified by DNA sequencing.

**Transfection of Heterogeneous Cells**—HEK293 cells were cultured and plated on polylysine-coated coverslips in 35-mm dishes 1 day before transfection. The plasmids for NR1 and NR2 subunits were transfected at a molar ratio of 1:1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After transfection, cells were grown in the presence of 0.5 mM ketamine and 1 mM kynurenic acid (Sigma). The transfected cells were used for electrophysiological recording and immunocytochemical staining 24-h later.

**Hippocampal Culture and Transfection**—Primary hippocampal neuron cultures were prepared from embryonic Sprague-Dawley (SD) rats aged embryonic day 18 according to the procedures previously described (21). The cultures were transfected at DIV5 with the constructs for NR subunits using Lipofectamine 2000 (Invitrogen). Immunofluorescent staining of the live neurons at DIV7 was performed under the same conditions as described above for the transfection of HEK293 cells. The neurons transfected with GFP-tagged NR subunits and surface stained were observed and imaged on a confocal microscope (FV500, Olympus).

**Electrophysiology**—Whole cell recordings from HEK293 cells were made at room temperature 24–48 h after transfection. The resistance of the patch pipette was between 5 and 8 MΩ. Electrical signals were amplified using a Multiclamp 700A amplifier (Axon, Foster City, CA) and the pCLAMP system (Version 8.2, Axon) was used for data acquisition and analysis. The extracellular recording solution contained (in mM): NaCl 145, KCl 3, HEPES 10, CaCl2 3, glucose 8, MgCl2 2 (310 Osm, pH adjusted to 7.30 with NaOH). Patch pipettes were filled with intracellular solution containing (in mM): potassium gluconate 136.5, KCl 17.5, NaCl 9, MgCl2 1, HEPES 10, EGTA 0.2 (310 Osm, pH adjusted to 7.20 with KOH). Recordings were made at −60 mV during the application of 100 μM glutamate and 20 μM glycine or 50 μM D-AP5, an antagonist of NMDA receptors.

**Immunofluorescent Staining**—GFP-tagged NR2 subunit-containing NMDA receptors expressed on the cell surface were detected using immunofluorescent staining in living cells as described previously (20). Briefly, the transfected HEK293 cells were incubated with rabbit anti-GFP antibody (Chemicon) for 7 min, rinsed three times in extracellular recording solution, and then incubated with Cy3-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) for another 7 min. After another brief wash in extracellular recording solution, cells were immediately fixed and imaged on a confocal microscope equipped with Fluoview imaging analysis software (FV500, Olympus). All processes were performed at room temperature.

**Quantitative Analysis of Surface Expression**—Surface expression of GFP-tagged NMDA receptors in HEK293 cells was measured as the percentage of cells positively stained in the population of cells with GFP expression. For each sample, more than 100 GFP-positive cells were counted from 3–4 different transfections. The surface expression in transfected hippocampal neurons was analyzed quantitatively by calculating the density of Cy3-stained puncta on the dendrites. Each individual secondary dendrite segment used for counting was at least 50-μm long. Several neurons per coverslip were selected on the basis of healthy morphology. Measurements of individual neurons from three coverslips per culture were averaged. The means were obtained from three different cultures. Data values were expressed as mean ± S.E. Statistical significance was calculated using unpaired Student’s t test.

**Detection of Fluorescence Resonance Energy Transfer (FRET) via Three Cube FRET Measurement**—The fluorescence imaging work station for FRET was described previously (19). 2 × 2 binning modes and a 200-ms integration time were used. Average background signal was determined as the mean fluorescence intensity from an area not expressing the constructs and was subtracted from the raw images before carrying out FRET calculations. The effective FRET efficiency was calculated using the FRET ratio (FR) measurement (22–24) in Equation 1, where
FR = F_D / F_A = \left( S_{\text{FRET}}(DA) - R_D \right) / S_{\text{CFP}}(DA) / R_A \times S_{\text{YFP}}(DA) \quad (\text{Eq. 1})

S_{\text{CUBE}}(\text{SPECIMEN}) \text{ denotes an intensity measurement, where CUBE indicates the filter cube (CFP, YFP, or FRET), and SPECIMEN indicates whether the cell is expressing donor (D; CFP), acceptor (A; YFP), or both (DA) as in Equations 2 to 4.}

\begin{align*}
R_D &= S_{\text{FRET}}(D) / S_{\text{CFP}}(D) \\
R_D &= S_{\text{YFP}}(D) / S_{\text{CFP}}(D) \\
R_A &= S_{\text{FRET}}(A) / S_{\text{YFP}}(A) \\
\end{align*} 

\quad (\text{Eq. 2}) 

\quad (\text{Eq. 3}) 

\quad (\text{Eq. 4})

RESULTS

The Three Amino Acids Following TM4 of the NR2 Subunit Are Required for the Surface Expression of NMDA Receptors in HEK293 Cells—To identify the region in the cytoplasmic domain of NR2 controlling surface expression of the NR1/NR2 receptor complex, we generated a series of NR2A and NR2B truncations tagged with GFP in the extracellular N terminus, named 2A2, 2AΔ3, 2AΔ4, 2AΔ5, 2AΔ59, 2BΔ2, 2BΔ3, 2BΔ4, 2BΔ5, and 2BΔ59. The number following Δ indicates the number of amino acids left after TM4 (Fig. 1, A and B). We then did live cell surface immunocytocchemical staining with a polyclonal anti-GFP antibody followed by a Cy3-conjugated secondary antibody, so positive labeling was indicated by numerous red puncta scattered on the cell surface (25). HEK293 cells to be tested were co-transfected with NR1/NR2A, NR1/NR2B, or NR2A, NR2B alone. As expected, only cells co-expressing NR1-1a exhibited red puncta, representing surface-expressed GFP-NR2B, whereas in cells without NR1-1a, no red puncta were observed (Fig. 1C).

To determine which motif in the proximal C terminus of the NR2B subunit is required for cell surface expression of NR1-1a/GFP-NR2B receptors, we transfected HEK293 cells with a series of truncations of this subunit (5 constructs, see above) in the presence of NR1-1a and studied the effect on the cell surface expression of the assembled receptor. We found that, with the exception of NR1-1a/2BΔ2, surface labeling was detected from cells transfected with all the truncations of NR2B (Fig. 2A), suggesting that removal all but three amino acids of the NR2B C terminus still produced receptors that reached the cell surface when co-expressed with NR1-1a. Strikingly, quantification of the percentage of cells with positive labeling (from 3 batches of different transfections) showed no significant difference among the experimental groups examined with expression of the different NR2B truncations (NR1-1a/GFP-NR2B, 73.0 ± 4.7%; NR1-1a/2BΔ59, 56.3 ± 3.3%; NR1-1a/2BΔ5, 60.0 ± 4.8%; NR1-1a/2BΔ4, 57.1 ± 5.5%; NR1-1a/2BΔ3, 53.3 ± 8.5%; Fig. 2B). This suggests that the successful detection of surface-expressed truncates of NR2B was not an exceptional phenomenon observed from a few transfected cells. Of note, the surface expression level of different truncates seemed to vary with the remaining length of the C terminus of the NR2B subunit. The surface expression level of NR2BΔ59 appeared to be no different from that of full-length NR2B based on the fluorescence intensity (Fig. 2A), which suggests that trafficking and delivery of the NMDARs was relatively unaffected by the loss of the last 585 amino acids of the C terminus. As for NR2BΔ5, NR2BΔ4, and NR2BΔ3, while there was no distinguishable difference between them, we noted an obvious reduction in surface expression level compared with cells expressing the NR1-1a/GFP-NR2B complex (Fig. 2A). These results strongly suggested that at least three amino acids after TM4 were required for the successful delivery of NR1-1a/NR2B receptors to the cell surface. Notably, with only one amino acid difference between NR2BΔ3 and NR2BΔ2, a distinctly different effect on NMDA receptor trafficking was produced.

Whole cell current responses were measured to determine the surface expression level of NMDARs in HEK293 cells with the different transfected NR2B truncations. We used focal
application of saturating doses of glutamate (100 μM) and glycine (10 μM). The evoked whole cell current represented the number of functional receptors on the cell surface. Representative currents through these truncation mutants evoked by a 500 ms glutamate application are shown in Fig. 2D. The average amplitudes of glutamate-evoked currents in cells expressing either NR1-1a/GFP-NR2B, NR1-1a/2BΔ59, 2BΔ5, 2BΔ4, or 2BΔ3 were 323.6 ± 28.5 (n = 6), 298.7 ± 31.8 (n = 4), 200.0 ± 18.3 (n = 4), 110.5 ± 45.2 (n = 5), or 153.5 ± 28.1 pA (n = 4), respectively (Fig. 2E). Consistent with the live cell surface immunostaining pattern, no responses were detected from cells transfected with the NR1-1a/2BΔ2 complex (n = 10). The average peak currents recorded from cells co-transfected with NR1-1a/2Δ3, NR1-1a/2BΔ4, and NR1-1a/2BΔ5 were significantly reduced compared with those recorded from cells co-expressing NR1-1a/full-length NR2B, whereas cells expressing NR1-1a/2BΔ59 showed a slight but insignificant decrease in peak currents. This strongly suggests that removal of most (at least 585 amino acids) of the NR2B C terminus does not affect the trafficking and surface expression of functional NR1-1a/NR2B complexes, while the short sequence proximal to TM4, especially the very first three amino acids, plays a crucial role in this process. However, the decreased surface expression level of NR2BΔ5, NR2BΔ4, and NR2BΔ3 suggests that very likely a specific region between 841 and 897 can facilitate the maximal surface expression of NMDARs.

It is well known that the sequences of the NR2A and NR2B subunits are highly homologous, but recent studies indicated that the functional role of the C terminus of NR2A may different from that of NR2B subunits. We then investigated the functional role of the NR2A C terminus by following a strategy similar to that used for examining the NR2B C terminus described above. The structures of the different NR2A truncations used in this study are illustrated in Fig. 1B. Similarly, all truncates but 2AΔ2 were expressed on the cell surface in the presence of NR1-1a (Fig. 3A). Compared with the 49.3 ± 6.9% of ER-retained NR1-1a/NR2B complexes, this strongly suggests that removal of most of the NR2B C terminus does not affect the trafficking and surface expression of functional NR1-1a/NR2B complexes, while the short sequence proximal to TM4, especially the very first three amino acids, plays a crucial role in this process. However, the decreased surface expression level of NR2BΔ5, NR2BΔ4, and NR2BΔ3 suggests that very likely a specific region between 841 and 897 can facilitate the maximal surface expression of NMDARs.

FIGURE 2. The three amino acids following TM4 of the NR2B subunit are required for the surface expression of NMDA receptors in HEK293 cells. A, surface and total expression of full-length and truncated GFP-NR2B in HEK293 cells. Green fluorescence microscopic images (left column) of cells co-transfected with full-length GFP-NR2B/NR1-1a or C terminus-truncated GFP-NR2B/NR1-1a, the corresponding live surface immunostaining (middle column; red), and the merged images (right column). A clear pattern of surface labeling was observed in all combinations of transfection except 2BΔ2/NR1-1a. Scale bar, 10 μm. B, summary of percentages of HEK293 cells with detectable surface labeling. All groups studied, except 2BΔ2/NR1-1a, showed similar percentages. Data are mean ± S.E. derived from three separate transfections (n = 50–200 per transfection). *, p < 0.05; **, p < 0.01, compared with GFP-NR2B/NR1-1a (two-tailed, unpaired Student’s t test). C, example image of a transfected HEK293 cell used for whole cell patch clamp recording. D, representative evoked currents recorded from HEK293 cells transfected with NR1-1a/NR2B or NR1-1a/2BΔ. Glutamate (100 μM) and glycine (20 μM) were co-applied by Y-tube to evoke whole cell currents (holding potential, −60 mV). E, summary data showing that the average peak amplitude of whole cell currents varied with the length of the C terminus in NR2B subunits. Cells co-expressing 2BΔ59/NR1-1a showed a slight but insignificant decrease in peak amplitude (pA), while all other counterparts showed profound decreases, compared with the control group transfected with full-length NR2B/NR1-1a. The numbers over the histogram indicate the number of cells responding out of the total number of recorded cells (i.e. 4/5 represents 4 responsive cells of 5).
three amino acids following the TM4 of NR2A was also necessary for the assembled receptor complex to leave the ER and be expressed on the cell surface. We then made the whole cell recordings to measure glutamate-evoked whole cell currents response by applying a focal saturating dose of glutamate and glycine. The glutamate-evoked currents were detectable in cells transfected with NR1-1α/NR2A (full-length) (131.9 ± 12.1 pA, n = 16), as well as with NR1-1α/2AΔ59 (89.8 ± 38.8 pA, n = 10), NR1-1α/2AΔ5 (99.2 ± 4.1 pA, n = 4), NR1-1α/2AΔ4 (54.5 ± 12.1 pA, n = 4), and NR1-1α/2AΔ3 (58.8 ± 15.5 pA, n = 5) (Fig. 3D). In agreement with the results from surface immunostaining, no glutamate-mediated currents were detected from HEK293 cells co-expressing NR1-1α and 2AΔ2. Taken together, these results suggest that the three amino acid segment after TM4 in the NR2 subunit is necessary for surface expression of the NR1-1α/NR2 receptor complex.

The Three Amino Acid Segment following TM4 of the NR2 Subunits Required for Surface Expression of NMDA Receptors Needs No Specific Sequence—Recently, Hawkins et al. (10) identified a trafficking motif (HLFY) following TM4 of the NR2B subunit as an ER export signal, because mutation of the four amino acids in the full-length NR2B subunit led to a complete loss of functional NMDA receptors in cell membrane. In agreement with their results, we found that the removal of all but three amino acids of the NR2B C terminus still produced functional NMDARs on the cell surface on co-expression with the NR1 subunit. Therefore, both of our findings can be interpreted similarly that the three amino acid segment following TM4, EHL, is critical for forming a functional receptor on the cell surface. However, we noted that mutations of four amino acids (HLFY) in the study of Hawkins et al. (10) were made in the full-length NR2B subunit, whereas our NR2B truncations were made by removal of all but a few amino acids after TM4. We asked the question whether the mutation of EHLF in our NR2B truncates (with removal of all the rest of the C-terminus but the five amino acids) would also lead to a complete loss of functional surface NMDARs. To address this question and to investigate more fully the function of the short tail after TM4 of the NR2B subunit, the five amino acid sequence EHLFY was mutated to AAAAA in the construct of 2BΔ5 (Fig. 1A). Unexpectedly, not only did 39.1 ± 7.7% of transfected cells have positive surface labeling (Fig. 2, A and B), but also the average amplitude of the whole cell current from NR1-1α/2BΔ5AAAAA was similar to that of NR1-1α/2BΔ5 (Fig. 2, D and E). Compared with the full-length NR2B, the whole cell current amplitude from this mutant was significantly decreased, suggesting that a smaller number of functional NMDAR complexes were delivered to the cell surface on co-expression with NR1-1α in HEK293 cells. The lack of change in surface expression with the mutation of the five amino acid segment immediately after TM4 in the C terminus of NR2B compared with 2BΔ5 suggests that the three amino acid seg-
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ment critical for surface expression of NMDA receptors requires no specific amino acid sequence but just a three amino acid length to ensure appropriate assembly and successful expression on the cell surface.

NR2BΔ2 Can Form Homomers and Heteromers on Co-expression with NR1-1a in Transfected HEK293 Cells—We have shown that 2BΔ2 failed to be expressed on the cell surface in the presence of NR1-1a (Fig. 2A). We speculated that the three amino acid domain after TM4 in the C terminus of NR2B was responsible for appropriate assembly. To test this, we examined the constitutive association between NR2BΔ2 and NR1-1a by using FRET in HEK293 cells. Several fluorescent protein-tagged NMDA receptor subunits were constructed, including CFP-NR1-1a, YFP-GluR1, CFP-NR2B, CFP-2BΔ2, and YFP-2BΔ2, and the FRET ratio (FR) was calculated as described under “Experimental Procedures.” Representative fluorescent images for NR1-1a/NR2B, NR1-1a/GluR1, NR1-1a/2BΔ2, 2B/2BΔ2, and 2BΔ2/2BΔ2 are shown in Fig. 4A. It has been suggested that the NMDA receptor subunit cannot form complexes with the AMPA receptor subunit, and this was verified in our system. Cells co-expressing CFP-NR1-1a and YFP-GluR1 showed no FRET signal, with FR values of about 1. When cells co-expressed CFP-NR1-1a and YFP-NR2B, FRET occurred, giving FR values significantly greater than 1 (FR = 3.5 ± 0.2, n = 30). Thus, our system was suitable for studies on the assembly of NMDA receptor subunits. When YFP-2BΔ2 was co-transfected with CFP-NR1-1a into HEK293 cells, significant FRET signals were detected (FR = 2.1 ± 0.10, n = 50, p < 0.01). This meant that NR2BΔ2 was still able to assemble with NR1-1a, indicating that the three amino acid domain after TM4 is only required for surface expression but not for assembly with NR1-1a. Interestingly, FRET also occurred when YFP-2BΔ2 was co-expressed with CFP-NR2B or CFP-NR2BΔ2 (FR = 1.5 ± 0.1, n = 37, p < 0.01 or FR = 1.8 ± 0.1, n = 21, p < 0.01), indicating the formation of 2BΔ2 oligomers.

Co-expression of NR1-4a or NR1-1aAAA Mutants Enables the Delivery of 2AΔ2 or 2BΔ2 to the Cell Surface—NR1-4a is the splice variant of the NR1 subunit with the shortest C terminus. The C terminus of NR1-1a is composed of C0, C1, and C2 exon cassettes, whereas the C terminus of NR1-4a is only composed of C0 and the C2’ cassette (26). As shown previously, two distinct motifs in the C terminus domain control the ER retention of the NR1 subunit: the RRR motif in the C1 cassette is an ER retention signal; the PDZ-interacting domain of the C2’ cassette (STVV) serves as an ER exit signal. Previous studies indicated that the NR1-4a subunit does not contain an ER retention motif, and can be expressed on the cell surface because the C terminus of NR1-4a contains an ER export motif (STVV) (3, 26–27) (Fig. 5A). In contrast, NR1-1a homomers fail to express on the cell surface because of the existence of an ER retention motif (RRR) in the C1 cassette (9) (Fig. 5A). In our experiments, no surface staining was found in HEK293 cells co-expressing NR1-1a/2AΔ2 (Fig. 3B). However, 2AΔ2 was detectable in transfected cells in the presence of NR1-4a (55.5 ± 3.4% of the cells expressed GFP and showed surface labeling with anti-GFP antibody (Fig. 5, B and E)). Furthermore, the NR1-1aAAA mutant in which the RRR motif of NR1-1a is substituted by the AAA residue can also express on the cell surface with 2AΔ2 (51.5 ± 2.8%; Fig. 5, B and E). Transfection with 2AΔ2 alone did not show any detectable surface labeling (data not shown). Taken together, these data indicated that co-expression of 2AΔ2 and NR1 subunit removal of the ER retention motif can be released from ER and reach the cell surface.

Similarly, NR1-4a/2BΔ2 or NR1-1aAAA/2BΔ2 heteromers were also detected on the HEK293 cell surface (58.2 ± 3.9%, 51.3 ± 0.5%; Fig. 5, B and E), while transfection with 2BΔ2 alone produced no detectable surface labeling (data not shown). These surface staining results indicated that the complexes formed by 2BΔ2 or 2AΔ2 subunits with deletion of the RRR motif of the NR1 subunits can be released from the ER and reach the cell surface.

We then performed whole cell recordings to further determine whether the complexes of NR1-4a/2AΔ2 or NR1-4a/2BΔ2 were functional. In the presence of 100 μM glutamate and 20 μM glycine, whole cell currents were evoked for both of the transfected NMDA complexes (Fig. 5, C and D). Currents were totally blocked by 50 μM AP5 (data not shown), suggesting that
both 2AΔ2 and 2AΔ2 were able to assemble with NR1-4a and form functional NMDA receptor channels on the cell surface.

Based on these results, it is likely that these mutants lost the ability to mask the ER retention signal in the C1 cassette of NR1-1a because of disruption of the three amino acid segment after TM4 in the C terminus of the NR2 subunit. In other words, our results suggest that the three amino acid segment after TM4 in the C terminus of the NR2 subunit can cause the C terminus of the NR1-1a to fold in such a way as to mask the ER retention signal of the RRR motif.

Surface Expression of NR2 Truncations in Cultured Hippocampal Neurons—In our final set of experiments, we investigated whether the three amino acid segment following TM4 of the NR2 subunit that is required for surface expression of the NR1-1a/NR2 complex in heterologous expression systems served an equivalent role in cultured hippocampal neurons. Hippocampal neurons grown under standard culture condition were transfected with GFP-NR2A, 2BΔ3/2BΔ2-GFP-NR2A’2AΔ5/2AΔ3 or 2AΔ2 truncates at DIV5. We measured the surface expression of the NR2 truncates containing receptors using anti-GFP antibody at DIV7. Total GFP fluorescence in transfected neurons was distributed throughout the somatodendritic compartment (Fig. 6, A and C). Red puncta identified GFP-tagged NMDA receptors on the plasma membrane of the somatodendritic compartment. As shown in Fig. 6, A and C, red puncta were clearly visible in neurons transfected with GFP-NR2A, GFP-NR2B (full-length), 2AΔ5, 2AΔ3, 2AΔ2, or 2BΔ3 (9.8 ± 0.3 per 10 μm for GFP-NR2A, 10.7 ± 0.7 per 10 μm for GFP-NR2B, 5.3 ± 1.0 per 10 μm for 2AΔ5, 2.9 ± 0.6 per 10 μm for 2AΔ3, 2.1 ± 0.2 per 10 μm for 2AΔ2, and 6.3 ± 0.3 per 10 μm for 2BΔ3; Fig. 6, B and D). Just as in heterologous cells, puncta were found on neurons transfected with 2AΔ3 or 2BΔ3 mutants, but significantly fewer than that in NR2A or NR2B transfected neurons (Fig. 6, B and D). Surprisingly, although neurons expressing 2BΔ2 did not show any surface staining at DIV7, neurons expressing 2AΔ2 had clearly detectable the red puncta on the cell surface (Fig. 6, A and C). It is known from previous studies that neurons have endogenous NR1 variants, so it was surprising that neurons expressing 2BΔ2 were not detected by red fluorescence at DIV7 in our system. Interestingly, neurons co-expressing NR1-4a with 2BΔ2 showed surface staining at DIV7 (3.1 ± 0.2 puncta per 10 μm; Fig. 6, C and D). However, NR1-4a and 2AΔ2 were co-expressed in cultured neurons, and surface levels were not increased compared with 2AΔ2 at DIV7 (2.0 ± 0.2 puncta per 10 μm; Fig. 6, A and B). Based on the above results in HEK293 cells, these data suggest that the 2AΔ2 or 2BΔ2 mutant maybe assemble with different NR1 splice variants. Neurons were transfected with cDNAs for the NR2 mutants at DIV5, and we measured the surface expression of the resulting receptors using anti-GFP antibody at DIV7.

**DISCUSSION**

Although NMDA receptors have been intensely studied with respect to their physiology and pharmacology, their cell biological properties, such as subunit assembly and trafficking, are only beginning to be addressed (28–30). Previous studies have shown that neither NR1 nor NR2 subunits form functional receptors when expressed alone in heterologous cells, because they are retained in the ER (31). ER retention is a common feature of the quality control mechanism for complex proteins, ensuring that unassembled or otherwise defective proteins are
not exported from the ER. Previous studies indicate the presence of ER retention motifs on the C terminus of both NR1 and NR2B subunits, but the mechanism by which the assembled complex overrides ER retention to reach the cell surface is largely unknown (10).

Based on the analysis of a series of NR2 truncations, we first found that a three amino acid tail (EHL) after TM4 of the NR2 subunit is required for surface expression of NMDA receptors. Both the live cell surface immunocytochemical staining and whole cell current recordings showed that a three amino acids tail after TM4 of the NR2 subunit permit assembly with NR1-1a and formation of functional NMDA receptor complexes at the cell surface, whereas having two amino acids at that location failed to do so. These results are consistent with previous studies (10) showing that successful export of the assembled NMDA receptors from ER cannot be controlled by a signal in the deleted part, which includes nearly all the C terminus. However, we observed that the whole cell current amplitude from the truncations, including NR2Δ5, NR2Δ4, and NR2Δ3, were significantly decreased compared with full-length and NR2Δ59, suggesting a lower surface expression level and/or an altered channel properties of these mutant receptors. Together these results suggest a more complex structure is responsible for maximal surface expression.

Interestingly, we found that the substitution of five alanine residues for EHLFY (NR2BΔ5AAAAA), neither affected the successful formation of the functional NMDA channels on the cell surface on co-expression with NR1-1a, nor changed the whole cell current response compared with NR2BΔ5. The lack of such changes suggests that the expression of functional NMDA receptors requires no specific amino acid sequence but only a short amino acid length to ensure appropriate assembly of a functional receptor. It appears, therefore, that the tail length in the region proximal to TM4 is more important for successful transport than the specific sequence of amino acids.

This result seems to contradict the finding of the previous study by Hawkins et al. (10), but this can be well explained by the different mutants used in our studies. We used truncations of the NR2 C terminus, while Hawkins used mutations restricted to four amino acids (HLFY) and retained all the C terminus of the NR2B subunit. The NR2 subunit C terminus contains a long cytoplasmic tail, which probably contains many potential sites regulating NR2 self-topology. In our system, we eliminated potential interference from the rest of the NR2 C terminus by removing almost all of it. In this case, ER retention of the NR1-1a/NR2 receptor was simply because of the ER retention motif of NR1-1a. A three amino acid tail immediately after TM4 of the NR2 subunit probably serves as a conformation-dependent signal that controls the release of the assembled receptor from the ER. To address this point more clearly and specifically, a three amino acid sequence EHL were mutated to AAA in subsequent studies to determine the formation of functional NMDA receptor channels on the cell surface.

Previous studies have shown that homodimer formation may be the first step in the assembly process of the NMDA receptor complex, because dimers of NR1 can be detected as intermediates in this process (32–33). However, the importance of homodimer formation in the processing of the NR2 subunit remains unclear. Our FRET results showed that removal of all but two amino acids after TM4 of the NR2B did not affect the assembly of NR1/NR2B oligomeric heteromers and/or NR2B oligomers intracellularly, suggesting that most of the C terminus of the NR2 subunit is not required for the assembly process.
in the formation of either NR1/NR2B heteromers or NR2B homomers.

A multisubunit protein complex containing an ER retention motif in its individual subunits commonly uses a mechanism involving mutual masking of the ER retention signal to allow the assembled complex to be exported from the ER. For example, the individual subunits of the ATP-sensitive potassium channel contain ER retention motifs that are masked after the assembly of the other subunits with the proper stoichiometry of the channel (34). As expected, our results clearly showed that NR1-1a/2Δ2 or NR1-1a/2Δ2 cannot be delivered to the cell membrane in heterologous cells, but NR1-1aAAA/2Δ2 or NR1-1aAAA/2Δ2 complex can be released from the ER and reach the cell surface. Therefore, it is very likely that a nonspecific, three amino acid tail following TM4 of the NR2 subunit can mask the RXR-based ER retention motif of NR1-1a C terminus by forming an appropriate conformation of the receptor complexes during the interaction with NR1-1a. Conformational changes during assembly can alter the exposure of motifs to interacting proteins and cause ER retention or export. For example, a conformation-dependent motif in a transmembrane domain of the nicotine receptor is masked by assembled receptors but is exposed on the unassembled subunits, causing ER retention (35). Our results offer the strong evidence that the length of the NR2 cytoplasmic tail controls ER retention of NMDA receptors. The exact nature of the intracellular trafficking pathway that leads to a conformational change of NR1-1a by the association of the NR2 subunit remains to be elucidated. Additional experiments are also required to study how a three amino acid tail of the NR2 subunit C terminus controls the formation of a proper conformational structure of the NR1/NR2 complex that allows export from the ER.

Based on our present study and previous work done by Hawkins et al., we may draw the conclusion that the HLFLY motif of the NR2 subunit may affect NR2 C terminus conformation to mask ER self-retention, but shielding ER retention of NR1-1a only requires a three amino acid long cytoplasmic tail of the NR2 subunit, which possibly induces a facilitated self-folding in the C terminus of NR1-1a to overcome its RRR ER retention. Although this conclusion will need further verification, we found other lines of supporting evidences from some previous studies. For example, NR1-1a truncation with a complete C terminus deletion can be delivered to the cell surface with the NR2A subunit (32), suggesting that the ER retention of the NR2A subunit is independent of the NR1-1a C terminus. In addition, another previous study demonstrated that a domain immediately after the transmembrane domain with nonspecific amino acid sequence is essential for the ER-to-Golgi transport of metalloendoproteinase meprin B (36).

Most interestingly, one recent study revealed a similar mechanism as ours when they were investigating the mechanism underlying the assembly, sorting, and trafficking of MHC class II molecule (37). Their study found a DRβ chain with a three amino acid cytoplasmic tail was sufficient to overcome the lip35 RXR motif located 41 residues away from the transmembrane- cytoplasmic domain junction in a way of sequence independent. As comparison, the RXR motif of NR1-1a is located 95 residues far away from the TM4 junction. These results suggest there is a new mechanism, which a nonspecific three amino acid cytoplasmic tail after TM of a protein can have a function to shield the RXR retention motif of its partner.

In addition, our results show that a small number of surface-expressed NMDARs containing 2AΔ2 was detected at DIV7 in cultured hippocampal neurons, while no 2BΔ2 containing NMDARs were detected on the cell surface. We think there are explanations for this. One likely reason is that, because of the existence of eight splice variants of NR1, the endogenous NR1 splice variants may have different preferences for assembling with NR2A versus NR2B subunit in cultured hippocampal neurons. Of course, further related studies are needed to confirm this hypothesis.

In conclusion, the mechanism by which the NR1 RXR retention motif is overcome during the subunit assembly of NMDA receptors remains obscure. Our study suggests there are more comprehensive mechanisms involved in the ER export of NMDA receptors.

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