Different Roles of C-terminal Cassettes in the Trafficking of Full-length NR1 Subunits to the Cell Surface*

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N,Methyl-d-aspartate (NMDA) receptors are glutamate-gated ion channels composed of NR1 and NR2 subunits. When expressed alone, the most prevalent NR1 splice variant and all NR2 subunits are retained in the endoplasmic reticulum (ER), whereas other NR1 splice variants reach the cell surface to varying degrees. Because similar trafficking patterns have been seen for single transmembrane domain chimeric proteins with appended C termini of NMDA receptor subunits, these chimeric proteins have been used as a model for studying the mechanisms underlying the ER retention and surface trafficking of NMDA receptors. Using this approach, an RRR motif in the C1 cassette has been identified as a major ER retention signal present in NR1 subunits, and the surface localization of other NR1 splice variants has been explained by the absence of the C1 cassette or by the presence of a PDZ/coatomer protein complex II-binding domain in the C2′ cassette. However, when we tested these conclusions using full-length NR1 constructs, a more complex role of the C-terminal cassettes in the trafficking of NR1 subunits emerged. Our experiments showed that two independent ER retention motifs in the C1 cassette, KKK and RRR, are the signals mediating ER retention of the full-length NR1 subunits and that the C2′ cassette has an additional inhibitory effect on the forward trafficking of NR1 subunits. On the other hand, C0 and C2′ cassettes had an enhancing effect on the trafficking of NR1 subunits to the cell surface. Our observations identify the unique roles of C-terminal cassettes in the trafficking of full-length NR1 subunits.

*N-Methyl-D-aspartate (NMDA)* receptors are glutamate-gated ion channels that play a central role in excitatory synaptic transmission as well as excitotoxicity (1, 2). The functional NMDA receptors are composed of NR1, NR2, and/or NR3 subunits. The NR1 subunit exists in eight different splice variants derived from a single gene, whereas NR2 subunits are products of four different genes, NR2A–D, and NR3 subunits are products of two different genes, NR3A and NR3B. All subunits have the same transmembrane topology with four membrane regions (M1–4), two extracellular regions, and an intracellular C-terminal region. Recent evidence suggests that abnormal surface expression and synaptic targeting of NMDA receptors play fundamental roles in the development of many disorders, including Alzheimer disease, Parkinson disease, and schizophrenia (1, 2). Although much is known about the biophysical properties of NMDA receptors, the mechanisms underlying their trafficking to the cell surface remain poorly understood.

Previous studies have shown that full-length NR1-1 and NR2 subunits are retained intracellularly when expressed by themselves in heterologous cells, whereas other NR1 splice variants reach the cell surface (3–5). Similar results have been obtained using single transmembrane domain chimeras with the appended C termini of NMDA receptor subunits, and thus, these chimeras have been used as a model for studying the mechanisms governing the trafficking of NMDA receptor subunits (5–9). Hence, the RRR ER retention motif has been identified as a major ER retention signal in the NR1-1 subunit (5–7), and the reason why the NR1-3 splice variant reaches the cell surface has been explained by the presence of the PDZ- and/or coatomer protein complex II (COPII)-binding domain in the far C terminus of C2′ cassette, which can negate the RRR motif (5–8). However, our recent study shows that the NR1 subunit truncated five residues after M4 traffics only slightly to the cell surface, likely due to the presence of an ER retention signal in the M3 domain (10). This indicates that the role of the C terminus in the trafficking of full-length NR1 subunits is more complex than previous experiments suggested.

In this study, we investigated the role of C-terminal cassettes in ER retention and forward trafficking of NR1 subunits. Using full-length and truncated yellow fluorescent protein (YFP)-NR1 constructs expressed in heterologous cells, we demonstrate that the C1 cassette contains two independent ER retention motifs, KKK and RRR, and that the C2′ cassette has an additional inhibitory effect on the surface trafficking of NR1 subunits. We also found that both C0 and C2′ cassettes have an enhancing effect on surface targeting of the NR1 subunit and, unexpectedly, that the C2′ cassette can partially negate C1 cassette-mediated ER retention, even when the PDZ-binding domain is not functional. These findings show that each C-terminal cassette has a unique role in the trafficking of NR1 subunits to the cell surface.

**EXPERIMENTAL PROCEDURES**

*Molecular Biology—*The YFP-NR1-1a expression vector, made previously by inserting a YFP gene between the third and fourth amino acid residues after the signal peptide, was used (11). The identical YFP-NR1-4a construct was kindly provided by Dr. S. Vicini (Georgetown University); the YFP-NR1 post-M4 (YFP-
NR1 \(_{\text{R3Sstop}}\) expression vector was described previously (10). All other constructs used in this study were generated using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Amino acid residues are numbered as published (12). The YFP-NR1-2a and YFP-NR1-3a expression vectors were constructed according to GenBank\textsuperscript{TM} accession numbers U08262 and U08265. For simplicity, YFP-NR1\(_{\text{R3Sstop}}\) and YFP-NR1-1a\(_{\text{R3Sstop}}\) are called YFP-NR1 C0-stop and YFP-NR1-1a C1-stop, respectively. The entire NR1 region following a YFP region of each construct was sequenced to verify the accuracy of DNA sequences.

**Cell Culture and Transfection**—COS-7 (African green monkey kidney fibroblast) cells were maintained in minimum essential medium with Earle’s salts (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) at 37°C in 5% CO\(_2\). The cells were transfected in 12-well plates in 2 ml of their media with 1.8 µg of cDNA mixed with 4 µl of Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 5 h, medium containing the DNA/Lipofectamine mixture was replaced with fresh medium. Cells were used for experiments within 36–40 h after transfection.

**Immunofluorescence**—COS-7 cells expressing YFP-NR1 subunits were washed in phosphate-buffered saline (PBS) and incubated with blocking solution containing PBS and 0.2% bovine serum albumin on ice for 15 min. The primary antibody (rabbit anti-green fluorescent protein (GFP) antibody; Millipore, Billerica, MA) diluted in blocking solution (1:500) was then added for 30 min, cells were washed twice in PBS, and the secondary antibody (Alexa Fluor 555-conjugated goat anti-rabbit IgG, Invitrogen) diluted in blocking solution (1:1,000) was applied for 30 min. The cells were washed twice in PBS, fixed in 4% paraformaldehyde in PBS for 20 min, and mounted with ProLong Antifade reagent (Invitrogen). Images from stained COS-7 cells were captured using a 40× oil objective on a Nikon Eclipse E1000 fluorescence microscope.

**Quantitative Assay of Surface and Total Expression**—Transfected COS-7 cells were washed in PBS and fixed for 20 min in 4% paraformaldehyde, and when total expression was measured, cells were treated with 0.25% Triton X-100 in PBS for 5 min. The cells were incubated for 1 h in PBS containing 10% normal goat serum without (surface) or with (total) 0.1% Triton X-100 and then for 1 h in the primary antibody (rabbit anti-GFP antibody; 1:500 for surface expression and 1:1,000 for total expression) in PBS with 3% normal goat serum. Cells were washed three times in PBS and incubated with the secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit IgG, 1:1,000; Amersham Biosciences) for 1 h. Cells were then washed three times in PBS and incubated for 30 min in 400 µl of ortho-phenylenediamine dissolved in phosphate/citrate buffer containing sodium phosphate (Sigma). The color reaction was terminated with 100 µl of 3 M HCl, and the optical density was determined at λ\(_{\max}\) using a Personal Densitometer SI (GE Healthcare). The background signal was measured in parallel wells transfected with empty vector and was subtracted from the experimental values obtained from wells containing YFP-NR1-expressing cells. Data are expressed as the mean ± S.E.; each value is an average of at least six different transfection reactions in three independent experiments performed under the same conditions. Statistical comparisons were made using an unpaired \(t\) test.

**RESULTS**

**C1 Cassette in the C terminus of the NR1 Subunit Contains Two ER Retention Motifs**—Previous studies from several laboratories reported that the full-length NR1-1 subunit does not reach the surface membrane when expressed in the absence of...
NR2 subunits (3–5). To identify the mechanism(s) underlying ER retention of the NR1-1 subunit, single transmembrane domain chimeras containing the C termini of NR1 subunits were employed, resulting in the identification of an RRR motif in the C1 cassette of the NR1-1 subunit as a major ER retention signal present on this subunit (Fig. 1A) (5–7). However, in these studies, the role of the RRR motif in ER retention of the full-length NR1-1 subunit was not tested.

In this study, we first investigated whether or not the C1 cassette contains any functional ER retention signal(s) when all of the preceding parts of the NR1 subunit are present. To do this, we constructed YFP-NR1 subunits truncated before (YFP-NR1 C0-stop) and after (YFP-NR1-1a C1-stop) the C1 cassette, expressed them in COS-7 cells (which lack native NMDA receptors), and compared the surface and total expression of these constructs with the wild type YFP-NR1-1a subunit using immunofluorescence and quantitative assay of surface and total expression (both performed with anti-GFP antibodies) (Fig. 1, B and C). As expected, the YFP-NR1-1a C1-stop subunit did not traffic to the cell surface, similar to the YFP-NR1-1a subunit, but the YFP-NR1 C0-stop subunit exhibited robust surface staining. This observation indicates that the C1 cassette contains ER retention signal(s). Next, we asked whether the RRR motif in the C1 cassette is a major ER retention signal in the full-length NR1-1 subunit (Fig. 1A). Surprisingly, substitution of this motif for alanine residues (YFP-NR1-1a RRR/AAA) did not result in surface targeting of the subunit, showing that the RRR motif is not the ER retention motif or the only ER retention motif responsible for the retention of the full-length NR1-1 subunit (Fig. 1, B and C). Because our experiments with deletion constructs suggested that the C1 cassette contains an ER retention signal, we mutated the other putative ER retention motif, KKK, present in

![Figure 2](image-url)

**FIGURE 2.** PDZ interaction but not phosphorylation can negate C1 cassette-mediated ER retention of the full-length NR1 subunit. A, shown is the the amino acid sequence of the C1 cassette with KKK and RRR ER retention motifs (gray) and protein kinase C (PKC; Ser897) and protein kinase A (PKA; Ser897) phosphorylation sites (underlined) demonstrated previously to be able to negate the RRR ER retention motif in the single transmembrane chimeric protein containing the C terminus from the NR1-1 subunit. B, representative COS-7 cells expressing the indicated NR1 constructs were immunostained for surface YFP. Surface and total expression is shown. COS-7 cells expressing the YFP-NR1-1a subunit were incubated with 100 nM phorbol 12-myristate 13-acetate (protein kinase C activator) for 90 min (YFP-NR1-1a + TPA). The YFP-NR1-1a + PDZ subunit was made by replacing the last six amino acid residues in the C terminus from the YFP-NR1-1a subunit with a PDZ-binding domain present in the C2’ cassette (SVSTVV). C, shown is the quantification of surface (black bars) and total (white bars) expression using quantitative colorimetric assay. Plotted data represent the mean ± S.E. (n = 6) in three experiments. **, p < 0.01 relative to the control (YFP-NR1-1a) using a t test.
the C1 cassette (YFP-NR1-1a KKK/AAA) and measured its surface targeting (13, 14). Although this construct tended to go to the surface more than the wild type or YFP-NR1-1a RRR/AAA constructs, we did not see a statistically significant difference compared with the wild type subunit. We then substituted both RRR and KKK motifs in the full-length subunit (YFP-NR1-1a KKK/AAA,RRR/AAA) and expressed it in COS-7 cells (Fig. 1, B and C). A quantitative assay of surface expression revealed significantly higher surface expression of the YFP-NR1-1a KKK/AAA,RRR/AAA construct compared with the wild type YFP-NR1-1a subunit; the total expression of all mutated YFP-NR1 subunits was not significantly different compared with the wild type YFP-NR1-1a subunit. Taken together, our data show that both RRR and KKK motifs work as independent ER retention motifs and are able to retain the full-length NR1-1 subunit even when one of them is not functional.

**PDZ Interaction but Not Phosphorylation Can Negate C1 Cassette-mediated ER Retention**—The C1 cassette contains four serine residues in close proximity to the RRR motif; two of them are known protein kinase C phosphorylation sites (Ser890 and Ser896), and one of them is a protein kinase A phosphorylation site (Ser897) (Fig. 2A) (15, 16). A previous study has shown that incubation of cells with the protein kinase C activator phorbol 12-myristate 13-acetate results in the appearance of the chimeric protein containing the NR1-1 C terminus on the surface membrane and that mutation of Ser896 and Ser897 to alanine residues completely abolishes phorbol 12-myristate 13-acetate-induced surface delivery (Ref. 6; but see also Refs. 5 and 7). A later study reported that the introduction of a negative charge mutation at Ser897 to mimic protein kinase A phosphorylation results in robust surface expression of the chimeric proteins (9). Thus, protein kinase C and protein kinase A phosphorylation at Ser896 and Ser897 could negate the...
RRR ER retention motif in the chimeric protein containing the NR1-1 C terminus. However, these conclusions were not confirmed using full-length NR1-1 subunits. Because we showed that in addition to the RRR motif, there is also a KKK motif responsible for retention of the NR1-1 subunit (Fig. 1), we asked whether the phosphorylation of these serine residues can negate the ER retention of the full-length subunit. First, we made single and double phosphomimic mutations in the full-length YFP-NR1-1a subunit by replacement of Ser<sup>896</sup> and/or Ser<sup>897</sup> with glutamic acid residues (YFP-NR1-1a S896E, YFP-NR1-1a S897E, and YFP-NR1-1a S896E,S897E) and performed experiments to determine their surface targeting (Fig. 2, B and C). In all cases, surface and total expression of these phosphomimic constructs was not significantly different compared the wild type YFP-NR1-1a subunit. Second, we incubated COS-7 cells expressing the YFP-NR1-1a subunit with 100 nM phorbol 12-myristate 13-acetate (protein kinase C activator) for 90 min and then quantified surface and total receptor expression (Fig. 2, B and C). These experiments showed no significant difference compared with control cells. Similarly, we expressed the YFP-NR1-1a subunit in COS-7 cells and tested the effect of a 90-min-long incubation of 20 μM forskolin (protein kinase A activator) on the surface and total expression of NR1 subunits. Our experiments showed no significant difference between control and forskolin-treated cells in the quantification of both surface and total expression (data not shown). Thus, our results show that protein kinase C and/or protein kinase A phosphorylation is not sufficient to negate the ER retention of the full-length NR1-1 subunit.

It has been shown previously using single transmembrane domain chimeric proteins that the far C terminus of the C2′ cassette, which contains a PDZ-binding domain, can negate the C1 cassette-mediated ER retention (5–7). To verify this observation, we replaced the last six amino acid residues in the C terminus of the YFP-NR1-1a subunit with a PDZ-binding domain present in the C2′ cassette (SVSTVV; YFP-NR1-1a+PDZ) and expressed this subunit in COS-7 cells (Fig. 2, B and C). As expected, adding the PDZ-binding domain to the C terminus of the NR1-1a subunit significantly increased surface but not total expression compared with the wild type subunit. This suggests that the PDZ-binding domain can negate both the RRR and KKK ER retention motifs present in the C1 cassette.

**Role of the C0 Cassette in Export of the Full-length Subunit to the Cell Surface**—Our experiments showed that the YFP-NR1 C0-stop subunit exhibits robust surface staining. However, we reported previously that the NR1 subunit truncated five amino acid residues after M4 (YFP-NR1<sub>838stop</sub>) exhibits only slight surface trafficking (10). This indicates that the C0 cassette enhances surface targeting of the full-length NR1 subunit. Next, we examined the surface trafficking of YFP-NR1 constructs truncated at different positions in the C0 cassette (Fig. 3A). These experiments showed that the YFP-NR1<sub>843stop</sub> subunit reaches the cell surface significantly more than the YFP-NR1<sub>838stop</sub> subunit; even more pronounced surface labeling was seen for YFP-NR1<sub>846stop</sub> and YFP-NR1<sub>851stop</sub> subunits (Fig. 3, B and C). Together, our data show that the proximal region of the C0 cassette plays a critical role in the export of the NR1 subunit to the cell surface. To determine whether any specific motif is responsible for the C0 cassette-mediated export, we made four different YFP-NR1<sub>851stop</sub> constructs, each containing three alanine residues replacing the original amino acid residues in the C0 cassette (Fig. 3A). A quantitative assay revealed significantly higher surface expression for all of these constructs compared with the YFP-NR1<sub>838stop</sub> subunit; interestingly, the constructs with replaced RRK and QMK trafficked to the surface more than the other two constructs (Fig. 3, B and C). The total expression of all truncated and mutated YFP-NR1 subunits was not significantly different compared with the YFP-NR1<sub>838stop</sub> subunit. In conclusion, our data show that the presence of the C0 cassette can enhance the surface trafficking of the NR1 subunit and that no particular motif is likely involved in this process.

**Roles of C2 and C2′ Cassettes in Export/Retention of Full-length NR1 Subunits**—As mentioned above, the full-length NR1-1 subunit is retained intracellularly, whereas it has been reported that the other NR1 splice variants traffic to the cell surface to varying degrees (Fig. 4A) (4, 5). To further explore the roles of the C2 and C2′ cassettes in trafficking of the full-length subunits, we decided to compare the surface labeling of all the NR1 splice variants with that of the YFP-NR1 C0-stop construct (Fig. 4, B and C). These experiments showed that the YFP-NR1-2a subunit, containing a C2 cassette, goes to the cell surface significantly less than the YFP-NR1 C0-stop subunit. On the other hand, both YFP-NR1-3a and YFP-NR1-4a, containing the C2′ cassette, trafficked to the cell surface significantly more than the YFP-NR1 C0-stop subunit. Taken together, these results show that the C2′ cassette has an inhibitory effect on the trafficking of the full-length NR1 subunit. In contrast, the C2′ cassette can enhance the surface trafficking of the full-length NR1 subunit in the absence or presence of the C1 cassette.

The mechanism of C2′ export function has been studied previously (8). This study showed that the last two residues (VV) of the far C terminus in the C2′ cassette can bind COPII and that this interaction but not a PDZ interaction is necessary for the robust export of the C2′ cassette-containing proteins to the cell surface. We studied the effect of selective disruptions of PDZ and COPII interactions on the surface targeting of YFP-NR1 constructs. We first deleted the last four amino acid residues in the YFP-NR1-4a subunit (YFP-NR1-4a ΔPDZ) to prevent both PDZ and COPII interactions. Second, we substituted the threonine in the PDZ-binding domain with arginine (TVV → RVV; YFP-NR1-4a RVV) so that the COPII interaction with the C2′ cassette remained intact, whereas the PDZ interaction was dis-
rupted (Fig. 5A). After the expression of these constructs in the COS-7 cells, we compared their surface expression with wild type YFP-NR1-4a and YFP-NR1 C0-stop subunits (Fig. 5, B and C). Indeed, the YFP-NR1-4a ΔPDZ construct trafficked significantly less to the cell surface than the YFP-NR1-4a construct, showing that disruption of both PDZ and COPII interactions decreases export function of the C2′ cassette. Interestingly, the YFP-NR1-4a RVV construct exhibited significantly less surface expression compared with the wild type YFP-NR1-4a, similar to the YFP-NR1-4a ΔPDZ construct. These data show that the intact PDZ interaction but not the COPII interaction can enhance the surface trafficking of the NR1-4 subunit. To further support this conclusion, we generated the YFP-NR1-4a construct with valine 3 replaced with alanine (TVV) so that a type 1 PDZ-binding domain (TXXV) is fully functional; however, a COPII interaction is disrupted. Indeed, surface and total expression of this subunit was not significantly different compared with the wild type YFP-NR1-4a subunit having an intact COPII interaction (data not shown). In addition, compared with the YFP-NR1 C0-stop construct, surface labeling of both YFP-NR1-4a ΔPDZ and YFP-NR1-4a RVV constructs was significantly higher, showing that the C2′ cassette has an export effect even when the PDZ-binding domain is not functional.

To further verify that the C2′ cassette has an enhancing effect on the trafficking of the NR1 subunit even in the absence of the functional PDZ-binding domain, we made YFP-NR1-3a ΔPDZ (with both the PDZ and COPII interactions disrupted) and YFP-NR1-3a RVV (with the PDZ interaction disrupted) constructs and quantified their surface expression.
trafficking together with wild type YFP-NR1-3a and YFP-NR1-1a subunits (Fig. 6). As expected, surface trafficking of both YFP-NR1-3a ΔPDZ and YFP-NR1-3a RVV subunits was significantly diminished compared with the YFP-NR1-3a subunit, confirming the conclusion that an intact PDZ interaction is necessary for the robust surface delivery of NR1 subunits. Unexpectedly, both YFP-NR1-3a ΔPDZ and YFP-NR1-3a RVV subunits trafficked to the cell surface significantly more than the YFP-NR1-1a subunit. This observation shows that even when the PDZ-binding domain is not functional, the C2’ cassette can negate the C1 cassette-mediated ER retention. In conclusion, our data support a model in which both C1 and C2 cassettes have an inhibitory effect, whereas both C0 and C2’ cassettes have an enhancing effect on the surface trafficking of the full-length NR1 subunits.

DISCUSSION

The number and composition of NMDA receptors present at surface membranes are highly regulated. Although the biophysical properties of the NMDA receptors on the surface were extensively studied, NMDA receptors must be first assembled in the ER, modified in the Golgi apparatus, and then sorted in the trans-Golgi network. However, these processes remain largely unexplored. In this study, we have focused on the characterization of the mechanisms governing the trafficking of the NR1 subunits to the cell surface. We found that two different ER retention motifs present in the C1 cassette, KKK and RRR, are responsible for the ER retention of the full-length NR1 subunits. This ER retention can be overcome by the presence of the C2’ cassette even when the PDZ-binding domain in the far C terminus of this cassette is not functional. We further demonstrated that the C0 cassette has an enhancing effect and that the C2 cassette has an inhibitory effect on the surface targeting of the full-length NR1 subunits.

ER Retention of Full-length NR1 Subunits Mediated by the C1 Cassette—Previous studies identified an RRR motif as a major ER retention site for the C1 cassette-mediated ER retention of the single transmembrane domain chimeric proteins with an appended C-terminal region from the NR1-1 subunit (5–7). Our finding that there are two independent ER retention motifs in the C1 cassette, KKK and RRR, shows that the trafficking of the full-length NR1 subunits is likely regulated somewhat different than the trafficking of the chimeric proteins. It also suggests that a more complex regulation than previously thought controls the ER retention of the NR1 subunits because the M3 domain of the NR1 subunit contains an additional ER retention signal (10). We cannot rule out the possibility that the C2 cassette, which plays an inhibitory role in the trafficking of NR1 subunits, plays an additional role in the C1 cassette-mediated ER retention of NR1 subunits. A recent paper has shown that the replacement of the RRR ER retention motif with three alanines is sufficient for a slight release of the full-length NR1-3 subunits to the cell surface of HEK-293 cells (17), which is not in agreement with our results. A similar mechanism of ER retention employing multiple ER retention motifs has been proposed for the kainate receptor subunit KA2 (18). The phosphorylation of serine residues adjacent to the RRR ER retention motif can suppress ER retention of single transmembrane chimeric proteins containing the C terminus of the NR1-1 subunit (6, 7, 9). Here, we have shown that protein kinase A/protein kinase C activation and replacement of Ser896 and/or Ser897 with phosphomimic residues do not negate the C1 cassette-mediated ER retention of the full-length subunit. This observation, supported by our previous study (5), is consistent with our finding that two ER retention motifs, KKK and RRR, are present in the C1 cassette, and thus, both signals must be masked before the subunit can leave the ER. Interestingly, the presence of a PDZ-binding domain in the C terminus of the NR1-1 subunit was able to negate both ER retention motifs in the C1 cassette and release the full-length subunit to the cell surface. Indeed, this finding is in agreement with the observation showing that the full-length NR1-3 subunit reaches the cell surface and indicates that other cassettes in the C terminus of the NR1 subunit play a
regulatory role in the surface trafficking of the NR1 subunits to the cell surface (4, 5).

Role of Other C-terminal Cassettes in Regulation of Surface Delivery of NR1 Subunits—We reported previously that the NR1 subunit truncated five amino acid residues after M4 is slightly transported to the cell surface (10). Surprisingly, when we truncated the NR1 subunit immediately after the C0 cassette, robust surface labeling was seen, indicating that the C0 cassette contains an export signal. The serial truncation and replacement of amino acid residues in the proximal region of the C0 cassette revealed that no specific motif is likely responsible for the export of the full-length subunit to the cell surface, although some significant changes in the surface delivery of these constructs were observed. These findings support the idea that the presence of the C0 cassette can negate the ER retention of the NR1 subunit mediated through the M3 domain. One possible mechanism is that C0 regulates the structure of the M4 domain and other surrounding regions critical for the release of the full-length NR1 subunit from the ER (10).

Interestingly, a 28-amino acid segment following M4 in the GluR62 subunit and a shorter segment immediately following M4 of the NR2B subunit have been shown to be critical for the release of receptors from the ER to the cell surface (19, 20).

We tested the effect of the C2 cassette on the trafficking of the NR1 subunits to the cell surface. Our experiments showed that the C2 cassette has an inhibitory effect on the surface targeting of the full-length NR1-2 subunit, which is consistent with previous observations showing that the NR1-2 subunit traffics less to the cell surface than the NR1-4 subunit and that the C2 cassette slows the ER export of the chimeric protein containing this cassette (4, 8). Finally, we studied the role of the C2’ cassette in the surface targeting of NR1 subunits. Previous data showed that the chimeric protein with an appended C terminus from the NR1-3 subunit reaches the cell surface but that deletion of the PDZ-binding domain from the C2’ cassette results in the ER retention of the chimeric protein (5, 6). Thus, the current conclusion is that the PDZ-binding domain can negate the RRR ER retention motif in the C1 cassette. Furthermore, it has been reported that an intact COPII but not PDZ interaction is required for the accelerated C2’-mediated protein trafficking from the ER (8). Because these conclusions have not been directly tested using full-length subunits, we asked the following questions. 1) Are intact PDZ and/or COPII interactions required for enhancement of the surface trafficking of the NR1-4 subunit mediated by the C2’ cassette? 2) Are intact PDZ and/or COPII interactions required for regulating the C1 cassette-mediated ER retention in the NR1-3 subunit? Our experiments showed that the disruption of PDZ and/or COPII interactions decreased the surface trafficking of both the NR1-4 and the NR1-3 subunits (Figs. 5 and 6). These results do not support the conclusion that a COPII interaction can enhance forward trafficking of the NR1 subunits, obtained using chimeric proteins (8). A PDZ interaction is likely mediated by the PSD-95 family proteins, which have been shown to be able to interact with the NR1-4 subunit in heterologous cells (5). At least one of the members of this family, SAP102, is endogenously expressed in COS-7 cells (21). Interestingly, compared with the NR1 subunit truncated after the C0 cassette or with the NR1-1 subunit, the NR1-4 and the NR1-3 subunits lacking PDZ/COPII domains reached the cell surface significantly more. Together, our findings show that the PDZ interaction but not COPII protein interaction can enhance the trafficking of the C2’ cassette-containing NR1 subunits to the cell surface and negate the ER retention mediated by the C1 cassette. In addition, our data indicate that the region of the C2’ cassette preceding the PDZ-binding domain contains another domain that is able to accelerate the surface trafficking of the NR1 subunit and negate the C1 cassette-mediated ER retention of NR1 subunits but to a lesser extent than the PDZ-binding domain.

Physiological Implications—Our results show that each cassette in the C terminus of NR1 subunits plays a unique role in the trafficking of the full-length subunit to the cell surface. What are the physiological implications of our findings? Given that NR1 subunits are expressed in stoichiometric excess in the central nervous system and have rapid turnover rates in cells, the precise timing of ER retention and trafficking of NR1 subunits within different compartments regulated by C-terminal cassettes can be a crucial step in the assembly of functional NMDA receptors (22). This view is supported by the observation that the NR2A and NR2B subunits associate differently with specific NR1 splice variants in the central nervous system (23) and that the C-terminal splicing of NR1 subunits regulates the number of surface functional NMDA receptors (4). Our preliminary results show that the disruption of both KKK and RRR ER retention motifs in the C1 cassette does not affect the surface targeting of NR1/NR2B receptors in heterologous cells. This observation shows that the NR1 C-terminal regulatory mechanisms can be negated after the assembly of NR1/NR2 receptors. Another interesting aspect of having different NR1 splice variants expressed in the central nervous system is that each variant can interact with a different combination of binding partners and that these interactions can tightly regulate intracellular trafficking of the NR1 subunits. For example, the C1 cassette can directly interact with calmodulin, yoatio, and neurofilament L-protein (24–26), and phosphorylation of the C1 cassette can regulate association with calmodulin (27). Indeed, both ER retention motifs present in the C1 cassette, KKK and RRR, can likely interact with different binding partners so that precise localization of the NR1 subunit can be regulated under various conditions. Finally, the importance of understanding the mechanisms underlying the trafficking of the NR1 variants is emphasized by observations that the C1 cassette regulates NMDA receptor-induced gene expression (28) and that the presence of the C2 but not the C2’ cassette is necessary for the growth and stabilization of dendritic spines (29).

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REFERENCES
1. Cull-Candy, S., Brickley, S., and Farrant, M. (2001) Curr. Opin. Neurobiol. 11, 327–335
2. Lau, C. G., and Zukin, R. S. (2007) Nat. Rev. Neurosci. 8, 413–426
3. McIlhinney, R. A., Le Bourdelle, B., Molnar, E., Tricaud, N., Streit, P., and Whiting, P. J. (1998) Neuropharmacology 37, 1355–1367
4. Okabe, S., Miwa, A., and Okado, H. (1999) *J. Neurosci.* 19, 7781–7792
5. Standley, S., Roche, K. W., McCallum, J., Sans, N., and Wenthold, R. J. (2000) *Neuron* 28, 887–898
6. Scott, D. B., Blanpied, T. A., Swanson, G. T., Zhang, C., and Ehlers, M. D. (2001) *J. Neurosci.* 21, 3063–3072
7. Xia, H., Hornby, Z. D., and Malenka, R. C. (2001) *Neuropharmacology* 41, 714–723
8. Mu, Y., Otsuka, T., Horton, A. C., Scott, D. B., and Ehlers, M. D. (2003) *Neuron* 40, 581–594
9. Scott, D. B., Blanpied, T. A., and Ehlers, M. D. (2003) *Neuropharmacology* 45, 755–767
10. Horak, M., Chang, K., and Wenthold, R. J. (2008) *J. Neurosci.* 28, 3500–3509
11. Prybylowski, K., Fu, Z., Losi, G., Hawkins, L. M., Luo, J., Chang, K., Wenthold, R. J., and Vicini, S. (2002) *J. Neurosci.* 22, 8902–8910
12. Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., Akazawa, C., Shigemoto, R., Mizuno, N., and Masu, M. (1993) *J. Biol. Chem.* 268, 2836–2843
13. Jackson, M. R., Nilsson, T., and Peterson, P. A. (1990) *EMBO J.* 9, 3153–3162
14. Nilsson, T., Jackson, M., and Peterson, P. A. (1989) *Cell* 58, 707–718
15. Tingley, W. G., Ehlers, M. D., Kameyama, K., Doherty, C., Ptak, J. B., Riley, C. T., and Huganir, R. L. (1997) *J. Biol. Chem.* 272, 5157–5166
16. Tingley, W. G., Roche, K. W., Thompson, A. K., and Huganir, R. L. (1993) *Nature* 364, 70–73
17. Kenny, A. V., Cousins, S. L., Pinho, L., and Stephenson, F. A. (2009) *J. Biol. Chem.* 284, 324–333
18. Nasu-Nishimura, Y., Hurtado, D., Braud, S., Tang, T. T., Isaac, J. T., and Roche, K. W. (2006) *J. Neurosci.* 26, 7014–7021
19. Hawkins, L. M., Prybylowski, K., Chang, K., Moussan, C., Stephenson, F. A., and Wenthold, R. J. (2004) *J. Biol. Chem.* 279, 28903–28910
20. Matsuda, I., and Mishina, M. (2000) *Biochem. Biophys. Res. Commun.* 275, 565–571
21. Davey, F., Hill, M., Falk, J., Sans, N., and Gunn-Moore, F. J. (2005) *J. Neurochem.* 94, 1243–1253
22. Huh, K. H., and Wenthold, R. J. (1999) *J. Biol. Chem.* 274, 151–157
23. Sheng, M., Cummings, J., Roldan, L. A., Jan, Y. N., and Jan, L. Y. (1994) *Nature* 368, 144–147
24. Ehlers, M. D., Fung, E. T., O’Brien, R. J., and Huganir, R. L. (1998) *J. Neurosci.* 18, 720–730
25. Ehlers, M. D., Zhang, S., Bernhardt, J. P., and Huganir, R. L. (1996) *Cell* 84, 745–755
26. Lin, J. W., Wyszynski, M., Madhavan, R., Sealock, R., Kim, J. U., and Sheng, M. (1998) *J. Neurosci.* 18, 2017–2027
27. Hisatsune, C., Umemori, H., Inoue, T., Michikawa, T., Kohda, K., Miki-shiba, K., and Yamamoto, T. (1997) *J. Biol. Chem.* 272, 20805–20810
28. Bradley, J., Carter, S. R., Rao, V. R., Wang, J., and Finkbeiner, S. (2006) *Science* 310, 1065–1076
29. Alvarez, V. A., Ridenour, D. A., and Sabatini, B. L. (2007) *J. Neurosci.* 27, 7365–7376