The intracellular C-terminal domain of the N-methyl-D-aspartate receptor (NMDAR) subunits 1 (NR1) and 2 (NR2) are important, if not essential, to the process of NMDAR clustering and anchoring at the plasma membrane and the synapse. Eight NR1 splice variants exist, four of which arise from alternative splicing of the C-terminal exon cassettes. Alternative splice variants may display a differential ability to interact with synaptic anchoring proteins, and splicing of C-terminal exon cassettes may alter the mechanism(s) of subcellular localization and targeting. The NR1-4 isoform has a significantly different C-terminal composition than the prototypic NR1-1 isoform. Whereas the NR1-1 C terminus is composed of C0, C1, and C2 exon cassettes, the NR1-4 C terminus is composed of the C0 and C2' cassettes. In the present study, we address the importance of the NR1-4 C-terminal exon cassettes (C0C2') in subcellular localization in differentiated pheochromocytoma (PC12) cells, in organotypic cultures of dorsal root ganglia, and also in heterologous cells. NR1-4-green fluorescent protein chimeras were created with deletion of either C0, C2', or both cassettes to address their importance in subcellular distribution and cell surface expression of the NR1-4 subunit. These experiments demonstrate that the NR1-4 splice variant found predominantly in the spinal cord uses the C0 cassette, to a large degree, to organize the subcellular distribution of this receptor subunit. Although the role of the C2' subunit is less clear, it may be involved in subunit clustering. However, this clustering is not always as efficient as that attributed to C0 alone or to the natural combination of C0C2'. Finally, although an intact C-terminal domain is necessary for interaction with the NR2A subunit nor surface expression of the NR1-4 subunit, the C-terminal domain fragment alone blocks surface expression of native NR1-4, in a dominant negative fashion, when the two are coexpressed.

Glutamate, the major excitatory neurotransmitter of the central nervous system, acts upon the N-methyl-D-aspartate receptor (NMDAR), a member of the ionotropic class of glutamate receptors (1). Functional NMDARs are heteromers composed of the two subunits NR1 and NR2 (2, 3). A third NMDAR subunit, NR3, has been described, but its biological significance is only just beginning to be investigated (4, 5). Although much is known about the physiological role that NMDARs play in long-term potentiation, learning, and memory (6, 7), much remains to be learned about the mechanisms by which these receptors are sorted, targeted, and anchored, both at the synapse and at extra-synaptic sites. To date, considerable research has focused on NMDAR C-terminal domain interactions, specifically those interactions between the NR2 subunit C-terminal threonine/serine, any amino acid, and valine (T/S)XV motif and PDZ domain-containing proteins located at the synapse (8–10). PDZ domains contain an 80–90-amino acid sequence that forms the binding site for proteins containing the C-terminal (T/S)XV motif (11). The post-synaptic density 95 (PSD-95) family of proteins contains 1–3 PDZ domains (12–14). Whereas most data implicate PSD-95 as the major protein that facilitates clustering of NMDAR on the post-synaptic density, a growing body of evidence supports the importance of cytoskeletal elements in the localization of this receptor at the synapse. The NR1 subunit has been shown to interact directly with the cytoskeletal proteins neurofilament (15) and tubulin (16). Furthermore, the NR1 C terminus may be cross-linked to the actin cytoskeleton via interaction with α-actinin-2, a member of the spectrin/dystrophin family of actin-binding proteins (17).

Although these findings only begin to explain how the NMDAR is clustered and anchored at the synaptic membrane, they do suggest that the C terminus is important, if not essential, to this process. Current research in this area has focused largely on the NR2 subunits and the NR1 subunit isoform, NR1-1. Eight NR1 splice variants exist, four of which arise from alternative splicing of C-terminal exon cassettes (18). Given the general consensus that the NR1 C terminus makes important cytoskeletal contacts, different splice variants might display a differential ability to interact with anchoring proteins. Furthermore, splicing of C-terminal exon cassettes may alter the mechanism(s) of subcellular localization and targeting.
used by NR1 isoforms. The NR1–4 splice variant has a significantly different C-terminal composition than the prototypic NR1-1 splice variant. The NR1-1 C terminus is composed of C0, C1, and C2 exon cassettes, and in contrast, the NR1–4 C terminus is composed of C0 and the alternatively spliced C2' cassette (18). A potentially important difference between the two variants lies within their extreme C termini as follows: the C1 and C2 cassettes are absent from NR1–4 and an amino acid frameshift following alternative splicing gives rise to the NR1–4 C2' cassette that terminates in a (T/S)5XV motif that is entirely absent from NR1-1 C termini (18). The presence of a (T/S)5XV motif in the NR1–4 C terminus suggests a potential for this subunit to interact directly with PDZ-containing proteins such as those belonging to the PSD-95 family.

In the present study we address the importance of the NR1–4 C-terminal exon cassettes (C0 and C2') in subcellular localization in differentiated PC12 cells and organotypic cultures of dorsal root ganglia (DRG). NR1–4–GFP chimeras were created with deletion of either C0, C2', or both cassettes to address their importance in subcellular distribution and cell surface expression of the NR1–4 subunit.

MATERIALS AND METHODS

Cell Culture and Transfection—Routine culturing and differentiation of PC12 cells (obtained from Dr. R. A. Bradshaw, University of California, Irvine, CA) with 50 ng/ml nerve growth factor (NGF) have been described previously (19). Differentiation of PC12 cells with NGF for all the reported experiments was allowed to proceed for at least 5 days before experimental use. HEK293 cells and COS7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 1% l-glutamine, and 0.1% penicillin/streptomycin in a 5% CO2 humidified atmosphere (all culture reagents were purchased from Invitrogen). Purified plasmid DNA (1 μg/well, prepared with a Qiagen Plasmid Maxi Kit (Mississauga, Ontario, Canada) and LipofectAMINE (10 μl/well; Invitrogen) were used to transfect differentiated PC12, HEK293, and COS7 cells as described in the manufacturer's instructions. After transfecting the cells for 5 h, 1 ml of Dulbecco’s modified Eagle’s medium was added to each well. After an additional 24 h at 37 °C, the media were removed from each well, and fresh media were added. A luciferase reporter plasmid (10 ng/mg of luciferase per well, provided by Dr. C. A. Strathdee, Robert's Research Institute, London, Ontario, Canada) was used to monitor transfection efficiency. Organotypic cultures were made from the DRG of postnatal day 7–9 Wistar rats as described previously (20). Marsh et al. (20) also describe the biologic gene transfer to organotypic cultures.

Stable Cell Line Production—HEK293 cells were transfected using LipofectAMINE (Invitrogen) and the plasmids pNRIC0C2/GFP or pNR1GPFCC0C2' in Opti-MEM media (Invitrogen) for 24 h. The transfection media were replaced with complete medium for a further 48 h. The cells were then passaged and seeded at single cell density into 60-mm tissue culture plates (Nunc) containing complete medium supplemented with 800 μg/ml geneticin (Invitrogen). Transgene expression and clone homogeneity were assessed using DNA sequencing to ensure that all fusion proteins were in frame and of the corresponding sequence. Vectors pNF-2 (NR1-1GFP, a generous gift from Dr. T. E. Hughes, Yale University School of Medicine, New Haven, CT) and pNR1–4, pNR2A, and pNR2D (generous gifts from Dr. S. Nakanishi, Kyoto University, Kyoto, Japan) were used as PCR templates. The NR2D and NR2A cDNAs were excised at sites digested at dilutions of 1:500, 1:1000, 1:2000, 1:4000, and 1:8000. Colony PCR was performed using the trans-blot SD semi-dry transfer cell (Bio-Rad) and the enhanced chemiluminescence (ECL) method according to the manufacturer's instructions (Amersham Biosciences). The same electrophoresis and immunoblotting conditions were applied for the analysis of biotinylated cell surface proteins precipitated with streptavidin beads.

Immunocytochemistry—For cell surface labeling of NR1–4, a mouse monoclonal anti-HA epitope antibody (clone 12CA5, Roche Molecular Biochemicals) was added to fixed, nonpermeabilized cells at a concentration of 1:2000. For colocalization of NR1–4–GFP with NR2A, rabbit polyclonal anti-NR2A/B antibody (Chemicon, Temecula, CA) was added at a concentration of 1:500. Following incubation at room temperature for 48 h, cells were washed twice with PBS and then incubated with biotin-conjugated donkey anti-mouse secondary antibody or biotin-conjugated donkey anti-rabbit secondary antibody (for anti-HA and anti-NR2A labeling, respectively, Jackson ImmunoResearch, Mississauga, Ontario, Canada) for 16 h, washed three times with PBS, and then incubated with rhodamine-streptavidin (Jackson ImmunoResearch) for 3 h. Cells were washed two more times with PBS and then stored at 4 °C until analyzed by confocal microscopy.

Immunofluorescence Microscopy—At 48 h post-transfection, fixed or live cells were imaged using the Zeiss LSM510 or Bio-Rad Radiance 2000 MP laser scanning confocal microscopes. Images were processed using LSM510 (Carl Zeiss Inc., North York, Ontario, Canada) or LaserSharp 2000 software (Bio-Rad), Adobe Photoshop 5.02 (Adobe Systems Inc., San Jose, CA), and Corel Draw 8 (Corel Inc., Kanata, Ontario, Canada).

Quantitation—To quantify clustering of NR1–4–GFP fusion proteins in differentiated PC12 cells, 10–15 neurites from transfected cells (from two separate transfections) were chosen randomly for image acquisition. Images were processed using Image Pro Plus (Media Cybernetics, Inc. Silver Spring, MD) to count clusters per neurite length. Gamma and contrast values were adjusted prior to counting to reduce background such that the same setting was applied to each image. Neurite length of ~40 μm was analyzed per image such that, for each construct, a total neurite length of 350–400 μm was assessed.

Biotinylation and Immunoprecipitation of Cell Surface Proteins—At 48 h post-transfection, HEK293 cells cultured in 100-mm plates were washed three times with ice-cold PBS, and surface proteins were biotinylated using 0.5 mg/ml Sulfo-NHS-LC-LC-Biotin (Pierce) in PBS for 30 min at 4 °C (21). Cells were washed once with cold PBS and then with 0.1% glycine/PBS for 10 min at 4 °C. Cells were washed two more times with cold PBS and then harvested using 0.5 ml of Nonidet P-40 lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.02% sodium azide) and then incubated with streptavidin for 2 h at 4 °C. Samples were then centrifuged 3 min at 2500 × g. This process was repeated one additional time. Pellets were washed four times in precipitation buffer and boiled in SDS sample buffer subjected to electrophoresis as described below.

Electrophoresis and Immunoblotting—At 48 h post-transfection, PC12 cells were harvested and lysed in a commercial cell culture lysis reagent (Promega, Madison, WI). Protein concentrations for each sample were then determined in triplicate using the Bradford assay (Bio-Rad). Proteins were separated by 12% SDS-PAGE, containing 8 μl urea, as described previously (22). Proteins were transferred to nitrocellulose (Amersham Biosciences) using the trans-blot SD semi-dry transfer cell (Bio-Rad). Immunoblotting was performed using the enhanced chemiluminescence (ECL) method according to the manufacturer's instructions (Amersham Biosciences). The same electrophoresis and immunoblotting conditions were applied for the analysis of biotinylated cell surface proteins precipitated with streptavidin beads.

The anti-NMDA R1 mouse monoclonal antibody (mAb) (clone 54.1, PharMingen, Mississauga, Ontario, Canada) that recognizes an epitope between amino acids 660 and 811 of the NR1-1 protein was used at a dilution of 1:500. Rabbit polyclonal anti-GFP antibody (Chemicon, Temecula, CA), rabbit polyclonal anti-NMDA R2A/B (Chemicon, Temecula, CA), goat polyclonal anti-NR2D (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA), mouse monoclonal anti-P-285 (PharMingen) antibody were used at a dilution of 1:5000.

Protein Half-life Determination—NR1CC0C2/GFP and NR1GPFCC0C2' stable cell lines were seeded into 6-well plates (Nunc) at 200,000 cells well−1. Additionally, 293 cells were seeded at 200,000 cells well−1 and...
transiently transfected with pNR1C0C2-GFP or pNR1–4. Transient transfections were supplemented with 10 ng of a plasmid encoding firefly luciferase under the regulation of the cytomegalovirus immediate early promoter. Cycloheximide (Calbiochem) was resuspended in PBS and applied to the wells to give a final concentration of 100 μg/ml. Cells were harvested in Reporter Lysis Buffer (Promega), and NR1 protein levels were assessed using SDS-PAGE and then Western-blotted with an α-NR1 antibody (PharMingen). Densitometry readings were derived from the resultant blots using a Bio-Rad GS700 Imaging Densitometer. Protein decay values were fitted with exponential regression curves, and the half-life was calculated as follows: $T_{1/2} = 0.693/k$, where $Y = span (t) + plateau$.

Statistical Analysis—Where applicable, data are presented as means ± S.E. Statistical analysis was performed using the one-way analysis of variance and Tukey's multiple comparison test, using GraphPad Prism (GraphPad Software, Inc. San Diego, CA).

RESULTS

To characterize the importance of the NR1–4 C terminus in NMDAR localization, NR1–4-GFP fusion chimeras were made with deletions in their C-terminal domains, C0 and/or C2 (Fig. 1). GFP was fused, in frame, to the extreme C terminus of NR1–4 or immediately following the fourth transmembrane domain, producing fluorescent NR1–4 receptor subunit proteins.

Immunoblot Analysis of NR1–4–GFP Fusions—To determine whether each NR1–4–GFP chimera expressed a protein of the predicted molecular weight, PC12 cells were transfected, harvested 48 h later, and immunoblotted using an anti-NR1 monoclonal antibody. Immunoblotting confirmed the presence and predicted molecular weight of each NR1–4–GFP chimera and HANR1–4–GFP chimera (not shown), as well as endogenous NR1 (Fig. 2A). Other NMDAR protein complex members, NR2A/B, NR2D, PSD-95, and α-actinin-2, were also detected in the PC12 cells used in our experiments (Fig. 2B). Fig. 2C demonstrates that the NR1–4 C-terminal domain fused to GFP was also the correct size when immunoblotted with anti-GFP antibody. Densitometry was used to quantify the level of NR1–4–GFP expression, after normalization for protein content. Transfection efficiency was normalized by measuring luciferase activity from a cotransfected luciferase reporter plasmid (n = 3, Fig. 2D). Expression levels for each chimera, relative to luciferase activity, were similar with the exception of NR1GFPC0C2 and NGFP that consistently exhibited 2-fold lower expression. The inclusion of the HA epitope tag at the N-terminal splice site did not affect expression levels (data not shown).

To verify that the fusion of GFP to NR1–4 did not destabilize the resulting protein, we treated HEK293 cells that stably expressed NR1C0C2-GFP and NR1GFPC0C2 with 100 μg/ml cycloheximide for varying times and analyzed the resultant protein levels via SDS-PAGE and then Western blot with NR1 antibody (PharMingen). NR1C0C2-GFP and NR1–4 proteins were similarly analyzed in transiently transfected 293 cells. The decay kinetics of these chimeras, with a representative blot, are displayed together in Fig. 2E. Both stably and transiently transfected NR1C0C2-GFP exhibited similar decay kinetics, although transient expression greatly increased the variability of the assay. Stably expressed NR1C0C2-GFP decayed with a half-life of 1.58 h ($r^2 = 0.9776$), whereas transiently expressed NR1C0C2-GFP decayed with a half-life of 2.08 h ($r^2 = 0.5811$). In contrast, stably expressed NR1GFPC0C2 decayed with a half-life of only 0.42 h ($r^2 = 0.9811$). Transiently expressed NR1–4 decayed with a half-life of 1.097 h ($r^2 = 0.6274$) that was intermediate between the two enhanced GFP fusion variants. Similar relationships were obtained using flow cytometry to characterize the decay kinetics of the fused enhanced GFP moiety (data not shown).

Confocal Microscopy Analysis of Subcellular Distribution of NR1–4–GFP Proteins—The subcellular distribution of each NR1–4–GFP chimera in transiently transfected, NGF-differentiated PC12 cells was examined by confocal microscopy 48 h post-transfection (Fig. 3). A full-length NR1–4–GFP chimera, NR1C0C2-GFP (with contiguous intracytoplasmic C termini), formed distinct receptor-rich clusters throughout cell bodies and neurites (Fig. 3, A and A’), respectively. A chimera with the (T/S)ixV restored at the end of the GFP C terminus of NR1C0C2-GFP yielded the same result (data not shown). This was comparable with the distribution patterns of endogenous NR1-1, as shown by immunocytochemistry for native NR1, suggesting that a GFP-tagged receptor behaves like an endogenous NR1 receptor (Fig. 3E). In the absence of C2’ or C0 cassettes (Fig. 3, B and C, respectively), chimeras tagged with GFP at their C terminus had no significant change in distribution, occurring in clusters throughout cell bodies as in Fig. 3A. However, the number of clusters typically observed in cell neurites expressing NR1C2’-GFP was decreased in comparison to cluster numbers in cells expressing NR1C0C2-GFP or NR1C0GFP, especially in neurites (compare Fig. 3, A’–C’). Cells expressing a fully truncated NR1–4–GFP chimera, lacking both C0 and C2’ cassettes, NGFP, lacked obvious clusters, and the chimera was typically distributed diffusely throughout cell bodies and neurites (Fig. 3, D and D’). We also quantified the effect of C-terminal deletion on formation of NR1–4–GFP fusion protein clusters in PC12 neurites (Fig. 3G). NR1C0C2-GFP and NR1C0GFP both displayed ~4–5 clusters per 10-μm length of neurite, whereas both NR1C2’-GFP and NGFP showed significantly reduced cluster number (n = 10). The distribution of GFP alone in PC12 cells is shown in Fig. 3F and was observed to be uniformly distributed in the cell body and in neurites as described previously (24). Cells expressing NR1–4-GFP chimeras with GFP fused immediately following TM4 typically showed very low fluorescence levels (data not shown). This occurred despite containing similar levels of protein expression as the NR1–4–GFP chimeras that had GFP fused at the extreme C termini (Fig. 2). This could be attributed to improper protein folding resulting from the placement of GFP between contiguous protein domains rather than at the free C terminus where protein folding would be less hindered. Furthermore, unlike NR1–4 chimeras with an intact TM4–C0 structure, the anti-NR1 54.1 mAb failed to bind to its epitope located in the extracellular loop between TM3 and TM4. Thus, NR1–4–GFP chimeras with GFP fused following TM4 were largely omitted from subsequent studies due to difficulty in analyzing cells with low fluorescence, the potential for altered confirmation in the TM3 and TM4 regions, and the lack of stability of these chimeras compared with chimeras with GFP fused to the extreme C termini.

To examine the distribution of NR1–4 deletion mutants in a more natural neuronal environment, C-terminally tagged NR1–4–GFP chimeras were also expressed in DRG explant cultures of sensory neurons following biolistic delivery of expression vectors. Similar to the distribution profiles exhibited in PC12 cells, NR1C0C2-GFP and NR1C0GFP formed discrete, receptor-rich clusters throughout cell bodies (Fig. 4, A and B) and neurites (Fig. 4, A’ and B’). This distribution was also very similar to the pattern of endogenously stained NMDAR in the DRG (Fig. 4D). In comparison to NR1C0C2-GFP and NR1C0GFP, NR1C2’-GFP typically displayed greatly reduced numbers of clusters in the cell body and in the neurites of DRG neurons (Fig. 4, C and C’). This pattern resembled that found in PC12 cells although the reduced clustering observed with NR1C2’-GFP appeared to be more dramatic. Finally, like in...
PC12 cells, the NGFP chimera that lacked both C0 and C2' cassettes was distributed uniformly throughout the cytoplasm with no obvious receptor clustering and was absent in neurites (Fig. 4E). Together the results from expression in PC12 cells and DRG suggest that the C0 domain is important in NR1–4 cluster formation. Those fusion proteins that lack the C0 domain display a greatly reduced ability to form clusters. Distributions of each chimera in heterologous cells such as HEK293
(see Fig. 6C), COS7, and RK13 cells (not shown) were similar to those observed in PC12 cells and DRG neurons.

**Analysis of Cell Surface Expression of NR1–4-GFP Chimeras**—To determine whether C-terminal deletion alters cell surface expression of NR1–4, PC12 cells or COS7 cells (data not shown) were transfected with NR1–4-GFP chimeras containing N-terminal (extracellular) HA tags and then fluorescently labeled using anti-HA antibody under nonpermeabilizing conditions (Fig. 5). The full-length HANR1C0C2/H11032GFP was localized at the cell surface in both PC12 cells (Fig. 5, A–C) and COS7 cells (not shown). Removal of C2’ (HANR1C0GFP, Fig. 5, D–F), C0 (HANR1C2’GFP, Fig. 5, G–I), or both (HANGFP, Fig. 5, J–L) had no influence on detection of these chimeras at the cell surface. Each chimera also displayed anti-HA immunoreactivity in neurites of differentiated PC12 cells (not shown).

Similar results using the mAb 54.1 to the extracellular loop between TM3 and 4 of NR1 were obtained with the similar chimeras lacking the HA epitope tag.

The microscopic observations of cell surface expression of NR1–4-GFP chimeras were confirmed by cell surface biotinylation of transfected HEK293 cells, followed by surface protein isolation with immobilized streptavidin. Surface and intracellular pools were then immunoblotted using anti-NR1 antibody (Fig. 6A). To determine whether an NR2 subunit was necessary for this localization at the membrane surface, the biotinylation experiment was performed in the presence or absence of NR2D or NR2A. The chimeras were found on the membrane fraction equally well in the presence or absence of coexpressed NR2D or NR2A. In agreement with cell surface labeling by anti-HA antibody, each NR1–4 chimera, regardless of C-terminal deletion, was detected at the cell surface in the streptavidin-isolated surface membrane fractions.
Together, these findings suggest that surface expression of NR1–4 can occur not only in the absence of a coexpressed NR2 subunit but also in the absence of an intact intracellular C-terminal domain. Exclusive biotinylation of cell surface protein was verified by showing that the cytoplasmic protein tubulin was not biotinylated, in each of the corresponding intracellular pools (Fig. 6B). Transfected HEK293 cells (Fig. 6C) expressed each chimera with similar fluorescent intensity, and distributions were similar to that seen in PC12 cells and DRG (Figs. 3 and 4, respectively). Specifically, NR1C0C2GFP and NR1C0GFP were distributed in discrete clusters, whereas NR1C2GFP and NGFP were not.

**Disruption of NR1–4 Surface Expression by Overexpression of NR1–4 C-terminal Fragments—**PDZ-containing proteins, such as the PSD-95 family, have been suggested to play a role in the trafficking of NR1 and NR2 subunits to the plasma membrane (25–27). To determine whether the NR1–4 C terminus is involved in trafficking of the subunit to the cell surface, we cotransfected PC12 cells and COS7 cells (Fig. 7 and 8) with an HA-tagged NR1–4 chimera lacking GFP (HANR1–4) as well as a GFP chimera containing only the NR1–4 C terminus (GFP-C0C2, Fig. 1). This was done in an attempt to disrupt the interaction of the NR1–4 subunit with potential trafficking proteins. To assess surface localization, transfected cells were fixed 48 h post-transfection and labeled using anti-HA antibody under nonpermeabilizing conditions. When expressed alone in PC12 cells and COS7 cells (not shown), HANR1–4 was found at the surface of cell bodies (Fig. 7A) and neurites (Fig. 7B). When transfected cells were permeabilized to allow anti-HA antibody access to cytoplasmic HANR1–4, clusters of HANR1–4 were detected throughout the cells (Fig. 7C) much like those expressing GFP-tagged NR1–4 (NR1C0C2’GFP, Fig. 3; HANR1C0C2’GFP, Fig. 5). However, when HANR1–4 and the C-terminal chimera, GFP-C0C2’, were cotransfected, we could no longer detect HANR1–4 at the cell surface (Fig. 7D–F), suggesting that the C-terminal fragment blocked surface expression of...
HANR1-4. Furthermore, a C-terminal chimera containing the last 13 amino acids of the C2' cassette, including the (T/S)XV motif, GFP-STVV (Fig. 1), also prevented surface expression of HANR1-4 (Fig. 7, G–I). Together, these results suggest that trafficking of the NR1–4 subunit to the cell surface may involve an interaction that occurs within the last 13 amino acids of the C2' cassette. These results are consistent with the role of the (T/S)XV motif in mediating cellular transport.
Little is known about the NR1–4 splice variant that is highly expressed in the spinal cord as most research has focused mainly on the prototypic NR1 splice variant, NR1-1. Furthermore, the role of individual C-terminal cassettes in NR1 receptor subcellular localization has only recently been more thoroughly investigated. We found that detection of surface expression was not dependent on the presence of the C-terminal cassette, C0 or C2'. However, the distribution of the NR1–4 receptor in an organized, clustered fashion within the cell requires at least C0.

**DISCUSSION**

We characterized the cellular distribution of a GFP-tagged NR1–4 subunit after C-terminal deletion of the C0 and/or C2’ cassette(s). Our studies show that a full-length NR1–4 receptor fused to GFP (NR1C0C2’GFP) and chimeras lacking C2’ (NR1C0GFP) or C0 (NR1C2’GFP) or both C0 and C2’ (NGFP), although displaying differing intracellular distributions, are nevertheless capable of cell surface expression. However, chimeras containing only the C-terminal domains (C0C2’) or the (T/S)xV motif by itself (STVV), when coexpressed with chimeras containing C2’ (HANR1–4, HANR1C0C2’GFP, and HANR1C2’GFP), greatly reduce the ability of these chimeras to reach the cell surface. However, these C-terminal chimeras did not interfere with the surface expression of the chimeras that lacked a C2’ cassette (HANR1C0GFP and HANGFP). This suggests that the C-terminal domain chimeras inhibit full-length NR1–4 from entering a trafficking pathway that leads to surface expression. Whether the C2’/STVV motif-mediated interactions represent a distinct pathway from that followed by those chimeras lacking the STVV motif by itself (STVV), when coexpressed with chimeras containing C2’ (HANR1–4, HANR1C0C2’GFP, and HANR1C2’GFP), greatly reduce the ability of these chimeras to reach the cell surface. However, these C-terminal chimeras did not interfere with the surface expression of the chimeras that lacked a C2’ cassette (HANR1C0GFP and HANGFP). This suggests that the C-terminal domain chimeras inhibit full-length NR1–4 from entering a trafficking pathway that leads to surface expression. Whether the C2’/STVV motif-mediated interactions represent a distinct pathway from that followed by those chimeras lacking the C2’ remains to be determined.

*FIG. 6. Cell surface biotinylation of HEK293 cells expressing NR1–4-GFP chimeras.* HEK293 cells were transfected with NR1–4-GFP chimeras, with or without NR2D cotransfection, and then incubated in PBS containing sulfo-NHS-Biotin, 48 h post-transfection, to label all cell surface protein. Cells were washed and harvested, and 100 μg of total cell protein was incubated with ultralink streptavidin beads to precipitate biotinylated surface protein. Protein was precipitated serially, indicated by lanes 1 and 2 (A). For each chimera, electrophoresis and immunoblotting were carried out using all of the streptavidin-precipitated surface protein fraction and 20% of the intracellular protein fraction. A, anti-NR1 immunoblot of surface (S) and intracellular (I) pools of each NR1–4-GFP chimera in the presence or absence of a coexpressed NR2D subunit. B, anti-tubulin immunoblot showing that tubulin, a cytoplasmic protein, is absent from biotinylated surface pools. Protein samples were taken from the corresponding intracellular fraction used in A. C, subcellular distribution of NR1–4-GFP chimeras in HER293 cells prior to biotinylation.
Several studies (16, 17, 28) have revealed the importance of NR1 C-terminal cassettes in making cytoskeletal attachments. The NR1 C0 cassette is necessary and sufficient for interaction with /H9251-actinin-2, a member of the spectrin/dystrophin family of actin-binding proteins (17). Matsuda et al. (28) showed that the interaction between F-actin and NR1 is mediated specifically by the C0 cassette and that F-actin disruption abolished NR1-1 receptor clustering in HEK293 cells. Depolymerization of F-actin in cortical and hippocampal neurons has also been shown to reduce the number of synaptic NMDA receptor clusters (29).

Our study demonstrates that the C0 cassette alone is sufficient to drive clustering of an NR1-GFP chimera in PC12 cells and DRG, especially in neurites. As the C0 cassette is shared by all NR1 splice variants, it is not surprising that it may be involved in receptor clustering.

A free C2 cassette did not appear to be necessary for clustering in PC12 cells. Both NR1C0GFP, which lacks a C2' cassette, and NR1C0C2/GFP, with a (T/S)XV motif that is blocked by C-terminal addition of GFP, clustered comparably to endogenous NR1 as well as to an HA-tagged NR1-4 subunit and the NR1C0C2/GFP-STVV chimera. In the absence of C0 (NR1C2/GFP), the distribution in these cell types revealed a more uniform distribution of green fluorescence with a greatly reduced ability to cluster in neurites of both PC12 cells and sensory neurons of DRG.

Half-life analysis of NR1GFP/C0C2' suggested that the insertion of GFP within a contiguous protein sequence rendered the resultant protein unstable, with a half-life approximately one-fifth shorter than the previously reported NR1-1 half-life (30), and approximately one-fourth shorter than stably expressed NRC0C2/GFP. Transiently expressed NR1-4 with no GFP insert also decayed faster than NRC0C2/GFP. One possible explanation for the increased turnover of the transiently expressed NR1-4 and NR1C0C2' with GFP-STVV is that the interaction with PDZ-containing proteins in the endoplasmic reticulum leads to rapid export of the newly synthesized protein to the plasma membrane via a PDZ-(T/S)XV interaction like that reported for the NR1-3 splice variant (27). If NR1-4 subunits with free (T/S)XV domains were more rapidly exported than NR1-4 subunits with occluded C termini, this might increase the decay kinetics of these proteins. Alternatively, C-terminal GFP may slow the degradation of these fusion proteins.

Our studies of the fusion proteins containing the GFP placed between TM4 and C0 indicated that these recombinant forms were less stable and had altered conformation because they could not be bound by the anti-NR1 antibody (clone 54.1). This antibody recognizes an epitope between TM3 and TM4, upstream of the C0 cassette. Our results strengthen the interpretation of the reduced excitotoxicity displayed by similar mutants as proposed by Rameau et al. (31). They speculated that...
removal of C0 negatively affects conformation and the channel opening properties thereby reducing excitotoxicity. The fact that the 54.1 mAb did not bind to our chimeras that lacked C0 or had GFP placed immediately downstream of TM4 reinforces this interpretation.

Surface biotinylation has been used by McIlhinney et al. (32) to show that, without coexpression of NR1-1 and NR2A, NR1-1 could not be detected in the biotinylated surface fraction. Okabe et al. (22) obtained similar results using an antibody to detect cell surface labeling of epitope-tagged NR1-1. Their results also showed that splice variants containing the C2 cas- sette, NR1-3 and NR1-4, localized at the cell surface in the absence of a coexpressed NR2 subunit. Accordingly, a GFP-tagged NR1-4 subunit should be expressed at the cell surface, as we demonstrated in the present study by antibody labeling and cell surface biotinylation. The discrepancy between the distributions of NR1-1 and NR1-4 at the cell surface, independent of NR2, is likely to lie in their C-terminal cassette composition, as NR1-1 contains the cassettes C1 and C2. These cassettes are not found in NR1-4, which contains the alternately spliced C2` cassette instead. Evidence from two groups (27, 33) working independently showed that the C1 cassette contains an endoplasmic reticulum retention signal and that surface expression occurred only when this site was suppressed by a C2` cassette, as is found in NR1-3. The authors further speculated that suppression of the endoplasmic reticulum retention signal is followed by a PDZ protein interaction with the (T/S)V motif within the C2` cassette which would then facilitate trafficking to the cell surface. We found that expressing C-terminal chimeras consisting of only C0C2` or only the last 13 amino acids of the C2` cassette (which includes the PDZ binding (T/S)V motif) could greatly reduce surface expression of an HA-tagged NR1-4 subunit. Furthermore, this reduction in surface expression was specific to C2`-containing chimeras. Those chimeras that lacked C2` (HANR1C0GFP and HANGFP) were not prevented from reaching the cell surface when expressed alone, chimera with deletions of C0, C2`, or both C0 and C2` were unhindered in their ability to reach the cell surface. It was surprising that the dominant negative effect

![Graph A](image1)

**FIG. 8.** Quantification of mean number of surface positive PC12 (A) or COS7 cells (B) transfected with each NR1–4 chimera alone or together with C-terminal chimeras. Cells were fixed 48 h post-transfection and surface NR1–4 chimeras were detected using anti-HA antibody. Data represent mean ± S.E. of numbers of surface positive cells per 153-mm² area. Asterisks (p < 0.01) indicate a significant reduction in surface expression as compared with chimera + empty vector (pBluescript). Diamonds (p < 0.01) indicate a significant reduction in surface expression compared with chimera + GFP.
occurred even in those chimeras in which the C terminus of the (T/S)XV motif was fused to GFP. The last 13 amino acids of C2’, including the (T/S)XV motif, were sufficient to mediate this effect. Thus, either a free (T/S)XV is not absolutely required for interaction with a PDZ domain or the blocking of the PDZ domain interaction greatly accelerates surface membrane turnover such that surface expression becomes extremely transient. It has been shown that uncoupling of PSD-95 from the NMDA receptor does alter surface membrane turnover (34).

Few data are available to suggest the process by which NR1 and NR2 subunits associate. McIlhinney et al. (32, 35) have shown that NR1-1 subunits depend upon NR2 subunits for cell surface expression. Conversely, Okabe et al. (22) have shown that the NR1–4 subunit can be targeted to the cell surface in the absence of an NR2 subunit, indicating that differences in C-terminal cassettes affect cell surface localization of the NMDAR. We attempted to determine whether the deletion of NR1–4 C-terminal cassettes had any effect on association with the NR2A subunit. Regardless of the C-terminal deletion, NR1–4-GFP chimeras were still able to colocalize and coimmunoprecipitate with NR2A subunits. Furthermore, those chimeras that were diffusely distributed and/or clustered poorly when expressed alone (NR1C2’/GFP, NGFP) were colocalized with NR2A and redistributed into punctate when coexpressed with NR2A. This suggests that although deletion in the C-terminal domain may affect the organization of NR1–4 in the cell, it does not prevent association with NR2A. This is consistent with recent evidence by Meddows et al. (36) showing that the molecular determinant of interaction between NR1 and NR2 subunits lies in the NR1 N terminus.

In summary, these experiments demonstrate that the C2’ cassette has a role in trafficking of the NR1–4 subunit to the plasma membrane, whereas the C0 cassette, to a large degree, is needed to organize the intracellular clustering of this receptor subunit, especially within neurites. The exact nature of the intracellular trafficking pathway that leads to the observed surface expression and clustering of NR1–4 remains to be elucidated. Additional experiments are also required to study the kinetics of cell surface expression and turnover of our NR1–4 chimeras which will help clarify the physiological role of NR1–4 intracellular regions.

Acknowledgments—We thank Marta Bielas and Shiva Nagalingam for their technical assistance in the making of various fusion proteins.
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