An Endoplasmic Reticulum Retention Signal Located in the Extracellular Amino-terminal Domain of the NR2A Subunit of *N*-Methyl-D-aspartate Receptors^{*}

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N-Methyl-D-aspartate (NMDA) receptors play critical roles in complex brain functions as well as pathogenesis of neurodegenerative diseases. There are many NMDA isoforms and subunit types that, together with subtype-specific assembly, give rise to significant functional heterogeneity of NMDA receptors. Conventional NMDA receptors are obligatory heterotetramers composed of two glycine-binding NR1 subunits and two glutamate-binding NR2 subunits. When individually expressed in heterogeneous cells, most of the NR1 splice variants and the NR2 subunits remain in the endoplasmic reticulum (ER) and do not form homomeric channels. The mechanisms underlying NMDA receptor trafficking and functional expression remain uncertain. Using truncated and chimeric NMDA receptor subunits expressed in heterogeneous cells and hippocampal neurons, together with immunostaining, biochemical, and functional analyses, we found that the NR2A amino-terminal domain (ATD) contains an ER retention signal, which can be specifically masked by the NR1a ATD. Interestingly, no such signal was found in the ATD of the NR2B subunit. We further identified the A2 segment of the NR2A ATD to be the primary determinant of ER retention. These findings indicate that NR2A-containing NMDA receptors may undergo a different ER quality control process from NR2B-containing NMDA receptors.

Ionotropic glutamate receptors (iGluRs)² mediate most of the excitatory neurotransmission in the central nervous system. They play key roles in complex brain functions as well as in the pathogenesis of neurodegenerative diseases. Based on pharmagrouped into three major subtypes: GluR1 to -4 subunits form α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, GluR5 to -7 and KA1 and -2 subunits make up kainate receptors, and NR1 together with NR2A to -D subunits comprise the NMDA receptors (1). All iGluR subunits share a unique membrane topology with a large extracellular NH2-terminal domain, three transmembrane segments (TM1 (transmembrane domain 1), TM3, and TM4), a P-loop region, and a cytoplasmic COOH terminus (2, 3). Based on the sequence homology to bacterial periplasmic binding proteins, the NH₂terminal domain of iGluRs can be divided into two domains in tandem: the amino-terminal domain (ATD), which includes the first 400 or so amino acids (4), and the following S1 domain preceding TM1, which forms the ligand-binding domain together with the extracellular loop between TM3 and TM4 (S2 domain) (5, 6).

cological properties and sequence similarities, iGluRs can be

Among iGluRs, NMDA receptors are special in that conventional NMDA receptors are obligatory tetrameric membrane proteins composed of two glycine-binding NR1 and two glutamate-binding NR2 subunits. The NR1 subunit is essential for the formation of functional NMDA receptor channel, whereas the NR2 subunit modifies channel properties, such as current kinetics and channel conductance (1). The major NR1 splice variant and the NR2 subunits are retained in the ER when expressed alone in heterogeneous cells. Only when expressed together do they form functional receptors on the cell surface (7-9). In the last decade, enormous progress has been made in understanding the phenomenology and mechanisms of functional plasticity of NMDA receptors. However, much less is known about the mechanisms underlying the ER retention of NMDA receptor subunits. Previous studies focused on the COOH terminus have shown that the NR1a subunit contains an ER retention signal, RRR, in the C1 cassette, whereas a motif, HLFY, found in the NR2B subunit immediately following the TM4 (10) or, at least, the presence of any two amino acid residues after NR2 TM4 (11) is required for the export of NR1-NR2 complexes from the ER. Recently, novel ER retention signals were identified in the TM3 of both NR1 and NR2B subunits. In addition, TM3 of both NR1 and NR2B and TM4 of NR1 are necessary for masking ER retention signals found in TM3 (12).

In the present study, we focused on the functional role of the ATD in the surface expression of NMDA receptors. Interest-



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² The abbreviations used are: iGluR, ionotropic glutamate receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; ATD, amino-terminal domain; FRET, fluorescence resonance energy transfer; GABA_A, γ-aminobutyric acid type A; NMDA, *N*-methyl-D-aspartate; NR, NMDA receptor; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; GFP, green fluorescent protein; ANOVA, analysis of variance; PDI, protein-disulfide isomerase.

ingly, we found an ER retention signal located in the ATD of the NR2A subunit but not in the corresponding domain of the NR2B. It is suggested that NR2A-containing NMDA receptors may undergo an ER quality control process different from that of NR2B-containing NMDA receptors.

EXPERIMENTAL PROCEDURES

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Plasmid Construction-The expression vectors for XFP-NR1a, XFP-NR2A, XFP-NR2B, and GABA α 1-YFP have been described previously, in which XFP indicates CFP, YFP, or GFP (13, 14). The ATD-deleted, XFP-tagged NR1a, NR2A, or NR2B subunits used in this study were constructed from XFP-NR1a, XFP-NR2A, and XFP-NR2B, respectively, using conventional DNA mutagenesis techniques. In this report, NR1a_{ΔATD} means that the first 390-amino acid residues of the NR1a subunit were deleted, NR2A_{ΔATD} means that the first 391 amino acid residues of the NR2A subunit were deleted, and $\text{NR2B}_{\Delta\text{ATD}}$ means that the first 392 amino acid residues of the NR2B subunit were deleted (Fig. 1A). NR2A_{ATD-2B} and NR2B_{ATD-2A} represent chimeras in which the ATD of the first subunit was substituted with the ATD of the second subunit (Fig. 4A). For example, NR2A_{ATD-2B} means that the ATD of the NR2A subunit was substituted with that of the NR2B subunit. NR2A_{$\Delta ATD-A1$}, NR2A_{$\Delta ATD-A2$}, or NR2A_{$\Delta ATD-A3$} were constructed by inserting an NR2A A1, A2, or A3 segment into the SacII site of NR2A_{$\Delta ATD}$, whereas NR2A_{$\Delta ATD-A2-2B$} was constructed by</sub> inserting the NR2B A2 segment into the SacII site of NR2A_{$\Delta ATD}$, as illustrated in Fig. 8A. pDisplay-GFP was con-</sub> structed by fusing GFP with the pDisplay vector between the XmaI and SacII sites by PCR amplification. The chimeras between the ATD of the NR1a, NR2A, or NR2B subunit and pDisplay-GFP were made by fusing the ATD of these subunits into the pDisplay-GFP at the SalI site and were named GFP- ATD_{1a} , GFP-ATD_{2A}, or GFP-ATD_{2B}, respectively (Fig. 5A). ATD_{1a} or ATD_{GluR2} was constructed by fusing the ATD of the NR1a subunit or the GluR2 subunit into the pDisplay at the SalI or SacII site (see Fig. 5A and supplemental Fig. 4A). GFP-A2_{2A} or GFP-A2_{2B} was constructed by fusing the A2 segment of the NR2A subunit or NR2B subunit into the pDisplay-GFP at the SacII site, respectively (Fig. 8C). NR2B_{A2-2A} means the A2 segment of the full-length NR2B subunit was substituted with that of the NR2A subunit (Fig. 8A), and GFP-ATD_{2B-A2-2A} means the A2 segment of the GFP-ATD_{2B} was substituted with that of the NR2A subunit (Fig. 8C), which was subcloned by using an In-FusionTM 2.0 Dry-Down PCR cloning kit (Clontech). According to the results from amino acid sequence alignment of the A2 segment of the NR2A and NR2B subunits, we selected 10 residues in the NR2A A2 segment with highly diverse side chain chemical natures (Fig. 8E). Each of them was replaced with the corresponding residue in the NR2B A2 segment. For example, I176Y means the isoleucine at position 176 of the NR2A A2 segment was replaced with a tyrosine (corresponding to Tyr¹⁷⁵ in the NR2B A2 segment). All site-directed mutagenesis manipulation was done using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). GFP-A22A-a, GFP-A22A-b, and GFP-A2_{2A}-c were constructed by fusing part of a (Ile¹⁵¹-Asp¹⁹²), b (Asn¹⁹³–Leu²³⁸), or c (Ile²³⁹–Asp²⁸²) of the NR2A A2 segment into the pDisplay-GFP at the SacII site, respectively

(Fig. 8*F*). For each construct, the amino acid number (relative to the first methionine in the open reading frame) at the appropriate junction is indicated in Figs. 1*A*, 4*A*, and 8, *A* and *F*. All constructs were verified with DNA sequencing.

Cell Culture and Transfection-HEK 293 cells and COS-7 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and antibiotics (all from Invitrogen), and transfected with appropriate plasmids $(3-4 \mu g/35$ -mm dish, $6-8 \mu g/60$ -mm dish) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfection mixture was replaced 3-5 h after transfection with fresh culture medium. Ketamine (0.5 mm; Sigma) and kynurenic acid (1 mM; Sigma) were added to the media to protect the cells from NMDA receptor-mediated toxicity. Cells were examined within 2 days after transfection in an extracellular solution composed of 145 mm NaCl, 5 mm KCl, 2 mм CaCl₂, 5 mм glucose, 0.01 mм glycine, and 5 mм HEPES at pH 7.4 with NaOH. Hippocampal neuronal cultures were prepared according to the protocol described previously (13). At 5 days *in vitro*, appropriate plasmids $(3-4 \mu g/35$ -mm dish) were added with 4 μ l of Lipofectamine 2000. After a 3-h incubation at 37 °C, cells were washed twice with serum-free medium, and the serum-containing culture medium was added. Expression of exogenous DNA was typically detected in 7 days in vitro neurons.

Surface Immunostaining and Quantitative Analysis-The methods used for surface immunostaining and quantitative analysis have been described previously (11, 13). Briefly, the transfected HEK 293 cells or hippocampal neurons were incubated with rabbit anti-GFP antibody (Chemicon) for 7 min, rinsed three times in extracellular 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 solution, cells were immediately fixed and examined through a $60 \times$, 1.4 numerical aperture oil immersion objective on a TE 2000 inverted microscope (Nikon, Tokyo, Japan) equipped with Metamorph version 5.0 software (Universal Imaging, West Chester, PA). All procedures were performed at room temperature. Surface expression of XFP-tagged NMDA receptor (NR) subunits in HEK 293 cells or hippocampal neurons was measured as the percentage of surface-stained cells (red) in the population of total cells transfected with XFP fusion proteins (green). For each sample, more than 200 XFP-positive HEK 293 cells or 50 XFP-positive hippocampal neurons were counted, and the means were obtained from three different cultures. The average intensities of surface (Cy3) and total fluorescence (GFP) were determined for regions outlined around the transfected HEK 293 cells or for regions outlined around the cell body of transfected hippocampal neurons. The optical signal level from nontransfected cells was considered as background. Intensity measurements are expressed in arbitrary units per unit area. For each sample, more than 60 XFP-positive HEK 293 cells or 30 XFP-positive neurons were counted, and the means were obtained from three different cultures. All data are presented as the mean \pm S.E. Differences were tested using Student's *t* test or a one-way ANOVA test followed by the Newman-Keuls multiple comparison test.

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Electrophysiology—The electrophysiological methods have been described previously (11). The extracellular recording solution contained 145 mm NaCl, 3 mm KCl, 10 mm HEPES, 3 mm CaCl₂, 8 mm glucose, 2 mm MgCl₂ (310 mosmol, pH adjusted to 7.30 with NaOH). Patch pipettes were filled with an intracellular solution containing 136.5 mm potassium gluconate, 17.5 mm KCl, 9 mm NaCl, 1 mm MgCl₂, 10 mm HEPES, 0.2 mm EGTA (310 mosmol, pH adjusted to 7.20 with KOH). Recordings were made at -60 mV during the application of 100 μ M glutamate and 10 μ M glycine or 50 μ M D-AP5, an antagonist of NMDA receptors.

Immunocytochemistry-Cultured COS-7 cells were fixed in 4% paraformaldehyde in PBS for 10 min and permeabilized in PBS containing 0.4% Triton X-100 and 5% bovine serum albumin for 30 min at room temperature. Cells were then incubated in primary mouse monoclonal PDI antibody (a marker for ER; Abcam), 58K antibody (a marker for Golgi; Abcam), 19 S S5A antibody (a marker for proteosome; Abcam), EEA1 antibody (a marker for early endosome; BD Biosciences), LAMP2 antibody (a marker for lysosome; Abcam), or NR1a (BD Biosciences) antibody, with or without primary rabbit polyclonal GFP antibody, in PBS containing 5% bovine serum albumin for 1 h. After washing three times with PBS, cells were incubated in antimouse Alexa-594-conjugated secondary antibody (Molecular Probes, Inc., Eugene, OR), with or without anti-rabbit Alexa-488-conjugated secondary antibody (Molecular Probes), in PBS containing 5% bovine serum albumin for another 1 h. After washing three times with PBS, cells were observed on a Fluoview FV1000 confocal microscope (Olympus). The primary antibody was used at 1:200 for PDI, 58K, LAMP2, EEA1, and GFP antibody and 1:100 for NR1a and 19 S S5A antibody, whereas the secondary antibody was used at 1:2000.

Detection of FRET Using Three-cube FRET Measurement— The fluorescence imaging work station for FRET and the FRET quantification method have been described previously (14). Briefly, the fluorescence imaging workstation consisted of a TE2000 inverted microscope (Nikon, Tokyo, Japan) equipped with a halogen lamp light source (100 watts), Dual-ViewTM (Optical Insights, LLC, Santa Fe, NM), and a SNAP-HQ cooled CCD camera (Roper Scientific, Trenton, NJ). MetaMorph version 5.0 software (Universal Imaging, West Chester, PA) was used to control the CCD camera and for analysis of the cell image data. Three-cube FRET filter cubes were as follows (excitation; dichroic; emission; company): YFP (S500/20 nm; Q515lp; S535/30 nm; Chroma); FRET (S430/25 nm; 455dclp; S535/30 nm; Chroma); and CFP (S430/25 nm; 455dclp; S470/30 nm; Chroma). Binning 2×2 modes and a 200-ms integration time were used. Average background signal was determined as the mean fluorescence intensity from a blank area and was subtracted from the raw images before carrying out FRET calculations. The FRET ratio (FR) was calculated with Equation 1 (14 - 16).

$$FR = \frac{S_{\text{FRET}}(\text{DA}) - R_{\text{D1}} \cdot S_{\text{CFP}}(\text{DA})}{R_{\text{A1}} \cdot S_{\text{YEP}}(\text{DA})}$$
(Eq. 1)

 S_{CUBE} (SPECIMEN) 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). $R_{D1} = S_{FRET}(D)/S_{CFP}(D)$, and $R_{A1} = S_{FRET}(A)/S_{YFP}(A)$.

Crude Membrane Preparation and Deglycosylation Analysis— To prepare a crude membrane suspension, the transfected HEK 293 cells were suspended and homogenized in lysis buffer (phosphate buffer, pH 7.4, containing 20 mM EDTA, 1 mM AEBSF, 50 μ g/ml leupeptin, and 10 μ g/ml aprotinin). Samples were then centrifuged (30,000 \times g for 30 min at 4 °C), and cell membranes were resuspended in 50 μ l of lysis buffer plus 1% SDS and 5% β -mercaptoethanol and boiled for 5 min before adding 1% Nonidet P-40 plus protease inhibitors as above. Samples were then partitioned into thirds; samples were treated with 0.4 units of N-glycosidase F (Roche Applied Science), 0.006 units of endoglycosidase H (Roche Applied Science), or control saline and incubated overnight. Reactions were terminated with $4 \times$ SDS-PAGE sample buffer and boiling for 5 min. Membrane proteins were resolved on a 6% SDS-polyacrylamide gel and visualized using immunoblot analysis with anti-GFP antibody.

RESULTS

Neither the NR1a ATD nor the NR2B ATD Is Required for the Formation and Surface Expression of NR1a/NR2B Receptors-In order to determine the role of the ATD in the trafficking of NMDA receptors, we generated a series of ATD deletions in fluorescent protein-tagged NR1a, NR2B, and NR2A subunits (Fig. 1*A*). We first used surface immunostaining to determine whether ATD deletion affected the trafficking of NR1a/NR2Bcontaining NMDA receptors to the cell membrane. Because the fluorescent protein was tagged to the extracellular side of each subunit, the expression of NR1a or NR2B in the plasma membrane could be detected in live cells by immunostaining of the fluorescent proteins. We used a polyclonal anti-GFP antibody followed by a Cy3-conjugated secondary antibody to label the receptors; expression in the plasma membrane was indicated by the presence of red puncta scattered on the cell surface. When we co-expressed CFP-NR1a $_{\Delta ATD}$ and YFP-NR2B, CFP-NR1a and YFP-NR2B_{ΔATD}, or CFP-NR1a_{ΔATD} and YFP-NR2B_{ΔATD}, we detected clearly positive surface staining (Fig. 1B). To further quantitatively analyze the ability of NMDA receptors containing ATD-deleted NR subunits to traffic to the plasma membrane, we measured the percentage of cells with positive immunolabeling over the number of transfected cells indicated by the presence of YFP fluorescence. The results showed that when NR1a was co-expressed with YFP-NR2B, 77.6 \pm 1.8% of cells had positive surface labeling. Similarly, when HEK 293 cells were co-transfected with NR1a $_{\Delta ATD}$ and YFP-NR2B, NR1a and YFP-NR2B $_{\Delta ATD}$, or NR1a $_{\Delta ATD}$ and YFP-NR2B $_{\Delta ATD}$, the percentage of cells with positive surface labeling decreased compared with NR1a/YFP-NR2B but was still high (57.8 \pm 5.5, 51.6 \pm 5.0, and 55.8 \pm 6.9%, respectively) (Fig. 1*C*). Subsequent intensity analysis revealed no significant differences in the fluorescence intensities of the surface labeling or total YFP among NR1a/YFP-NR2B, NR1a $_{\Delta ATD}$ /YFP-NR2B, NR1a/YFP-NR2B_{ΔATD}, and NR1a_{ΔATD}/YFP-NR2B_{ΔATD} (Fig. 1D). These data indicate that the ATD of NR1a or NR2B subunits was not required for the successful delivery of NR1a/NR2B receptors to

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the cell surface, although the ability of these receptors to be trafficked to the cell membrane was reduced to some degree. We further investigated the expression and trafficking of NR1a or NR2B subunits with the ATD deleted in cultured hippocampal neurons (supplemental Fig. 1). These experiments showed that YFP-NR1a_{ΔATD} or YFP-NR2B_{ΔATD} expressed alone in hippocampal neurons was able to express in the cell membrane, probably by co-assembly with endogenous NR2 or NR1 subunits.

To determine whether the surface-expressed complexes were functional, we recorded NMDA receptor currents in response to local application of saturating doses of glutamate (100 μ M) and glycine (10 μ M) in HEK 293 cells. Representative current traces through these deletion mutants evoked by a 500-ms glutamate application are shown in Fig. 1E, and the mean peak current amplitudes are shown in Fig. 1F. In close agreement with the live cell surface immunostaining results, currents were obtained from cells transfected with NR1a/YFP-NR2B, NR1a_{$\Delta ATD}/YFP-NR2B$, NR1a/YFP-NR2B_{$\Delta ATD}, and</sub></sub>$ NR1a_{$\Delta ATD}/YFP-NR2B_{<math>\Delta ATD}$; the average amplitudes of the glu-</sub></sub> tamate-evoked currents in picoamperes were 326.86 ± 69.20 (n = 6), 149.36 \pm 19.24 (n = 7), 114.54 \pm 15.55 (n = 7), and 172.84 ± 22.34 (*n* = 7), respectively. These data strongly suggested that deletion of the whole ATD did not eliminate the formation of functional channels between NR1a and NR2B subunits.

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Surface Expression of NMDA Receptors Composed of ATDdeleted NR1a and Full-length NR2A Was Abolished—When HEK 293 cells co-expressing CFP-NR1a $_{\Delta ATD}$ and the fulllength YFP-NR2A subunit were analyzed for cell surface expression by immunoassay, no positive staining was detectable (Fig. 2A). This was true even for cells exhibiting clear intracellular CFP or YFP fluorescence signals, indicating strong expression of NR subunit proteins. This result is consistent with previous findings that the mutant NR1a subunit missing ATD cannot form receptors with the NR2A subunit in the plasma membrane (17). However, when CFP-NR1a subunits were co-expressed with YFP-NR2A $_{\Delta A\,TD}$ subunits or when CFP-NR1a_{$\Delta ATD}$ subunits were co-expressed with YFP-</sub> NR2A_{$\Delta ATD}$ </sub> subunits, we found positive surface staining (Fig. 2A). In addition, significant surface staining was also observed when YFP-NR2A_{ΔATD} was expressed in hippocampal neurons (supplemental Fig. 1), which indicated that ATD-deleted NR2A subunits were able to co-assemble with endogenous NR1 subunits and express in the surface membrane. We also measured the percentage of transfected cells with positive surface staining. When HEK 293 cells were co-transfected with NR1a/YFP-

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NR2A, 66.0 \pm 3.6% of cells had positive surface labeling. When NR1a/YFP-NR2A_{ΔATD} or NR1a_{$\Delta ATD}/YFP-NR2A_{<math>\Delta ATD$} was co-</sub> transfected, the corresponding percentages were 46.0 ± 4.2 and $60.1 \pm 1.2\%$, respectively (Fig. 2*B*). However, when NR1a_{$\Delta ATD}/$ </sub> YFP-NR2A was co-transfected, the corresponding percentage was 2.4 \pm 0.7%, which was significantly lower than that for NR1a/YFP-NR2A (Fig. 2B) and was not statistically different from the percentage when YFP-NR2A was expressed alone $(2.9 \pm 1.4\%)$. The total intensity of YFP fluorescence showed no significant difference among NR2A or ATD-deleted NR2A, whereas the fluorescence intensity of the surface staining of $NR1a_{\Delta ATD}/YFP-NR2A$ was significantly different from that of NR1a/YFP-NR2A (Fig. 2C). These results thus showed that $NR1a_{\Delta ATD}/YFP-NR2A$ co-expression did not yield any surface protein, whereas the other combinations did. In agreement with the live cell surface immunostaining, no current response was detected from cells transfected with NR1a_{$\Delta ATD}/YFP-$ </sub> NR2A (Fig. 2, D and E). In contrast, significant currents were recorded in cells expressing NR1a/YFP-NR2A, NR1a/YFP-NR2A_{ΔATD}, or NR1a_{$\Delta ATD}/YFP-NR2A_{<math>\Delta ATD}$ </sub>; the average ampli-</sub> tudes of glutamate-evoked currents in picoamperes were 147.12 \pm 13.17 (n = 4), 107.15 ± 25.90 (n = 4), and 139.10 ± 31.11 (n = 6), respectively (Fig. 2, D and E). Taken together, these results indicated that NR1a/NR2A receptors with NR2A ATD deleted were still delivered to the surface membrane, whereas NR1a/NR2A receptors with NR1a ATD deleted could not be delivered to the surface membrane.

The Heteromers between the ATD-deleted NR1a and the Full*length NR2A Were Formed but Retained in the ER*—We further compared the surface expression of full-length NR1a or ATDdeleted NR1a subunits co-expressed with GFP-tagged NR2A or NR2B subunits in HEK 293 cells using FACS analysis. We found that co-expression of NR1a/GFP-NR2A or NR1a/GFP-NR2B led to robust surface expression, with the percentage of GFPpositive cells showing surface expression being 68.0 or 71.7% (supplemental Fig. 2A), respectively. In addition, when ATDdeleted NR1a and GFP-NR2B were co-expressed, the percentage was decreased but still relatively high (50.3%). However, the percentage of GFP-positive cells showing surface expression of NR1a_{$\Delta ATD}/GFP-NR2A$ was quite low (4.1%) and was similar to</sub> that of the HEK 293 cells expressing GFP-NR2A alone (3.8%) (supplemental Fig. 2A). Taken together, results from FACS measurements were quantitatively consistent with the previous immunostaining and functional data, revealing that NR1a_{AATD}/NR2B were able to express in the surface membrane but not NR1a_{$\Delta ATD}/NR2A$.</sub>

One possible reason for NR1a_{$\Delta ATD}/GFP-NR2A$ unable to express in the cell membrane was that ATD-deleted NR1a sub-</sub>



FIGURE 1. **ATD of NR1a or NR2B subunit is not required for the formation of functional NR1a/NR2B receptors.** *A*, schematic diagram of the NR subunit deletion constructs. *ATD* represents the first ~400 amino acids, and *XFP (dotted box)* represents the fluorescent protein CFP or YFP. The *hatched box* corresponds to the signal peptide, the *dotted line* corresponds to the deleted ATD region, and the *black boxes* correspond to TM. The *number above* each NR subunit deletion construct indicates the residues deleted. *B*, HEK 293 cells expressing the indicated cDNAs were surface-stained with anti-GFP antibodies. CFP was tagged to the NR1a or ATD-deleted NR1a subunit, whereas YFP was tagged to the NR2B or ATD-deleted NR2B subunit (*two left columns*). The *right column* was from surface staining using anti-GFP antibody and Cy3-conjugated secondary antibody. *Scale bar*, 10 μ m. *C*, the percentages of cells with detectable surface labeling were determined for >200 YFP-expressing cells for each combination of cDNAs in three experiments. The *gray boxes* represent YFP. The *bar graphs* represent mean \pm S.E. **, *p* < 0.01, ANOVA. *D*, data represent mean \pm S.E. of fluorescence intensities per unit area obtained for surface (*black*) or total YFP (*white*) expression. More than 60 YFP-expressing cells for each combination of cDNAs were analyzed in three experiments. *E*, representative glutamate-evoked currents recorded from HEK 293 cells. Glutamate (100 μ M) and glycine (10 μ M) were added to evoke currents. *F*, summary data showing the average peak amplitudes of the whole-cell currents when co-expressing NR1a or ATD-deleted NR2B or ATD-deleted NR2B. The *numbers above* the *histogram bars* indicate the number of cells responsing of the cotal number of recorded cells (*i.e. 6/6* represents six responsive cells of six).





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FIGURE 3. **NR1a**_{AATD} and full-length NR2A subunits are assembled but mainly retained in the ER. *A*, ATDdeleted NR1a subunits form homomers or heteromers with full-length NR2A subunits analyzed by three-cube FRET measurement. *Black boxes* indicate CFP, and *gray boxes* indicate YFP, *FR* is expected to be 1 when there is no FRET and above 1 when FRET does occur. **, p < 0.01 versus CFP-NR1a_{AATD}/ α 1-YFP, unpaired t test. *B*, glycosylation properties of glutamate receptor subunits. GFP-NR2A, when co-expressed with the wild-type NR1a subunit, showed detectable sensitivity but was not completely sensitive to Endo H, indicating that under such conditions, GFP-NR2A entered Golgi. In contrast, when co-expressed with NR1a_{AATD}, GFP-NR2A was completely sensitive to Endo H, indicating that under such conditions, GFP-NR2A was retained in the ER. *C*, immunocytochemical analysis revealed that when GFP-NR2A was co-expressed with NR1a_{AATD}, it mainly colocalized with NR1a_{AATD} and the ER marker (PDI) but not with the Golgi (58K) (*insets* show the *boxed regions* in greater detail). *D*, when GFP-NR2A was co-expressed with NR1a, it mainly colocalized with NR1a but not with the ER marker (PDI). The colocalization of the NR2A subunits and the Golgi (58K) was also clearly observed (*insets* show the *boxed regions* in greater detail). *Scale bars*, 20 μ m (10 μ m for the *insets*).

units were not able to co-assemble with NR2A subunits. In previous studies, we analyzed the assembly of native NMDA receptor subunits using a three-cube FRET measurement (14). With the same approach, we found that FRET signals were produced when CFP-NR1a_{$\Delta ATD} and YFP-NR1a_{<math>\Delta ATD}$ or CFP-</sub></sub> NR1a_{AATD} and YFP-NR2A were co-transfected into HEK 293 cells, with FRET ratios (FR) of 1.43 \pm 0.04 (n = 35) or 1.47 \pm 0.09 (n = 30), respectively (Fig. 3A). These values were significantly different from the *FR* value $(1.01 \pm 0.02, n = 32; p < 0.01)$ obtained from the co-expression of CFP-NR1a and $\mbox{GABA}_{\mbox{\tiny A}}$ α 1-YFP (14). In addition, when CFP-NR1a_{AATD} and GABA_A α 1-YFP were co-transfected, the *FR* value was 1.09 \pm 0.09 (*n* = 38) and showed no significant difference from that obtained from the co-expression of CFP-NR1a and GABA_A α 1-YFP (14). The results confirmed that the deletion of ATD did not affect the assembly of NR1a and NR2A subunits.

Another potential reason for

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NR1a_{$\Delta ATD}/GFP-NR2A$ unable to</sub> express in the cell membrane was that the ATD-deleted NR1a subunits were assembled with NR2A subunits, but they were retained in the ER. To test this possibility, we examined the subcellular localization of the NR1a $_{\Delta ATD}$ /NR2A receptors using confocal microscopy (Fig. 3, C and D). After NR1a_{$\Delta ATD} and</sub>$ GFP-NR2A were co-transfected into COS-7 cells, NR2A colocalized extensively with NR1a and the ER marker (PDI) but not with the Golgi marker (58K) (Fig. 3C). In contrast, when GFP-NR2A was co-expressed with NR1a in COS-7 cells, NR2A colocalized with the Golgi marker (58K) (Fig. 3D). We further tested the colocalization of GFP-NR2A when co-expressed with NR1a_{$\Delta ATD}$ </sub> with the proteosome, endosome, and lysosome markers. The data showed that NR2A under such conditions was not colocalized with these markers (supplemental Fig. 3). To confirm the ER retention of NR1a_{\Delta ATD}/NR2A, we examined its glycosylation state. Immunoblots with GFP antibody showed that GFP-NR2A, when co-expressed with the wild-type NR1a subunit, was not completely sensitive to

was not completely sensitive to Endo H, indicating that under such conditions, GFP-NR2A entered Golgi (Fig. 3*B*). In contrast, when co-expressed with the ATD-deleted NR1a subunit, GFP-NR2A was completely sensitive to Endo H, indicating that under such conditions, GFP-NR2A was retained in the ER (Fig. 3*B*). Taken together, these biochemical and immunocytochemical results provide strong evidence that, when co-expressed with NR1a Δ ATD, the NR2A subunits were almost exclusively retained in the ER and failed

NR2A ATD but Not NR2B ATD Possesses an ER Retention Signal—The results described above suggest that the NR2A ATD may play a role in controlling the trafficking of NMDA receptors that is different from the NR2B ATD. To further test this possibility, we exchanged the ATDs between NR2A and NR2B and tested whether these chimeras could form functional

to reach the Golgi or other degradation apparatus.

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FIGURE 2. **NR1a**_{AATD}/**NR2A** receptor complexes are not delivered to surface membrane. *A*, HEK 293 cells co-expressing indicated cDNAs were surfacestained with anti-GFP antibodies. CFP was tagged to the NR1a or ATD-deleted NR1a subunit, whereas YFP was tagged to NR2A or ATD-deleted NR2A subunit (*left two columns*). The *right column* was from surface staining using anti-GFP antibody and Cy3-conjugated secondary antibody. *Scale bar*, 10 μ m. *B*, the percentages of cells with detectable surface labeling were determined for >200 YFP-expressing cells for each combination of cDNAs in three experiments. The *gray boxes* represent YFP. The *bar graphs* represent mean \pm S.E. **, p < 0.001, ANOVA. *C*, Surface (*black*) and total YFP (*white*) expression. More than 60 YFP-expressing cells for each combination of cDNAs were analyzed in three experiments. **, p < 0.001, ANOVA. *D*, representative glutamate-evoked currents recorded from HEK 293 cells. Glutamate (100 μ M) and glycine (10 μ M) were added to evoke currents. *E*, summary data showing the average peak amplitude of the whole-cell currents when co-expressing NR1a or ATD-deleted NR1a and NR2A or ATD-deleted NR2A subunits. The *numbers above* the *histogram bars* indicate the number of cells responding of the total number of recorded cells.

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FIGURE 4. Surface expression and functional analysis of NMDA receptors containing NR2 chimeras with ATDs exchanged between NR2A and NR2B subunits. *A*, schematic diagram of the NR2 chimeric constructs. *Gray bar*, NR2A subunit; *white bar*, NR2B subunit. *Hatched boxes*, signal peptide; *black boxes*, transmembrane domain. *B*, HEK 293 cells co-expressing indicated cDNAs were immunostained for surface XFP. *Scale bar*, 10 μ m. *C*, summary data showing percentages of HEK 293 cells with detectable surface labeling. **, p < 0.01, unpaired *t* test. *D*, summary data showing the average peak amplitude (*pA*) of the whole-cell currents when co-expressing NR1a or ATD-deleted NR1a subunits and NR2A_{ATD-2B} or NR2B_{ATD-2A} subunits.

channels in the cell membrane (Fig. 4A) with wild type or ATDdeleted NR1a subunits. The results showed that when the YFP-NR1a subunit was co-transfected with NR2AATD-2B or NR2B_{ATD-2A}, significant positive surface labeling was detected: 63.0 ± 4.3 and $64.3 \pm 2.3\%$, respectively (Fig. 4, *B* and *C*). However, when YFP-NR1a_{$\Delta ATD}$ was co-expressed with NR2B_{ATD-2A},</sub> no surface expression was observed: $0.7 \pm 0.7\%$ (Fig. 4, *B* and *C*). In contrast, when YFP-NR1a $_{\Delta \mathrm{ATD}}$ was co-expressed with NR2A_{ATD-2B}, positive surface labeling was again detected: $39.3 \pm 6.4\%$ (Fig. 4, *B* and *C*). Furthermore, glutamate-evoked currents were recorded from YFP-NR1a/NR2BATD-2A, YFP-NR1a/NR2A_{ATD-2B}, or YFP-NR1a_{$\Delta ATD}/NR2A_{ATD-2B} co-trans-</sub>$ fected cells; the mean current amplitudes in picoamperes were $107.63 \pm 21.50 \ (n = 4), 113.50 \pm 33.61 \ (n = 4), and 148.45 \pm 100.000 \ (n = 4)$ 59.25 (n = 4), respectively (Fig. 4D). These results again indicated that it is the NR2A ATD that caused the NR1a $_{\Delta ATD}/$ NR2A receptors to fail to express on the cell surface.

centage of GFP-positive cells containing surface expression of GFP-ATD_{2A} was quite low (3.3%). Quantitative fluorescence measurements showed a dramatic difference in the intensity of surface labeling between GFP-ATD_{2A}, GFP-ATD_{1a}, or GFP-ATD_{2B}; the intensities of total GFP fluorescence were not significantly different among these GFP-tagged ATD-pDisplay chimeras (Fig. 5D). More interestingly, when co-expressing ATD_{1a} and GFP-ATD $_{2A}$, most cells showed detectable surface staining, with 70.8 \pm 3.1% positive surface labeling (Fig. 5, *B* and C). However, no positive surface staining was detected when co-expressing $\text{ATD}_{\text{GluR2}}$ and $\text{GFP-ATD}_{\text{2A}}$ (3.03 \pm 1.6%) (supplemental Fig. 4), which further indicated that the ER retention signal of NR2A ATD was specifically masked by NR1a ATD. In addition, when the GFP-ATD_{1a}, GFP-ATD_{2B}, or GFP-ATD_{2A} was expressed in hippocampal neurons, similar results were obtained. Clear surface staining was detected when neurons expressed GFP-ATD_{1a} or GFP-

To further test the role of the

ATD of NR subunits in membrane

trafficking, we constructed a series

of chimeras in which the ATD of the

NR subunit was fused to the trans-

membrane domain of an unrelated

membrane protein: pDisplay-GFP

(Fig. 5A). The pDisplay vector con-

tained an NH₂-terminal murine

Igκ-chain leader sequence, which

directs the biosensor protein to the

secretory pathway, and a COOHterminal transmembrane domain of

platelet-derived growth factor re-

ceptor, which targets the biosensor

protein to the plasma membrane. This plasmid has been used in the

study of the role of the GluR2 ATD in spine growth (18). As expected,

the expression of GFP-ATD_{1a} or

GFP-ATD_{2B} in HEK 293 cells

resulted in robust surface labeling in

nearly all GFP-expressing cells (Fig.

5, *B* and *C*). However, the majority

of HEK 293 cells expressing GFP-

ATD_{2A} alone were not surface-la-

beled (Fig. 5*B*), and the positive surface labeling was 2.6 \pm 0.5% (Fig.

5*C*). We further measured the surface expression of these chimeras of pDisplay-GFP and ATD from different NR subunits using fluorescence-activated cell sorting analysis (supplemental Fig. 2*B*) and found

that expression of GFP-ATD_{1a} or

GFP-ATD_{2B} led to robust surface

expression with the percentage of

GFP-positive cells showing surface

expression being 83.1 or 81.8%,

respectively. However, the per-



leucine-, isoleucine-, and valine-

binding proteins and the ATD of

metabotropic glutamate receptors

(4) (Fig. 8A). A1 and A3 correspond to the regions forming lobe-1, and

A2 and A4 correspond to those forming lobe-2. A1, A2, and A3

combined comprise about 90% of

the ATD sequence, whereas A4 is relatively short and may function as

a linker between ATD and S1. We

therefore focused our attention on

the A1-A3 segments. We inserted

the A1, A2, or A3 segment of the

NR2A subunit into NR2A_{$\Delta ATD}$ (Fig.</sub>

8A) and named the products

NR2A_{$\Delta ATD-A1$}, NR2A_{$\Delta ATD-A2$}, or

NR2A_{$\Delta ATD-A3$}, respectively. We co-

expressed these chimeras with the

YFP-tagged NR1a subunit or with

the YFP-tagged, ATD-deleted NR1a subunit. We found that when

 $NR2A_{\Delta ATD-A1}$ was co-expressed

with YFP-NR1a or YFP-NR1 $a_{\Delta ATD}$,

significant positive surface labeling

was detected in 51.0 \pm 3.5 and

 $48.3 \pm 6.1\%$ of the YFP-positive cells,

respectively. When NR2A_{$\Delta ATD-A3$} was

co-expressed with YFP-NR1a or

YFP-NR1a_{$\Delta ATD}$, significant positive</sub>

surface labeling was also detected in

 42.3 ± 1.1 and $46.0 \pm 7.9\%$, respectively. However, when NR2A $_{\Delta ATD-A2}$

was co-expressed with YFP-

NR1a_{$\Delta ATD}$, the percentage of posi-</sub> tive surface labeling was significantly

reduced to only 7.4 \pm 0.3%, indicating

that the surface expression of the

YFP-NR1a_{$\Delta ATD}/NR2A_{<math>\Delta ATD-A2$} com-</sub>

plex was almost completely abol-

ished. When NR2A $_{\Delta ATD-A2}$ was co-

expressed with YFP-NR1a, the



FIGURE 5. NR2A ATD possesses an ER retention signal, and NR1a ATD is necessary for overcoming this signal. A, diagram of chimeric constructs between ATD and pDisplay-GFP. Dotted box, fluorescent protein GFP. Hatched box, signal peptide; black box, transmembrane domains of platelet-derived growth factor receptor. B, HEK 293 cells transfected with indicated cDNAs were surface-stained with anti-GFP antibodies. Scale bar, 20 μ m. C, the percentages of cells with detectable surface labeling were determined for >200 GFP-expressing cells for each indicated cDNA in three experiments. The *bar graphs* represent mean \pm S.E. **, p < 0.01, ANOVA D, surface (black) and total GFP (white) expression. More than 60 GFP-expressing cells for each indicated cDNA were analyzed in three experiments. **, p < 0.01, ANOVA.

 ATD_{2B} , whereas virtually no surface staining was observed when they expressed GFP-ATD_{2A} (Fig. 6, A-C).

We next compared the intracellular distribution of GFP- ATD_{2A} with GFP-ATD_{2B} in COS-7 cells. The immunostaining results showed that GFP-ATD_{2A} exclusively co-localized with the ER marker (PDI) but not with the Golgi marker (58K) (Fig. 7). In contrast, the GFP-ATD $_{2B}$ co-localized with both markers (Fig. 7). Together with previous data, our results indicate that the NR2A ATD but not the NR2B ATD contains an ER retention signal. In addition, the assembly of NR2A ATD with NR1a ATD is able to negate this ER retention signal.

The ER Retention Signal Is Located in the A2 Segment of the NR2A ATD—To further clarify the location of this ER retention signal in the NR2A ATD, we divided NR2A ATD into four segments, termed A1-A4, according to homology with the

percentage significantly increased to 23.3 \pm 1.6% (Fig. 8*B*). We further inserted the A2 segment of the NR2B subunit (Fig. 8A) into NR2A $_{\Delta ATD}$, named NR2A $_{\Delta ATD-A2-2B}$, and co-expressed this chimera with the YFP-tagged NR1a subunit or with the ATD-deleted NR1a subunit. We found that the percentages of positive surface labeling were 57.7 \pm 7.8 and 58.7 \pm 5.9%, respectively (Fig. 8*B*), which indicated that the A2 segment of NR2B has no ER retention function. More interestingly, when we substituted the A2 segment of NR2B with that of the NR2A subunit (named NR2B_{A2-2A}) and co-expressed it with YFP-NR1a, the percentage of positive surface expression was 51.6 \pm 8.0%, whereas when YFP-NR1a_{\Delta ATD}/NR2B_{A2-2A} were co-expressed, the percentage was 2.3 \pm 0.2%. These results indicate that the A2 segment of the NR2A subunit, but not that of the NR2B subunit, acts as a dominant ER retention signal in the NR1a/NR2A complex.

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FIGURE 7. **NR2A ATD is retained in the ER while NR2B ATD is delivered to the Golgi.** GFP-ATD_{2A} or GFP-ATD_{2B} was transfected into COS-7 cells. Cells were immunostained with PDI as a marker for ER or 58K as a marker for Golgi and were imaged using confocal laser-scanning microscopy (Olympus). ATD_{2A} colocalized with the ER marker but not with the Golgi marker, whereas ATD_{2B} colocalized with both the ER and Golgi markers (*insets* show the *boxed regions* in greater detail). *Scale bars*, 20 μ m (10 μ m for the *insets*).

We further tested the ER retention signal using chimeras of different segments of the NR2A or NR2B ATD and pDisplay (Fig. 8C). When the A2 segment of NR2B ATD was fused into the pDisplay-GFP and transfected into HEK 293 cells, significant surface staining was observed (76.9 \pm 6.0%) (Fig. 8D). However, almost no surface staining was seen when the A2 segment of NR2A ATD was fused into the pDisplay-GFP, with the percentage of positive surface labeling being 1.0 \pm 1.0%. We further constructed a chimera in which the A2 segment of the NR2B ATD was substituted with the A2 segment of the NR2A ATD (named GFP-ATD_{2B-A2-2A}) (Fig. 8C). When GFP-ATD $_{\rm 2B-A2-2A}$ was expressed alone in HEK 293 cells, it was almost all retained in the ER, with the positive surface labeling being 5.5 \pm 2.2% (Fig. 8D). However, when co-expressed with ATD_{1a}, GFP-ATD_{2B-A2-2A} was transported to the cell surface, with the positive surface labeling being $67.5 \pm 3.3\%$ (Fig. 8D). In addition, when these constructs were expressed in hippocampal neurons, similar results were obtained (Fig. 6, D-F). When GFP-A2_{2A} or GFP-ATD_{2B-A2-2A}

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was expressed in hippocampal neurons, no surface staining was detected. However, when GFP-A2_{2B} or ATD_{1a}/GFP - $ATD_{2B-A2-2A}$ was expressed in hippocampal neurons, clear surface staining was detected. This result was in close agreement with the previous results and confirmed that the A2 segment of the NR2A subunit but not the A2 segment of the NR2B subunit contained an ER retention signal.

The A2 segment of NR2A or NR2B has ~130 amino acid residues. To further screen for any possible retention motif in this segment, we generated a series of point mutations in GFP- ATD_{2A} (Fig. 8E). Ten amino acids that are different between the NR2A A2 segment and the NR2B A2 segment according to sequence alignment were chosen for mutagenesis (Fig. 8E). Mutants were screened by surface staining using anti-GFP antibody in HEK 293 cells. Unfortunately, no mutation resulted in a significant increase in the surface labeling of GFP-ATD_{2A}. The percentages of surface labeling for I176Y, F186K, M200L, A213S, S225P, D234E, L238Y, E242V, F253Y, and K270S were $1.7 \pm 1.7, 1.7 \pm 1.7, 0.8 \pm 0.4, 0.5 \pm 0.5, 1.3 \pm 1.3, 0.7 \pm 0.6,$ 1.9 ± 1.1 , 3.2 ± 0.6 , 1.3 ± 1.3 , and $2.8 \pm 1.6\%$, respectively. These results suggest that either these residues are not involved in the ER retention of NR2A ATD or each of them alone is not sufficient for this retention.

In order to locate the ER retention signal, we split the A2 segment into three parts, named A2_{2A}-a (Ile¹⁵¹–Asp¹⁹²), A2_{2A}-b (Asn¹⁹³–Leu²³⁸), and A2_{2A}-c (Ile²³⁹–Asp²⁸²), and fused these parts individually into pDisplay-GFP (Fig. 8*F*). Unexpectedly, surface staining showed that all three of these chimeras were expressed on the surface membrane, and the percentages of surface labeling were 79.5 ± 1.5% for A2_{2A}-a, 75.6 ± 4.9% for A2_{2A}-b, and 77.3 ± 1.6% for A2_{2A}-c (Fig. 8*G*). Fluorescence intensity analysis of surface labeling or total GFP expression showed no significant difference among these three chimeras (Fig. 8*H*). Together with the point mutation results, these findings suggest that it is likely that the overall structure or conformation of the A2 segment, instead of an isolated sequence, may be involved in the ER retention of the NR2A ATD.

DISCUSSION

NMDA receptors play important roles in both normal brain function and neurological and psychiatric disorders. The number and composition of heteromeric NMDA receptors on the cell surface determine its activity in response to particular stimuli. Therefore, understanding the assembly and trafficking of the NMDA receptor is pivotal for understanding the mechanisms of its diverse functions. In this study, we found that the NR2A ATD contains an ER retention signal, and the NR1a ATD is necessary for overcoming this retention signal. In contrast, the NR2B ATD has no such retention signal. Our findings indicate that NR2A-containing NMDA receptors have an ER quality control mechanism different from that of NR2B-containing NMDA receptors.



FIGURE 6. **NR2A ATD was not trafficked to the surface membrane in hippocampal neurons, and its A2 segment was responsible for ER retention.** Hippocampal neurons transfected with the indicated cDNAs at 5 days *in vitro* were surface-stained with anti-GFP antibody at 7 days *in vitro* (A and D). Scale bar, 20 μ m. The percentages of neurons with detectable surface labeling were determined for >50 GFP-expressing neurons for each combination of cDNAs in three experiments (B and E). Gray boxes, GFP; bar graphs, mean \pm S.E. Intensity per unit area represents mean \pm S.E. of the fluorescence intensity per unit area obtained for surface expression (*black*) or total GFP expression (*white*), and more than 30 GFP-expressing neurons for each combination of cDNAs in three experiments were analyzed (C and F). **, p < 0.01, ANOVA.



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NR2A ATD Contains an ER Retention Signal—ER retention is a common feature of the quality control mechanism for complex proteins, ensuring that unassembled or otherwise defective proteins are not released from the ER (19, 20). The NMDA receptor is a heteromeric complex. Most NR1 splice variants and NR2 subunits are retained in the ER when expressed alone. When expressed together, they assemble efficiently and form functional receptors in the cell membrane. This indicates that more restrictive mechanisms are required for the ER quality control of NMDA receptors. In the present study, we found that the ATD of the NR2A subunit contains an ER retention signal (Fig. 2). We narrowed down this ER retention signal to the A2 segment of the NR2A ATD, which contains about 100 amino acids (Fig. 8). ER retention effects were abolished when the A2 segment was further divided into three parts. In addition, mutagenesis of selected amino acids in the A2 segment of NR2A ATD failed to revert the ER retention. Together, these findings suggest that the A2 segment contains a multiple sitedependent or a conformation-dependent ER retention motif. It is unlikely that a specific motif within the A2 segment is responsible for retention. ATD of the NMDA receptor is facing the extracellular or ER luminal side. There are precedents of ER retention associated with extracellular domains. For example, the extracellular domains of T cell antigen receptor α or β chains are involved in ER retention (21, 22). Kv1 α subunits also contain a dominant ER retention signal in their extracellular pore region (23); an ER protein with a domain structurally resembling that of snake dendrotoxins may be involved in ER retention. Previous studies have shown that the NR1a subunit contains

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Previous studies have shown that the NR1a subunit contains two ER retention motifs, RRR and KKK, in its C1 cassette (24, 25) and potential ER retention signals in its TM3, and the NR2B subunit possesses ER retention signals in its TM3 as well as its COOH terminus (10, 12, 26). The COOH terminus of the NR2A subunit also has been shown to play a role in ER retention (10). We found that indeed the ATD-deleted NR2A subunits, when expressed alone in heterogeneous cells, were retained in the ER (data not shown), indicating an ER retention signal located in the other part of the NR2A subunit. Taken together, these studies are consistent with the idea that multiple ER retention signals are present in the NR subunits.

The ER Retention Signal in NR2A ATD Is Masked by NR1a ATD—Recently, increasing evidence indicates that the ATD is not essential for assembly or gating of NMDA receptors but rather plays a modulatory role (27–31). Here, using truncated NR subunits combined with FRET measurements, we found that deletion of the ATD did not eliminate the homo- or heterooligomerization of NR subunits in living HEK 293 cells (Fig. 3A). These results further indicate that the ATD is not a crucial

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element for subunit assembly. In addition, our results suggested a novel mechanism for the modulatory role of ATD. We showed that the ER retention signal in NR2A ATD was masked by the NR1a ATD but not the GluR2 ATD (Fig. 5 and supplemental Fig. 4), suggesting that the NR1a ATD can interact specifically with the NR2A ATD and negate the ER retention signals in the NR2A ATD.

The mechanism underlying the masking of the NR2A ATD by the NR1a ATD is still unclear. Also, the specific region of NR1a ATD involved in this process remains to be identified. The ER retention mechanism observed in Kv1 α channels (23) suggests that it is possible that certain ER protein may bind to the NR2A ATD, in particular the A2 segment, and lead to its retention in the ER. The assembly of the NR1a ATD with the NR2A ATD may cause conformational changes of the NR2A ATD that result in its release from ER retention. It is also possible that the assembly of the NR1a ATD with the NR2A ATD may increase the overall structural stability of the NR2A subunit (32). A recent study (31) using a shorter NR1a deletion (350 amino acids instead of 390 amino acids in our study and 380 amino acids in the studies of Meddows and co-workers (17)) revealed different results that the ATD-deleted NR1a subunit can assemble with the NR2A subunit into functional receptors in Xenopus oocytes. We attribute this discrepancy to the different lengths of deletion our two groups used. In addition, these results suggest that the region of the NR1a ATD after 350 amino acids is important in masking the ER retention signal of the NR2A ATD.

NR2A ATD Contains an ER Retention Signal; NR2B ATD Does Not-NMDA receptors containing different NR2 subunits exhibit different channel properties, such as current kinetics and conductance (33-35). However, the structurefunction relationships of NMDA receptors are still uncertain. In the present study, we identified an ER retention signal that occurs in the NR2A ATD but not in the NR2B ATD. The finding provides evidence that the ER quality control mechanism of NR2A subunits is different from that of NR2B subunits. How this difference affects the functional heterogeneity of NMDA receptor subtypes remains to be determined. Nonetheless, these results suggest that the NR2A subunit may have more restrictive "structural checkpoints" than the NR2B subunit does in the ER quality control pathway. One potential consequence of this difference is to ensure the proper folding of NR2A subunits and proper assembly of the NR1a/NR2A receptor subtype. Alternatively, it is possible that the difference provides a potential mechanism to regulate the ratio of the expression of NR2A-containing NMDA receptors and NR2B-containing NMDA receptors through modulation of the preference of assembly of different NR2 subunits with NR1 subunits.

FIGURE 8. **A2 segment of NR2A ATD contains an ER retention signal.** *A*, schematic diagram of the NR2A_{ΔATD-A1,A2,A3}, NR2A_{ΔATD-A2-2B}, and NR2B_{A2-2A}, *Gray bar*, NR2A subunit; *white bar*, NR2B subunit; *hatched box*, signal peptide. The *numbers above* the NR2A or NR2B subunits indicate the residues deleted. *B*, the percentages of cells with detectable surface labeling were determined for >200 GFP-expressing cells for each combination of indicated cDNAs in three experiments. The *bar graphs* represent mean \pm S.E. **, *p* < 0.01, unpaired *t* test. *C*, schematic diagram of chimeras between A2 segments of NR2A or NR2B subunits and pDisplay-GFP. *Dotted box*, GFP. *D*, the percentages of cells with detectable surface labeling were determined for >200 GFP-expressing cells for each indicated cDNA in three experiments. The *bar graphs* represent mean \pm S.E. **, *p* < 0.01, unpaired *t* test. *C*, schematic diagram of chimeras between A2 segments of NR2A or NR2B subunits and pDisplay-GFP. *Dotted box*, GFP. *D*, the percentages of cells with detectable surface labeling were determined for >200 GFP-expressing cells for each indicated cDNA in three experiments. The *bar graphs* represent mean \pm S.E. **, *p* < 0.01, ANOVA. *E*, the amino acid sequence alignment of the A2 segment of the NR2A and NR2B subunits. *Triangles*, sites at which mutations were introduced to the A2 segment. *F*, the A2 segment of NR2A ATD was further split into three parts named A2_{2A}-a, A2_{2A}-b, and A2_{2A}-c. This *panel* shows the chimeras of these sections with pDisplay-GFP. *G*, the percentages of cells with surface staining were determined for >200 GFP-expressing cells for each indicated cDNA in three experiments. The *bar graphs* represent mean \pm S.E. *H*, surface (*black*) and total GFP (*white*) expressions. More than 60 GFP-expressing cells for each indicated cDNA were analyzed in three experiments.



Recently, several studies have indicated that under special conditions, such as inflammatory or degenerative diseases of the central nervous system, matrix metalloproteinases cleave the NR1a subunit at the extracellular NH_2 -terminal domain and modify NMDA receptor function (36, 37). Tissue-type plasminogen activator is also known to bind to and then cleave the ATD of the NR1 subunit at arginine 260, which serves as a necessary step to enhance NMDA receptor signaling in neurons (38, 39). Based on these observations, it is possible that a similar mechanism may also exist in the ER lumen, where various extracellular and intracellular stimuli can regulate the interaction of the NR1a ATD with the NR2A ATD and control the trafficking of the NR1a/NR2A receptor subtype.

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