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Turnover Analysis of Glutamate Receptors Identifies a Rapidly Degraded Pool of the *N*-Methyl-D-aspartate Receptor Subunit, NR1, in Cultured Cerebellar Granule Cells*

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The number, composition, and location of receptors in neurons are critically important factors in determining the neuron's response to neurotransmitters. The functional expression of receptors appears to be regulated both generally, at the level of transcription or translation, and locally, at the level of the individual synapse. A key component in the regulation of any protein is its turnover rate, which, measured in half-lives, ranges from a few minutes to several days. In the present study, we measured the turnover rates of subunits of *N*-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, the two major ionotropic glutamate receptors, using cultured cerebellar granule cells. Turnover rates for NR1, NR2A/B, GluR2/3, and GluR4 subunits were determined by pulse-chase labeling of cells with [^{35}S]methionine. Half-lives were found to be 18 ± 5 h and 23 ± 8 h for the AMPA receptor subunits GluR2/3 and GluR4, respectively, and 16 ± 5 h for NR2A. The NR1 subunit showed a biphasic decay with half-lives of 2 and 34 h for the rapidly and slowly degraded populations, respectively. Splice variants of the NR1 subunit with different carboxyl-terminal cassettes, C2 and C2′, showed similar biphasic degradation patterns. To further characterize the rapidly degraded pool of NR1, surface receptors were labeled by biotinylation, and half-lives of the biotinylated proteins were determined. All surface NR1 was slowly degraded with a pattern similar to that of NR2A, GluR2/3, and GluR4, suggesting that the rapidly degraded pool is confined to the cytoplasm and not assembled with NR2 subunits. A significant amount of NR1 was not immunoprecipitated by NR2 subunit-specific antibodies after solubilization with deoxycholate. This unassembled pool, but not the assembled one, was greatly diminished following treatment of cycloheximide for 5 h, indicating that the rapidly degraded pool of NR1 is not assembled with NR2. These results show that NMDA and AMPA receptors have similar turnover rates, but NMDA receptors have a separate pool of NR1 subunits that is rapidly degraded and accounts for most of the intracellular pool.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and three distinct subtypes of ionotropic glutamate receptors mediate fast excitatory transmission (1). AMPA[1] receptors and NMDA receptors are the principle ionotropic receptors, while kainate receptors are widely distributed but less abundant (2, 3). AMPA receptors are composed of four homologous subunits, GluR1, -2, -3, and -4, which assemble into a receptor complex, probably containing five subunits (2, 4). While homomeric AMPA receptors are functional and appear to exist in neurons, most native AMPA receptors consist of two or more different subunits (5–7). For NMDA receptors, two classes of subunits, NR1 and NR2A-D, have been cloned (2, 8). In addition, various splice variants have been identified for the NR1 subunits (9–11). The NR1 subunit may be functional by itself, but it is generally thought that native NMDA receptors are heteromeric complexes with NR1 serving as an obligatory subunit (12, 13). Unlike AMPA receptors, all NMDA receptors are calcium-permeable, and gating is voltage-dependent.

NMDA and AMPA receptors are expressed in the same neuron and often are co-localized at postsynaptic membranes, where they are anchored through their C termini to PDZ (PSD-95/discs large protein/zona occludens protein 1) domain-containing proteins in the postsynaptic density (14–19). The number and type of receptors at the postsynaptic membrane will determine the nature as well as the magnitude of the response to release of the neurotransmitter, glutamate. Two general mechanisms appear to control the number and composition of glutamate receptors at the postsynaptic membrane. The first is the control of the expression of a receptor at the levels of transcription and translation. Expression of mRNA for all glutamate receptors is developmentally and regionally regulated (20–23). Selective changes in expression of receptor subunits also occur under certain pathophysiological conditions and may have a critical impact on neurons. For example, it has been proposed that a selective decrease in GluR2 mRNA and protein following ischemia generates calcium-permeable AMPA receptors, which eventually lead to neuronal degeneration due to excess calcium influx through their channels (24). Receptor expression is also regulated at the level of the individual synapse such that different synaptic populations of a neuron may contain different glutamate receptors (25–27). The synaptic receptor composition may vary under different physiological conditions; for example, it has been proposed that long term potentiation is generated by the conversion of synapses that previously did not contain AMPA receptors or contained electrophysiologically silent AMPA receptors to synapses that contain functional AMPA receptors through the activation of

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[1] The abbreviations used are: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; NMDA, *N*-methyl-D-aspartate; PAGE, polyacrylamide gel electrophoresis; SNAP25, synaptosomal associated protein of 25 kDa; Sulfo-NHS-LC-biotin, sulfosuccinimidyl-6-(biotinamido)hexanoate; Sulfo-NHS-SS-biotin, sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; HBSS, Hanks' buffered saline solution.

NMDA receptors (28, 29). A similar up-regulation of AMPA receptors may occur during synapse development (30, 31).

A critical parameter in a protein's response to changes in its rate of synthesis or degradation is the turnover rate of the protein. Turnover rates, usually expressed in half-lives, vary widely for proteins with half-lives ranging from less than 1 min to many days (32). A protein with a short half-life is much more effectively regulated by changing synthesis or degradation rates than a protein with a long half-life. Therefore, if glutamate receptors have short half-lives, changes in synthesis, resulting from either changes in transcription or translation, as well as changes in degradation could be effective in rapid regulation of levels of synaptic receptors. In the present study, we investigated the turnover characteristics of two key ionotropic receptors, AMPA and NMDA receptors, in cultured cerebellar granule cells. These cultures have the advantage of representing a nearly homogenous population of neurons that express several glutamate receptors, including functional AMPA and NMDA receptors. We demonstrate that subunits of both receptors have similar and relatively long half-lives, with values of about 20 h, as measured both by pulse-chase and surface biotinylation. However, a pool of the NR1 subunit, which is not assembled with NR2 and represents about half of the total NR1, is rapidly degraded with a half-life of about 2 h.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture media, sera, and supplies were purchased from Life Technologies, Inc.; cytosine arabinoside from Sigma; methionine and cysteine-free medium from NIH Media Service branch; [^{35}S]methionine and Enlightening from NEN Life Science Products; protein A-agarose beads, Ultralink Plus immobilized streptavidin beads, Sulfo-NHS-LC-biotin, and NHS-SS-biotin from Pierce; Kodak X-Omat AR films from Eastman Kodak Co.; autoradiographic ^{14}C-labeled microscales and horseradish peroxidase-conjugated secondary antibodies from Amersham Pharmacia Biotech; SDS electrophoresis gels from Novex; and horseradish peroxidase-conjugated streptavidin from Southern Biotechnology Associates.

Cerebellar Granule Cell Culture—Cultures of cerebellar granule neurons were prepared as described by Gallo *et al.* (33). Briefly, cerebella were obtained from 7–8-day-old Sprague-Dawley rat pups. They were chopped by using a tissue slicer and treated with 0.025% trypsin for 15 min at 37 °C. Following trypsinization, tissue was dissociated by passage through fire-polished pipettes, and tissue debris was separated from dissociated cells by sedimentation. Cells were centrifuged and suspended in the plating medium, which consisted of basal medium Eagle, 25 mM KCl, 10 μg/ml gentamycin, 2 mM glutamine, 10% fetal calf serum, and were plated on 3.5-cm culture plates coated with poly-L-lysine at a density of 2.5×10^6 cells/dish. After 19–20 h, proliferation of glial cells was inhibited by treatment with 10 μM cytosine arabinoside. All experiments were performed on 8–9-day *in vitro* cells.

Pulse-chase Labeling of Cultured Cells—Cells were washed twice with prewarmed Hanks' balanced salt solution (HBSS) containing 25 mM KCl, incubated for 30 min at 37 °C in depletion media, which consisted of methionine- and cysteine-free basal medium Eagle, 25 mM KCl, 2 mM glutamine, 10 μg/ml gentamycin, 5% dialyzed fetal calf serum, and then pulse-labeled with 250 μCi of [^{35}S]methionine (1175 Ci/mmol) in depletion medium for 20 min at 37 °C. Some experiments were performed in medium lacking glutamine. After 20 min, cells were washed once with basal medium Eagle containing 2 mM methionine and then were incubated with conditioned medium containing 2 mM methionine and 2.5 mM HEPES for variable times. KCl (25 mM) was included in all media throughout the experiment. To block glycosylation, tunicamycin was added to a final concentration of 1 μg/ml to the medium 12 h before pulse labeling and was included during pulse labeling. To harvest, cells were washed twice with cold Dulbecco's phosphate-buffered saline, scraped into phosphate-buffered saline containing protease inhibitor mixture (1 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 μM leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin), and centrifuged at $2000 \times g$ for 15 min. Pellets were stored frozen at −70 °C until use.

Antibodies—Monoclonal antibodies (54.2) to NR1 subunits and NR2B subunits were purchased from Pharmingen (San Diego, CA) and Transduction Laboratories (Lexington, KY), respectively. Antibodies specific to NR2A, NR2B, and NR2C subunits were generated against polyhistidine fusion proteins containing the C-terminal region of each subunit encompassing 934–1203 for NR2A, 935–1856 for NR2B (34), and 1110–1242 for NR2C subunits. The NR2C antibody was specific to NR2C subunits as tested by Western blot analysis using human embryonic kidney (HEK293) cells transfected with different NR2 subunits. Other antibodies to splice variants of NR1, NR2A/B, GluR1, GluR2/3, and GluR4 subunits were generated against synthetic peptides which correspond to the sequences at the C termini, and characterization and demonstration of the specificity of the antibodies was made in previous studies (5, 35, 36).

Immunoprecipitation and Fluorography—Cell pellets were suspended in buffer A (50 mM Tris, pH 7.5, 150 mM NaCl, 0.02% NaN_3, 1 mM EDTA, 20 μM leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 2 μg/ml pepstatin, 2 μg/ml aprotinin) by trituration and solubilized with 2% SDS in buffer A by incubating for 3 min at 90 °C. The soluble extract was obtained by centrifugation at $100{,}000 \times g$ for 30 min at 15 °C, and 100 μl of the soluble extract was diluted 6-fold with 2% Triton X-100 in buffer A. To retain NMDA receptor subunit associations, cells were solubilized with 1% deoxycholate at pH 9 in buffer A (36) (200 μM phenylmethylsulfonyl fluoride was used instead of 1 mM ABESF). After centrifugation, the soluble extract was diluted 10-fold with 0.1% Triton X-100. SDS or deoxycholate soluble fractions were incubated overnight at 4 °C with 50 μl of protein A-agarose beads to which 10 μg of polyclonal antibodies or 2.5 μg of monoclonal antibodies were preattached. Immunoprecipitated pellets were washed three times with buffer A containing 2% Triton X-100, 10% glycerol, and the above protease mixture, followed by two washes with buffer A containing 0.1% Triton X-100 and protease inhibitor mixture. Immunoprecipitated proteins were eluted with SDS-sample buffer (60 μl) by incubating at 90 °C for 3 min and subjected to SDS-PAGE on 4–20% gradient gels. For fluorography, electrophoresed gels were fixed with the solution containing 30% methanol and 10% glacial acetic acid for 30 min and treated with fluorophore (Enlightening) for 30 min. Gels were dried onto Whatman filter papers and exposed to films (Kodak X-Omat AR) at −70 °C. Fluorograms were scanned with a Molecular Dynamics densitometer, and densities were normalized using standard curves generated with autoradiographic ^{14}C-labeled microscales.

Deglycosylation of the Subunits—Membrane homogenates from cultured cells or cerebellum were suspended in 10 mM NaH_2PO_4 buffer containing 10 mM EDTA, 0.2 mM leupeptin, and 10 μg/ml pepstatin and solubilized with 1% SDS containing 5% β-mercaptoethanol by incubating at 90 °C for 2–3 min. The soluble fraction was diluted with 1% β-octyl glucopyranoside in 10 mM NaH_2PO_4 containing the above protease mixture to a final concentration of 0.1% SDS and incubated with endoglycosidase H (15 milliunits) or *N*-glycosidase F (3 units) overnight at 37 °C. An equal volume of 2× SDS-sample buffer was added for SDS-PAGE analysis.

Gel Electrophoresis and Western Blot Analysis—Membrane homogenates or soluble extracts of granule cells or adult rat cerebella were subjected to SDS-PAGE using 4–20% gradient gels. Proteins were transferred to nitrocellulose membranes, and the membranes were blocked with Tris-buffered saline containing 0.1% Tween (TBST) and 5% nonfat dry milk overnight at 4 °C, incubated with primary antibodies in TBST for 1.5 h, and washed three times for 15 min each. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h and washed three times, and bound antibodies were visualized by the chemiluminescence detection method.

Biotinylation of Cell Surface Proteins—Cultured granule cells were washed four times with HBSS, and surface proteins were biotinylated with Sulfo-NHS-LC-biotin or NHS-SS-biotin (1 mg/ml) in HBSS for 30 min at 4 °C. Cells were washed with HBSS four times and incubated in conditioned medium for 15 min at 37 °C in a humidified incubator before harvest. For determination of the cytoplasmic pool of the receptors, cells were harvested immediately after washing. KCl (25 mM) was included in all HBSS solutions for washing and biotinylation. Cells were harvested at various times as described above, by using phosphate-buffered saline containing 0.1 M glycine and protease mixture. Cells were stored at −70 °C. Pellets were solubilized with 2% SDS, diluted with 2% Triton X-100, immunoprecipitated using appropriate antibodies as described above, and subjected to SDS-PAGE. After transferring the gel to nitrocellulose membranes, membranes were blocked with 5% nonfat dry milk in TBST, incubated for 1.5 h with horseradish peroxidase-conjugated streptavidin (1:10,000) in TBST containing 0.5% milk, and washed three times for 15 min each. Biotinylated proteins were visualized by chemiluminescence. When NHS-SS-biotin was used, Ultralink-immobilized streptavidin beads were used to precipitate biotinylated proteins, and subunit proteins were detected by Western blot analysis.

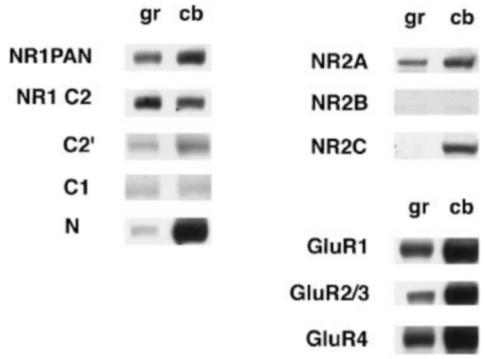

FIG. 1. **Expression of glutamate receptor subunits in cultured cerebellar granule cells.** Membrane homogenates were prepared from adult cerebellum (*cb*) and cerebellar granule cells (*gr*) of 8–9 days *in vitro* and analyzed by SDS-PAGE, followed by Western blot analysis using subunit-specific antibodies. NR1 PAN recognized all NR1 subunits. NR1 C2, C2′, C1, and N are splice variants of NR1 subunits containing C2, C2′, C1, and N cassettes, respectively. Subunits were visualized by the chemiluminescence detection method. For a given sample, the same amount of protein was loaded for each lane for Western blot analysis.

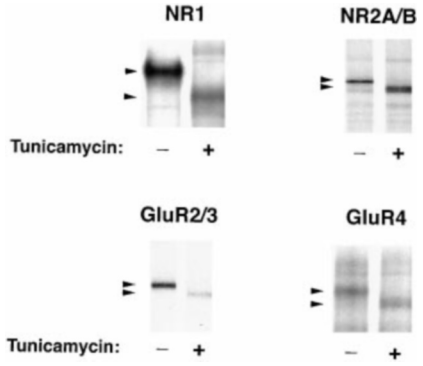

FIG. 2. **Treatment with tunicamycin demonstrates that pulse-labeled subunits are obtained as mature glycosylated subunits.** Cerebellar granule cells were pulse-labeled with 250 μCi of [^{35}S]methionine and solubilized with 2% SDS. After diluting SDS with 2% Triton X-100, glutamate receptor subunits were immunoprecipitated using subunit-specific antibodies and subjected to SDS-PAGE analysis. Gels were dried and analyzed by fluorography as described under "Experimental Procedures." For deglycosylation, cells were incubated with tunicamycin (final concentration of 1 μg/ml) for 12 h before and during pulse labeling. Glycosylated and deglycosylated subunits are indicated by *arrowheads*.

RESULTS

Expression of Glutamate Receptors in Cerebellar Granule Cells—Cultured cerebellar granule cells, 8–9 days *in vitro*, were chosen for this study because it has been shown previously that these cells express functional AMPA and NMDA receptors (37–41). Using antibodies selective for AMPA and NMDA receptor subunits and NR1 splice variants, Western blot analysis shows that multiple subunits and splice variants are expressed in these cultures (Fig. 1). It has been previously shown that NR2A and NR2C are the predominant NR2 subunits in granule cells *in vivo* in the adult animal, while NR2B is the major subunit in granule cells at early developmental stages (20, 23). This developmental change of the subunit expression also occurs in cultured granule cells (42). The pattern of NR2 subunit expression in the present study is consistent with previous findings of mRNA analysis, where NR2A was the major subunit expressed and NR2B and NR2C were expressed in a lower abundance at 7–8 days *in vitro* (37, 41). Since all four alternatively spliced cassettes of NR1 are found in cultured granule cells, it is possible that they are differentially assembled and that multiple functionally distinct NMDA receptors are expressed. NR1 C2-containing receptors appear to be more abundant than those containing the other C-terminal cassette, C2′, as determined by using different antibodies selective for the two cassettes. This was confirmed in a separate experiment where NR1 PAN antibodies were used to probe receptors immunoprecipitated with NR1 C2 and C2′ antibodies (data not shown). At least three AMPA receptor subunits were expressed in granule cells, GluR1, GluR2/3, and GluR4 (the antibody to GluR2/3 recognizes both GluR2 and GluR3 (5); mRNA for GluR2 and GluR3 subunits have been shown to be expressed (42)). GluR1 is not expressed in granule cells *in vivo* in adult rats but is expressed in cultured cells, as previously reported by Hack *et al.* (39), who showed a greater than 3-fold increase in GluR1 between 2 and 9 days in culture, while GluR2/3 and GluR4 remained relatively constant. Because of these rapidly changing levels of GluR1, this subunit was not included in the turnover analyses.

Pulse Labeling and Turnover of NMDA and AMPA Receptor Subunits—To measure the degradation rates of receptor subunits, cells were pulse-labeled with [^{35}S]methionine and chased in conditioned medium containing unlabeled methionine. Sufficient radioactivity was incorporated under these conditions to permit quantitation of receptor subunits immunoprecipitated with antibodies to NR1, NR2A, GluR2/3, and GluR4. Incubation with 2% SDS at 90 °C was used to ensure optimal solubilization of subunits; NMDA receptor subunits, in particular, have been reported to be only partially solubilized from the brain with nonionic or weaker ionic detergents (36, 43). Under the conditions used in the present study, about 90% of NR1 and 80% of NR2A immunoreactivities were present in the soluble fraction (data not shown).

After a 20-min incubation with [^{35}S]methionine, the predominant species of NMDA and AMPA receptor subunits appeared to be mature, fully glycosylated forms (Fig. 2), since molecular weights of pulse-labeled subunits are similar to those of subunits that are expressed in cultured cells and the brain as determined by Western blotting (data not shown). Treatment of the culture with tunicamycin before and during pulse labeling resulted in the complete loss of the mature subunit and the appearance of lower molecular weight bands that migrate at a position consistent with that of the deglycosylated forms of subunits (44–47). The absence of significant lower molecular weight unglycosylated forms indicates a rapid glycosylation of the subunits, and this appears to occur for nicotinic acetylcholine receptor subunits as well (48, 49). To characterize the glycosylation properties of NMDA and AMPA receptor subunits, membrane homogenates of cultured cells and the cerebellum were analyzed by Western blotting after treatment with endoglycosidase H, which recognizes *N*-linked high mannose carbohydrates or *N*-glycosidase F (Fig. 3). Treatment with endoglycosidase H generated low molecular weight species of NR1 subunits, which comigrate with those that were treated with *N*-glycosidase F, indicating glycosylation of NR1 subunits is of the high mannose type. NR2A subunits showed significant sensitivity but were not completely sensitive to endoglycosidase H. GluR2/3 and GluR4 subunits appeared to be resistant; however, there was a slight shift in molecular weights, indicating that some glycosylation moieties have high mannose structures (Fig. 3).

Quantitation of radioactivity associated with the receptor subunits from 0–48 h after the pulse labeling gave half-lives of 18 ± 5 h and 23 ± 8 h for the AMPA receptor subunits GluR2/3 and GluR4, respectively, and 16 ± 5 h for NR2A (Fig. 4, *A* and *B*). For the NR1 subunit, at least two different half-lives were detected, with the majority of the NR1 subunit being degraded rapidly with a half-life of 2 h. Although it was difficult to

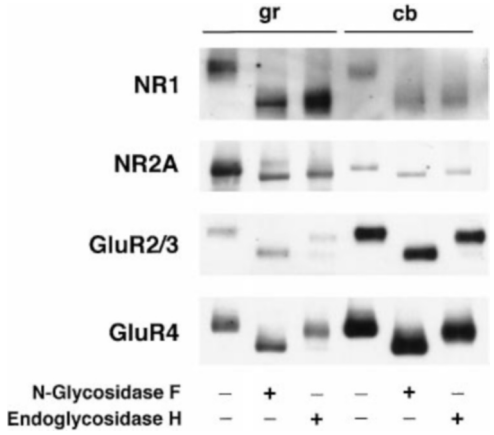

FIG. 3. **Glycosylation properties of glutamate receptor subunits.** Membrane homogenates from cultured cells (*gr*) and cerebellum (*cb*) were solubilized with 1% SDS in the presence of 5% β-mercaptoethanol. Soluble extracts, after dilution with 1% β-octyl glucoside, were incubated with endoglycosidase H or *N*-glycosidase F and analyzed by SDS-PAGE and Western blotting. Migration of glycosidase-treated samples is compared with that of the control samples, which were treated identically but without enzymes.

accurately measure the half-life of the slowly degrading pool due to low radioactivity remaining, we estimate a half-life of about 34 h for the slow pool. After 14 h, only about 2% of the total radioactivity remains; since most of the rapidly degraded pool is gone at this time, we conservatively estimate that less than 10% of the total pulse-labeled NR1 is associated with the slowly degraded pool. The biphasic decay of NR1 could reflect different degradation rates for NR1 splice variants, since the relative abundance of C2 and C2′ would be consistent with a larger, rapidly degraded pool of C2 and a minor, slowly degraded pool of C2′. The differential assembly of NR1 splice variants with NR2 subunits has been proposed by Sheng *et al.* (50). To determine if the C2 and C2′ cassette-containing variants of NR1 have similar or different degradation properties, immunoprecipitation was done using antibodies selective for these cassettes. As shown in Fig. 5, similar degradation patterns were obtained for the two variants.

Degradation of Glutamate Receptors on the Cell Surface—The rapidly degraded pool of NR1 could represent unassembled subunits that are not expressed on the cell surface or, less likely, homomeric NR1 receptors expressed on the cell membranes. We measured the degradation of surface receptors by determining the amount of biotinylated NMDA and AMPA receptor subunits remaining 0–14 h after biotinylation of surface proteins. These results (Fig. 6*A*) show similar degradation properties for NR1 and NR2A as well as for the AMPA receptor subunits and suggest that functional NMDA receptors have long half-lives similar to those of AMPA receptors and, also, support an intracellular location for the rapidly degraded pool of NR1. Biotinylation of surface proteins, but not cytoplasmic proteins, was verified by showing that SNAP25, which is present in the cytoplasm, is not biotinylated (Fig. 6*B*).

NR1 Subunits in the Rapidly Degraded Pool Are Not Associated with NR2—Using pulse labeling and co-immunoprecipitation, we were not able to clearly demonstrate that degradation of NR1 is dependent on association with NR2 subunits due to low amounts of radioactivity in the co-immunoprecipitating bands. To determine if the rapidly degraded and slowly degraded pools of NR1 are differentially associated with NR2, the co-immunoprecipitation of the subunits was investigated by Western analysis after treatment of cultures with cycloheximide. Rapidly degraded proteins are lost more quickly than slowly degraded proteins in the presence of a protein synthesis inhibitor such as cycloheximide, and since degradation continues in the absence of synthesis, this approach has been used to estimate degradation rates (51, 52). Receptors were solubilized with deoxycholate using conditions that were previously shown to preserve complexes of NR1 and NR2 subunits (36). The solubilized receptor complex was immunoprecipitated with an antibody to NR2, and the amount of co-immunoprecipitating NR1 was determined. As shown in Fig. 7, NR1 is found in both the bound and unbound fractions in untreated cultures. Its presence in the unbound fraction was not due to insufficient first round immunoprecipitation, since no NR2 immunoreactivity is detected in the unbound fraction. However, after treatment with cycloheximide, the amount of unbound NR1 is greatly diminished (a decrease of 76%, $n = 2$), while the amount in the bound fraction is not changed. These data show that the unassembled fraction of NR1 is selectively affected by the block of protein synthesis and indicate that the rapidly degraded pool of NR1 is not assembled with NR2.

Estimation of the Size of the Rapidly Degraded Pool of NR1—While our pulse labeling data indicate that a relatively small amount of the newly synthesized NR1 is associated with the slowly degraded pool, the total amount of the slowly degraded pool is expected to be much larger because of its relatively slow rate of degradation. Two approaches were taken to estimate the amount of NR1 associated with the two pools. First, we estimated the relative amounts of NR1 associated with the surface and intracellular pools, based on our findings that surface NR1 subunits are not rapidly degraded. Intact cells were biotinylated, and the biotinylated surface receptors were removed from SDS-solubilized whole cell extract using streptavidin beads. By quantifying the unbound, nonbiotinylated receptors, our results show that 60% of the NR1 is intracellular, while only 10% of the NR2A is intracellular (Table I). Under the conditions which were used, we detected no biotinylated proteins in the unbound fraction after incubation with streptavidin beads (data not shown). Based on these findings, the rapidly degraded pool of NR1 would account for 60% or less of the total pool of NR1. Unless surface expression itself, rather than assembly with NR2, is the determining factor in the degradation rate of NR1, we would expect that some slowly degraded NR1 is also intracellular. Since a rather small amount of NR2 is intracellular, we can conclude that the intracellular pool of NR1 that is assembled with NR2 is also relatively small.

A second approach was to block protein synthesis with cycloheximide and measure the remaining NR1 at a time such that most of the rapidly degraded NR1 is lost. After treatment of granule cells with cycloheximide, changes of NMDA and AMPA receptor subunits were determined by Western blot analysis. The major effect was on NR1 with 40% reduction after 5 h, while NR2 did not change (Table II). The AMPA receptor subunits were only slightly affected under these conditions. These results suggest that about 40% of NR1 subunits are associated with the rapidly degraded pool. Therefore, based on these two results, most of intracellular NR1 subunits are in a pool with short half-lives.

DISCUSSION

In the present study, we characterized the turnover properties of AMPA and NMDA receptor subunits in cultured cerebellar granule neurons. Our studies show that, with the exception of a pool of NR1, the AMPA and NMDA receptor subunits are degraded relatively slowly with half-lives of about 20 h. These values, which are similar to that recently obtained for GluR1 in cultured spinal cord neurons (31 h for 11 day cultures) (53), suggest that regulation of synthesis is not an efficient mechanism for rapid modulation of the levels of AMPA

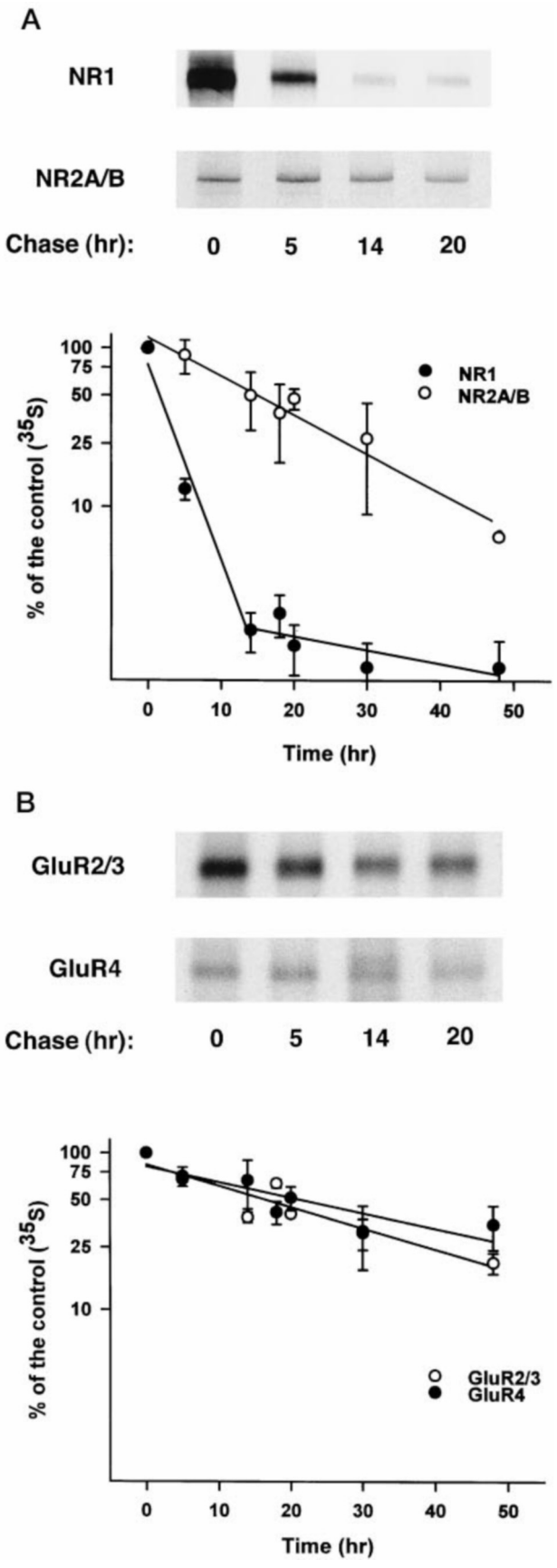

FIG. 4. **Turnover rates of NMDA and AMPA receptor subunits determined by pulse-chase labeling.** After pulse labeling with [^{35}S]methionine, cells were harvested at the indicated times. Harvested cells were solubilized and treated for fluorography as described in the legend to Fig. 2. Fluorograms were scanned for densitometric analysis using ^{14}C-labeled microscales as standards to quantify radioactivity. Values were expressed as percentages of the control (radioactivity at time 0), and half-lives are mean ± S.E. from 3–7 separate experiments. *A*, half-lives of NR1 subunits were estimated to be 1.7 ± 0.3 h and 34 h (three separate determinations for each time point), and those of NR2A subunits were 16 ± 5 h (for 5, 14, 18, 20, 30, and 48 h; 5, 4, 2, 5, 2, and 1 separate determination(s), respectively). *B*, degradation of GluR2/3 and GluR4 subunits displays half-lives of 18 ± 5 and 23 ± 8 h, respectively (for 5, 14, and 20 h, two separate determinations; for 18, 30, and 48 h, three separate determinations, respectively).

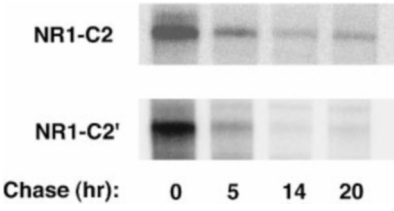

FIG. 5. **Turnover rates of NR1 splice variants.** Antibodies made against the two different C-terminal cassettes of NR1, C2 and C2′, were used to immunoprecipitate corresponding subunits from SDS-solubilized extracts of pulse-labeled cells at the indicated times. Following SDS-PAGE and fluorography, radioactive bands were analyzed by densitometric analysis as described in Fig. 4. Half-lives for the rapid degradation phase of C2 and C2′ cassette-containing NR1 subunits were estimated as 3.0 ± 0.4 h (n = 3) and 2.3 ± 0.3 h (n = 2), respectively.

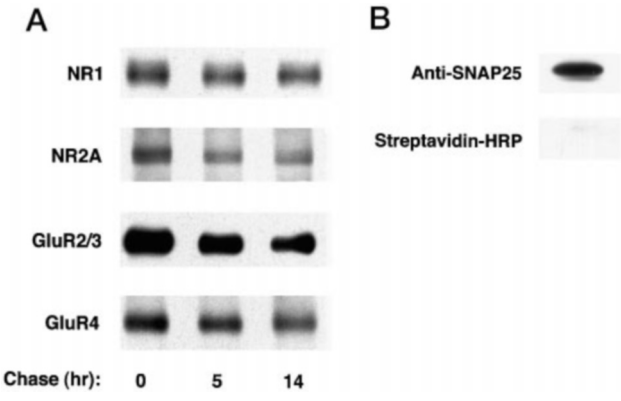

FIG. 6. **Turnover of biotinylated surface glutamate receptors.** *A*, surface proteins of cerebellar granule cells were biotinylated using Sulfo-NHS-LC-biotin or Sulfo-NHS-SS-biotin, and, after solubilization with 2% SDS, they were treated as described under "Experimental Procedures." Biotinylated proteins were detected by chemiluminescence. Half-lives of the surface glutamate receptors were estimated as 20 ± 4 and 21 ± 7 h for NR1 and NR2A/B, and 17 ± 4 and 21 ± 5 h for GluR2/3 and GluR4 subunits, respectively. These values are from two or three separate experiments. Data shown were obtained using Sulfo-NHS-LC-biotin. *B*, as a control for biotinylation of intracellular proteins, SNAP25 was immunoprecipitated from soluble extracts of biotinylated cell membranes, and immunoprecipitates were analyzed as described for *A*.

and NMDA receptors in neurons. This serves as indirect evidence supporting local mechanisms, such as insertion of receptors from an intracellular pool, in cases where rapid changes in the number of synaptic receptors have been proposed to occur, such as long term potentiation (28, 29, 54). Our studies using pulse labeling and treatment with cycloheximide identified a large pool of NR1 that is rapidly degraded with a half-life of about 2 h. This pool of NR1 is not assembled with NR2 based on co-immunoprecipitation analysis. Biotinylation of surface proteins indicates that the rapidly degraded pool of NR1 is not expressed on the cell surface and therefore would not form functional homomeric receptors. This is consistent with results on transfected cells showing that only NR1 and NR2 subunits that are assembled are expressed on the cell surface (55). These findings support the interpretation that NR1 becomes metabolically stable when it assembles with NR2. Since we find that

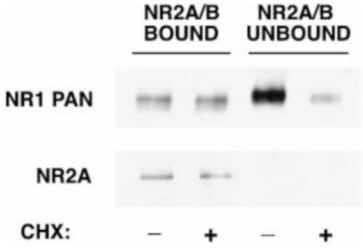

FIG. 7. **Effect of cycloheximide on unassembled and assembled pools of NMDA receptors.** After treatment of cultured cells with cycloheximide (*CHX*) (50 μg/ml) or Me_2SO vehicle (0.02%) for 5 h, NMDA receptors were solubilized with deoxycholate as described under "Experimental Procedures." Soluble extracts were used for immunoprecipitation with antibodies specific to NR2A/B subunits. The unbound fraction was reimmunoprecipitated with antibodies to NR1 C2 subunits. Immunoprecipitates were probed with NR1 PAN or NR2A antibody. Changes in immunoreactivity were quantitated by the chemiluminescence detection method using a standard curve generated from serial dilutions of unbound NR1, which was not treated with cycloheximide.

only a small percentage of NR2 is intracellular, assembled receptors may become quickly associated with the plasma membrane.

The function of the rapidly degraded pool of NR1 subunits is unclear. The pool may simply represent misfolded or unfolded subunits that are retained in the endoplasmic reticulum and degraded (56). A number of other ion channel proteins, which are formed from multiple subunits, have been shown to have subunits that are rapidly degraded and/or do not assemble with other subunits. The α-subunit of the muscle nicotinic acetylcholine receptor is synthesized in excess of the other subunits, and the unassembled α-subunits undergo rapid degradation (57). Similarly, unassembled Kv1.2 subunits of potassium channels as well as the α-subunit of the voltage-sensitive sodium channel are rapidly degraded (58, 59), although the α-subunits of sodium channels have a metabolically stable pool of free subunits that has a half-life similar to those of the assembled subunits (58).

Since all our studies have been done using cultured cells, a key question is whether or not a similar pool of NR1 is found in neurons *in vivo*. Although turnover analyses have not been done on intact brain, there is considerable evidence supporting the presence of a relatively large pool of unassembled NR1 in brain. Analysis of detergent solubility of rat brain membranes showed that more NR1 subunits than NR2 can be solubilized with 1% Triton X-100 in the presence of high salt (43, 60). Subfractionation shows that this Triton-soluble pool is associated with the microsomal fraction, while the synaptic membrane fraction contains little Triton-soluble NR1 (36). Immunocytochemical studies also showed a large intracellular pool of NR1 (61). The NR1 subunit, unassembled with NR2, could exist either as an assembled homomeric complex or unassembled as a single subunit that is ready to combine with NR2 subunits. The fact that NR1 mRNA alone can produce a functional NMDA receptor in oocytes indicates that under some conditions NR1 can form a homomeric receptor complex (8, 11). However, a functional homomeric receptor cannot be formed in transfected cell lines, although it appears that the NR1 subunit can form a receptor complex that has the binding sites for the co-agonist, glycine (62, 63). Size fractionation of the Triton-soluble fraction of NR1 from rat brain indicated a peak at 125,000 daltons, which fits the size of a single unassembled NR1 subunit (60). However, those studies cannot rule out the possibility that the complex was disrupted into individual subunits with detergent treatment.

The most likely functional role of a large, rapidly degraded pool of receptor subunits in neurons would be to serve as a reserve for assembly into receptor complexes when the synthesis of its co-assembling subunit is increased. Since unassembled NR1 appears not to be expressed on the surface of a neuron, the presence of an excess pool of NR1 implies that the expression of NR2 subunits controls the number of functional NMDA receptors. NR2 subunits define the functional properties of the NMDA receptor, and NR2 subunits are more finely regulated than NR1 subunits with respect to regional and developmental expression (20, 23, 35, 64). A recent study indicates that NR2 expression during development may be regulated by synapse formation (65). Therefore, a large pool of unassembled NR1 could be a reserve awaiting changes in expression of NR2 subunits arising, for example, during synapse development or other changes in synaptic strength. None of the studies addresses the subcellular location of the NR1 pool other than indicating that it is not expressed on the cell surface. An interesting possibility is that in neurons, unassembled NR1 subunits are present in dendrites and assembly with NR2 occurs immediately prior to insertion into the postsynaptic membrane. Such a mechanism would allow assembly and surface expression to be controlled locally, by synaptic activity, for example. The presence of an endoplasmic reticular structure, as well as the translational machinery associated with the endoplasmic reticulum in dendrites lends support to this idea (66, 67). The fact that we do not see a similar pool of AMPA receptors with rapid turnover rates may suggest that these two subtypes are differently regulated, perhaps reflecting the different functional roles of these receptors. A key point may be the fact that AMPA receptors can form functional homomeric receptors, which have been identified in neurons, while NMDA receptors appear to require both NR1 and NR2 subunits (2, 6).

The relatively long half-lives of NMDA and AMPA receptors indicate that the number and composition of postsynaptic receptors is likely to be finely controlled at the level of the individual synapse. While receptors are believed to be anchored

TABLE I

Estimation of the cytoplasmic pool of the glutamate receptor subunits

Cell surface proteins of cerebellar granule cells were biotinylated as described under "Experimental Procedures." After solubilization with 2% SDS, biotinylated proteins were separated from nonbiotinylated proteins using streptavidin beads. The unbound fraction was used for Western blot analysis to estimate the cytoplasmic pool of the receptors. Quantification was made by the chemiluminescence detection method using a standard curve generated from serial dilutions of the original soluble extracts. Data were expressed as mean ± S.E. for three or four determinations.

Subunits	Cytoplasmic pool
	%
NR1	60 ± 2 (n = 4)
NR2A	10 ± 9 (n = 4)
GluR2/3	39 ± 4 (n = 3)
GluR4	55 ± 9 (n = 3)

TABLE II

The effect of cyclohexamide on the expression and degradation of the glutamate receptor subunits

Granule cells were treated with cyclohexamide (50 μg/ml in 0.02% Me_2SO vehicle) for 5 h. Cells were pelleted and subjected to SDS-PAGE followed by Western blot analysis. Subunits were detected by chemiluminescence. Data were expressed as percentage of the control (from Me_2SO vehicle-treated cells) and are mean ± S.E. from four or five determinations.

Subunits	Control
	%
NR1	60 ± 6 (n = 4)
NR2A	106 ± 12 (n = 4)
GluR2/3	93 ± 9 (n = 4)
GluR4	79 ± 9 (n = 5)

at the synapse by specific proteins (68, 69), little is known about the mechanisms involved in adding and removing receptors that are critical factors for determining turnover rates. Our results together with earlier findings on the surface biotinylation of receptors (54), indicate that surface receptors are stable and have turnover rates that are similar to those of the entire pool of receptors, except NR1. Therefore, half-lives of glutamate receptors appear to reflect the half-lives of the surface pool of the receptors, suggesting that degradation may be closely linked to the removal of the receptor from the postsynaptic membrane. The C terminus of some glutamate receptor subunits can be cleaved by the calcium-sensitive enzyme, calpain (70), and since AMPA and NMDA receptors are believed to be anchored to the postsynaptic density through their C termini, this cleavage would irreversibly free the receptor from its anchor and may begin the process of internalization and degradation. In support of such a mechanism, we have observed a rapid loss of AMPA and NMDA receptor C termini in cultured granule cells after treatment with agonist or removal of conditioned medium (71).

Additional information will be required to determine whether or not this is related to the normal mechanism of receptor removal from the postsynaptic membrane.

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