Cellular/Molecular

Selective Activation Induced Cleavage of the NR2B Subunit by Calpain

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Although activation of calcium-activated neutral protease (calpain) by the NMDA receptor has been suggested to play critical roles in synaptic modulation and neurologic disease, the nature of its substrates has not been completely defined. In this study, we examined the ability of calpain to cleave the NMDA receptor in cultured hippocampal neurons. Activation of the NMDA receptor by agonist application led to rapid calpain-specific proteolysis of spectrin and decreased levels of NR2A/2B subunits. Cleavage of the NR2A/2B subunit created a 115 kDa product that retained the ability to bind ^{125}I-MK-801 and is predicted to be active. Increases in levels of this product appeared within 5 min of NMDA receptor activation and were stable for periods of >30 min. Subtype-specific antibodies demonstrated that the NR2B subunit was cleaved in these primary cultures, but the NR2A subunit was not. An inhibitor of calpain blocked both the decrease of intact NR2B and the increase of the low molecular weight form, whereas neither caspase nor cathepsin inhibitors had an effect on these events. Cell surface biotinylation experiments demonstrated that the 115 kDa fragment remained on the cell surface. This NR2B fragment was also found in the rat hippocampus after transient forebrain ischemia, showing that this process also occurs *in vivo*. This suggests that calpain-mediated cleavage of the NR2B subunit occurs in neurons and gives rise to active NMDA receptor forms present on the cell surface after excitotoxic glutamatergic stimulation. Such forms could contribute to excitotoxicity and synaptic remodeling.

Key words: **glutamate; calpain; protease; excitotoxicity; NMDA; stroke**

Introduction

Glutamate, the major excitatory neurotransmitter in the CNS, acts at several postsynaptic receptors. One of these, the NMDA receptor, is a cation channel permeable to calcium and sodium. Calcium entering through the NMDA receptor activates many cellular enzymes such as calpain, protein kinases, and neuronal nitric oxide synthase (Lynch and Guttmann, 2002). NMDA receptors play a key role in excitotoxicity, in which their sustained activation leads to excess neuronal cell death (Lynch and Guttmann, 2002).

A series of cDNAs encoding the subunits of the NMDA receptor (designated NR1, NR2A–2D) have been cloned. In most situations, both an NR1 and an NR2 subunit are required for receptor function, with different combinations of subunits producing receptors with distinct properties (Dingledine et al., 1999; Lynch and Guttmann, 2002). Compared with NR1, NR2 subunits possess an extra 50 kDa of protein in the intracellular C terminus. Receptors containing C-terminally truncated NR2 subunits are relatively similar electrophysiologically to native receptors, but mice having receptors lacking only the C-terminal region of NR2A or NR2C have the same abnormal phenotype as animals lacking the entire subunit. This suggests that many NMDA receptor functions require intracellular events mediated by the region within the C terminus (Sprengel et al., 1998).

The C-terminal region of the NR2 subunit binds intracellular anchoring proteins, is necessary for regulation by second messengers, and is a potential substrate for the calcium-activated neutral protease calpain (Kim et al., 1996; Bi et al., 1998a,b; Grant et al., 1998; Zheng et al., 1999; Guttmann et al., 2001, 2002). Calpain regulates numerous proteins, including cytoskeletal components, integral membrane proteins, and receptors, and plays a critical role in long-term potentiation, synaptic plasticity, and excitotoxicity (Wang, 2000). Calpain I (hereafter called calpain) typically cleaves substrates in a limited manner, leading to the formation of large products that remain active (Hell et al., 1996; Johnson and Guttmann, 1997).

The proposed roles of calpain in the brain are directly connected to NMDA receptor activation (Vanderklish et al., 1996). Neuronal calpain is physiologically activated primarily by the NMDA receptor, reflecting its high calcium permeability and relative lack of desensitization (Adamec et al., 1998); however, the physiological substrates of calpain are not well understood. The NR2A subunit is cleaved by calpain *in vitro* and in heterologous systems at two specific sites in the C-terminal region, sug-

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gesting that calpain directly modulates NMDA receptor activity, function, or localization (Bi et al., 1998a,b; Guttmann et al., 2001). Studies examining the cleavage of NMDA receptors in neurons have been equivocal, with relatively long agonist exposures being needed to demonstrate cleavage of NR2 subunits (Bi et al., 1998a,b). Although calpain cleavage *in vitro* is limited to the C-terminal region, neuronal studies have not clearly identified truncated forms of the receptor, and the NR2 subtype specificity is unknown (Bi et al., 1998a,b; Dingledine et al., 1999). To address these questions, we have characterized rapid cleavage of the NR2B subunit in hippocampal neurons after glutamatergic stimulation, identified the region of the protein that is cleaved, and determined that these subunits remain on the cell surface after cleavage. These results address mechanisms of NMDA receptor turnover and may have implications for excitotoxicity.

Materials and Methods

Materials. Glutamate, glycine, ketamine, aprotinin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), and anti-actin were from Sigma (St. Louis, MO); minimal essential medium, penicillin–streptomycin, and glutamine were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Hyclone Laboratories (Logan, UT). Dizocilpine (MK-801) was from Sigma/RBI (Natick, MA). ^{125}I-MK-801 was from Perkin-Elmer Life Sciences (Boston, MA). Human embryonic kidney (HEK) 293 cells were from American Type Culture Collection (Manassas, VA). Immobilized monomeric avidin and sulfo-*N*-hydroxysulfosuccinimide (NHS)-biotin were from Pierce (Rockford, IL). Antibodies from Chemicon International (Temecula, CA) included AB 1516 (NR1, C-terminal, amino acids 909–938) and AB 1557 (NR2B specific, C-terminal, amino acids 984–1104). An N-terminal antibody to amino acids 25–130 of NR2C (A-6475) was from Molecular Probes (Eugene, OR) (Guttmann et al., 2001). This antibody recognizes NR2A and NR2B (both 170 kDa) as well as NR2C (135 kDa) (Guttmann et al., 2001). An NR2A-specific antibody (AB 6313) to the C-terminal 200 amino acids was from Upstate Biotechnology (Lake Placid, NY). AB38, which recognizes calpain-cleaved spectrin, was produced as described previously (Roberts-Lewis et al., 1994). Calpain inhibitor III (CalI3) (MDL 28170; Z-Val-Phe-CHO), caspase inhibitor II (CaspI2) (Ac-Val-Ala-Asp-CHO), cathepsin inhibitor I (Cath I1)(Z-Phe-Gly-NHO-Bz), and porcine calpain I were purchased from Calbiochem (San Diego, CA). Rabbit calpastatin cDNA was a generous gift from Dr. Masatoshi Maki (Nagoya University, Nagoya, Japan). The cDNA for NR2B was from mouse (Lynch et al., 1994; Gallagher et al., 1997).

Preparation of primary neuronal cultures. Embryonic rat hippocampal neurons were prepared by previously described techniques (Wilcox et al., 1994) and include a heterogeneous mixture of pyramidal and nonpyramidal neurons of multiple morphologies. Hippocampal tissue was harvested from Sprague Dawley rat pups (Charles River) on embryonic day 17, gently minced and trypsinized [0.027%; 37°C; 7% CO_2 for 20 min], and then washed with 1× HBSS. The tissue was triturated in warm media (10% defined, heat-inactivated fetal bovine serum), 10% Hams F12 medium, and 80% DMEM with penicillin–streptomycin), and cells were plated at a density of 6×10^5 viable cells per 35 mm culture dish onto poly-L-lysine-coated coverslips. Neurons were maintained at 37°C, 5% CO_2 and fed with neurobasal media with B27 by addition of 50 μl of media into a 2 ml well. Non-neuronal cell growth was inhibited with cytosine arabinoside at 7–10 d *in vitro*. Cells were used after 2–4 weeks in culture.

Incubation in agonists and drug treatments. For studies of calpain cleavage, cells were rinsed twice with 1× PBS and then preincubated for 30 min in HBSS with inhibitors or vehicle. The solution was removed and replaced with an identical solution containing glutamate, glycine (100 μM each for routine experiments), and any inhibitors or vehicles. Drugs were dissolved in stock solutions at 1000× or greater in appropriate vehicles. For studies using MK-801 or ifenprodil, drugs were dissolved in water and used at final concentrations of 100 and 10 μM, respectively. Calpain inhibitor III, caspase inhibitor II, and cathepsin inhibitor I were dissolved in DMSO and used at 10, 3, and 10 μM, respectively. Final DMSO concentration was ≤0.1%. For glutamate dose–response curves, the concentration of glutamate was varied from 1 to 100 μM, with the glycine concentration remaining 100 μM. In selected experiments, cell death was monitored using exclusion of trypan blue (Anegawa et al., 1995). After incubation in agonist, the solution was removed, and cells were immediately scraped into 1× Laemmli stop buffer without bromophenol blue, EGTA, or dithiothreitol (DTT) (Guttmann et al., 2001). Samples were boiled for 5 min, and protein concentrations were determined using the bicinchoninic assay (Pierce). Bromophenol blue and DTT were then added, and the samples were then stored at −20°C until used.

NR2B degradation in vitro *by purified calpain I.* Twenty-four hours after transfection of HEK293 cells, cells were rinsed with PBS and then scraped into 40 mM HEPES, pH 7.2, containing 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml pepstatin. Samples were briefly sonicated on ice and stored in aliquots at −80°C until use. For *in vitro* proteolysis, the homogenates were incubated in the presence of calpain I (2.5 U/ml) for 30 min in a buffer containing 1 mM $CaCl_2$, 5 mM dithiothreitol, and 40 mM HEPES, pH 7.2.

Calpain activity assay with Suc-LLVY-AMC. Twenty-four hours after transfection, cells were rinsed with serum-free medium, and the medium was replaced with serum-free media containing 100 μM glutamate and 100 μM glycine in the presence or absence of 100 μM MK-801 in addition to 80 μM Suc-LLVY-AMC. Cells were then placed in a 5% CO_2 incubator at 37°C. After a 40 min incubation, the plates were read in a Victor^2 fluorescence plate reader (Perkin-Elmer Wallac, Turku, Finland) at wavelength settings of 390 and 460 nm for excitation and emission, respectively. This assay is linear with cell number, and the activity measured is representative of calpain activity observed with protein substrates (Guttmann et al., 1997, 2002; Johnson and Guttmann, 1997).

^{125}I-MK-801 binding. Cell membranes were prepared as described previously (Lynch et al., 1994). Briefly, the membrane fractions were homogenized in assay buffer (20 mM HEPES, pH 7.5, 100 μM glutamate, 100 μM glycine, and 300 μM $MgCl_2$) and incubated at 37°C for 30 min. Homogenates were then centrifuged, and the pellet was resuspended in assay buffer. This process was repeated two more times. Membrane suspensions were then assayed in saturating glycine (100 μM), glutamate (100 μM), spermidine (100 μM), $MgCl_2$ (100 μM), and 300 pM ^{125}I-MK-801 (Lynch et al., 1994). Membranes were harvested onto polyethyleneimine-coated glass fiber filters (Schleicher & Schuell, Keene, NH), and the radioactivity was quantified using a Beckman (model 5500B) gamma counter.

Truncated forms of NR2. Truncated forms of NR2A and NR2B were made by standard molecular biological approaches. NR2B (del 1170–1482) was produced by cleaving the cDNA of full-length ε2 in pRK7 with *Apa*I, isolating the major piece, and ligating it to itself. This yields a form of ε2 containing the first 1170 amino acids of wild-type followed by missense mutations for 20 amino acids before a premature stop codon. NR2B (del 1034–1370) was created by cleavage of ε2 in pRK7 with Esp I (Cel II) and ligation of the major piece back to itself. Truncated forms of NR2A (at amino acids 1279, 1051, and 895) were produced as described previously (Grant et al., 1998; Guttmann et al., 2001). Truncated forms were confirmed by restriction mapping and DNA sequencing.

Transfection of HEK293 cells. HEK293 cells were grown on tissue culture grade dishes (Corning; Corning Glassworks, Corning, NY) in MEM media containing 5% horse serum and 5% fetal bovine serum supplemented with 2 mM glutamine and 100 U/ml penicillin–streptomycin incubated in 5% CO_2 at 37°C. Transfection of HEK293 cells was accomplished by calcium phosphate precipitation as described previously (Grant et al., 1997). Treatments were added 24 hr after transfection. Ketamine (300 μM) was added to the media during transfection to prevent NMDA receptor activation as described previously (Grant et al., 1998).

Western blotting. Thirty micrograms of total protein was loaded on a 7% polyacrylamide gel (10% for experiments examining actin). After SDS gel electrophoresis, proteins were transferred to nitrocellulose, blocked with 3% fetal bovine serum, and incubated with primary anti-

body. Blots were then incubated with appropriate HRP-conjugated secondary antibodies and developed with enhanced chemiluminescence (Pierce).

Biotinylation. After treatment, cultures were placed on ice and rinsed twice with a cold rinsing solution containing PBS, pH 7.5, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ (Sims et al., 2000). Cells were then gently agitated at 4°C for 20 min in rinsing solution containing 1 mg/ml NHS-biotin. Cells were rinsed twice in quenching solution (rinsing solution with 100 mM glycine added) and agitated at 4°C in quenching solution with 10 μM MK-801 for 20 min. Cells were washed and agitated for 1 hr at 4°C in RIPA buffer [150 mM NaCl, 1 mM EDTA, and 100 mM Tris HCl, pH 7.4; 1% (v/v) Triton X-100, 1% (w/v) deoxycholate, 0.1% (w/v) SDS] containing protease inhibitors (1 μg/ml leupeptin, 250 μM PMSF, 1 μg/ml aprotinin, 1 mg/ml trypsin inhibitor, and 1 mM iodoacetamide). Samples were harvested and centrifuged at 12,400 rpm for 20 min at 4°C. Separate aliquots of the lysate were taken for total lysate fraction, protein quantification, and incubation in avidin beads overnight at 4°C. After overnight incubation, samples were centrifuged at 12,400 rpm for 15 min in a microfuge, and the supernatant (the intracellular fraction) was removed. Sample solubilizing buffer (4×; 8%SDS, 240 mM Tris, pH 6.8, 0.04% bromophenol blue, 40% glycerol, 4% 2-mercaptoethanol) was added to both the total lysate and intracellular fractions. Avidin beads were then washed four times with RIPA buffer and incubated in 2× sample solubilizing buffer for 30 min at room temperature. Samples were then centrifuged for 5 min at 12,000 rpm, and the supernatant (the extracellular fraction) was removed and stored at −20°C. Thirty micrograms of each fraction was subjected to SDS-PAGE and Western blot analysis.

Image quantitation. Autoradiograms generated from Western blots were scanned using an Epson scanner in conjunction with Adobe Photoshop (Guttmann et al., 2001). Data was quantitated using imaging densitometry and analyzed using the NIH Image program. For quantitation of calpain-generated receptor products, values were normalized to maximal cleavage level for statistical analysis. For degradation of NMDA subunits, samples were normalized to the 30 min 100 μM MK-801 control condition on a given blot.

Statistical analysis. Statistical differences were determined by ANOVA or *t* tests using Instat software and considered significant at $p < 0.05$. For comparisons in groups of more than two samples, ANOVA was initially used, followed by serial *t* tests if the ANOVA value was significant.

Transient forebrain ischemia. All animal procedures were performed in accordance with *NIH Guidelines for the Care and Use of Laboratory Animals* and were approved by our Institutional Animal Care and Use Committee. Male Long–Evans rats (400–500 gm) were subjected to 10 min of transient forebrain ischemia using an established model initially described by Smith et al. (1984), modified by Gionet et al., (1992), and used routinely in our laboratory (Neumar et al., 2001; Zhang et al., 2002).

Briefly, rats were anesthetized with 1–1.5% halothane, orotracheally intubated, and mechanically ventilated with 30% O_2 and 70% N_2O. P_{CO_2} values were kept between 35 and 45 mmHg, and temperature was maintained at 37.0–37.5°C throughout the procedure. Femoral arterial and venous catheters were placed by cut-down, and carotid arteries were exposed using aseptic technique. Transient forebrain ischemia was initiated by the combination of bilateral carotid occlusion and hypovolemic hypotension to a mean arterial blood pressure (MABP) of 30 mmHg. Hypovolemic hypotension was achieved by rapidly withdrawing blood from the femoral arterial catheter into a heparinized syringe and maintained during the ischemic period by withdrawal or infusion of blood through the femoral venous catheter. Once an MABP of 30 mmHg is achieved, both carotid arteries were reversibly occluded with surgical aneurysm clips. The duration of ischemia was timed from when the aneurysm clips were placed. After 10 min of ischemia the aneurysm clips were removed and shed blood was reinfused. Rats were maintained on mechanical ventilation for 1 hr, after which vascular catheters were removed, surgical wounds were closed, and animals were extubated. Sham (uninjured) animals were subjected to anesthesia and surgical preparation without bilateral carotid occlusion and hypovolemic hypotension.

Rats were killed 48 hr after injury, and brains were rapidly microdissected on an ice-chilled plate. The dorsal hippocampus was homogenized in 10:1 v/w homogenization buffer containing 10 mM HEPES, pH 7.4, 320 mM sucrose, 2.0 mM EGTA, 0.5 mM $MgSO_4$, 2.0 mM 2-mercaptoethanol, 2.0 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin, and 0.1 mM PMSF 0.1 μg/ml Z-VAD-FMK. To generate synaptosomal fractions, hippocampal homogenates were centrifuged at 900 × *g* for 10 min two times, and the pooled supernatant was centrifuged at 17,000 × *g* for 45 min two times. The 17,000 × *g* pellet was then resuspended in homogenization buffer, layered over a 20/40% discontinuous sucrose gradient, and centrifuged at 63,700 × *g* for 45 min. The band at the interface was then centrifuged at 20,000 × *g* for 30 min, and the pellet was resuspended in homogenization buffer. Protein concentration was determined by the Bradford method. Samples with diluted 1:1 in 2× Laemmli buffer and boiled for 5 min and then snap-frozen in dry ice–ethanol slurry until time of analysis.

Results

Activation of calpain in hippocampal cultures

To establish NMDA receptor-mediated calpain activation in hippocampal neurons, we incubated neuronal cultures in glutamate and glycine, followed by harvesting and Western blotting with an antibody (AB38) directed at the calpain-generated spectrin degradation product (Roberts-Lewis et al., 1994). The spectrin degradation fragment was produced rapidly, within 5 min of agonist application, and levels were markedly elevated after 30 min of agonist treatment. This increase was inhibited by the inclusion of the NMDA receptor antagonist MK-801 (100 μM) ($p < 0.0001$ for 0 min compared with 30 min; and for MK-801 with 30 min; $n = 13$) (Fig. 1*A*,*B*). CalI3 also prevented the increase of the degradation product, whereas CasI2 and CathI1 did not (Fig. 1*C*). No inhibition was observed in agonist plus vehicle treatment (data not shown). This confirmed that NMDA receptor activation resulted in calpain activity in this preparation. Although both NR2A and NR2B subunits were present in this population (Fig. 1*D*), NMDA receptor-mediated calpain activity was not significantly inhibited by 10 μM ifenprodil, an NR2B-selective antagonist (Fig. 1*C*) (Williams, 1993; Lynch et al., 1994, 1995). This suggested that receptors containing NR2B alone (commonly felt to be mostly extrasynaptic) are not necessary for activating calpain in this preparation (Stocca and Vicini, 1998; Tovar and Westbrook, 1999; Li et al., 2002).

Cleavage of NR2 subunits in cultured hippocampal neurons

We then investigated whether NR2 subunits might be rapidly cleaved under conditions that clearly activate calpain. Hippocampal neurons were incubated in glutamate and glycine and analyzed by Western blots using an N-terminal antibody (A-6475) that recognizes NR2A, NR2B, and NR2C subunits in transfected cells (Guttmann et al., 2001). In hippocampal neurons, this antibody recognized an incompletely resolved doublet species at 170 kDa (designated NR2A/2B) that comigrated with NR2A and NR2B identified by subtype-selective antibodies (Fig. 1*D*). No 135 kDa species characteristic of NR2C was observed, consistent with the lack of NR2C in forebrain (Nakanishi, 1992; Guttmann et al., 2001). We observed a decrease in full-length NR2A/2B immunoreactivity after 30 min agonist treatment ($p = 0.016$ for 30 min vs 0 min; $n = 13$) with the simultaneous increase in levels of a low molecular weight (LMW) species of 115 kDa (0 vs 30 min = 0.0044; $n = 12$) (Fig. 2*A*). Levels of this LMW species increased within 5 min of agonist application and were stable over time. The increased levels of the LMW fragment correlated temporally with the decrease of full-length NR2A/2B. In addition, there was a strong correlation between decreases of full-length NR2A/2B and the increases in levels of the LMW fragment in individual experiments ($R_s = -0.70$; $p = 0.007$; $n = 13$), further showing that the decrease of full-length and the increase of

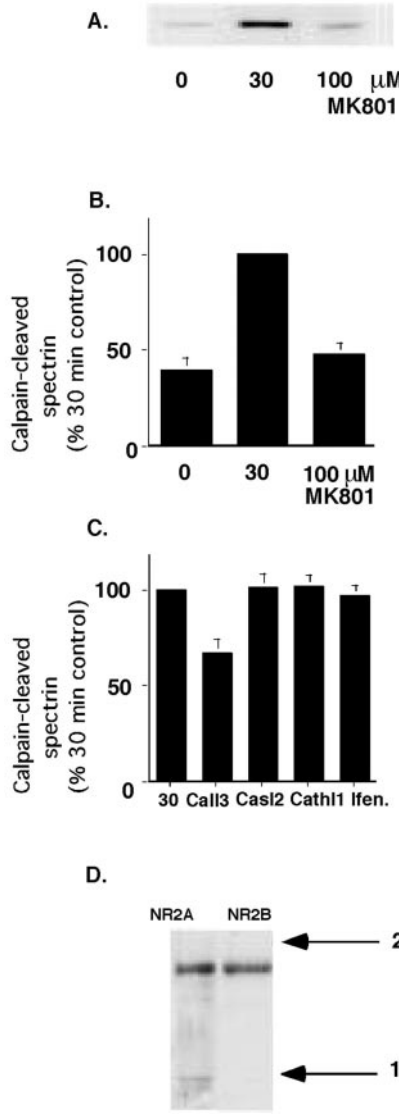

Figure 1. NMDA receptor-mediated calpain activation in hippocampal neurons. Thirty micrograms of total protein were separated by SDS-PAGE and probed for the calpain-produced spectrin degradation product (AB38). A representative Western blot and bar graph (*A*, *B*) demonstrate elevated levels of this product after stimulation of NMDA receptors with 100 μM glutamate and 100 μM glycine for 30 min, and inhibition of this effect by 100 μM MK-801 ($p <$ 0.0001 for 0 min compared with 30 min; and for MK-801 with 30 min; $n = 13$). The production of the spectrin degradation product was also inhibited by the presence of CaII3 in the treatment conditions, but not by CasI2 or CathI1 ($p = 0.0009$ by ANOVA; $p = 0.0014$ for CaII3 vs 30 min; $p =$ n.s. for CasI2 and CathI1; $n = 10$) (*C*). When NR2B-containing receptors were selectively inhibited by ifenprodil (Ifen.), no change was found in production of the spectrin degradation product as shown in *C*. Blots probed with subunit-specific antibodies demonstrated the presence of both NR2A (AB 6313) and NR2B (AB 1557) in this population, as shown in *D*. These results suggest that receptors containing only 2B are not required for activating calpain in hippocampal neurons. Error bars on bar graphs indicate SEM.

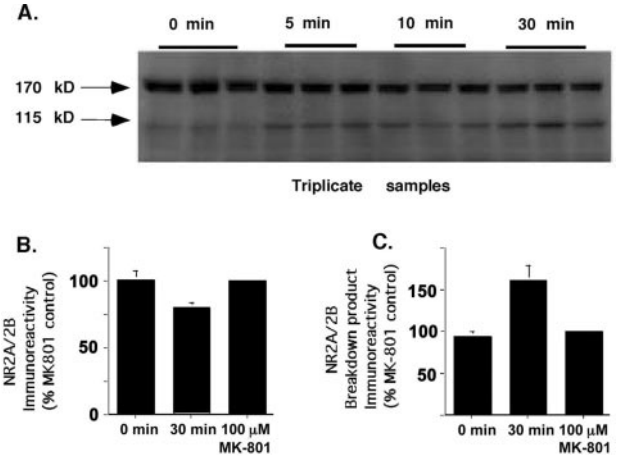

Figure 2. Effects of calpain activation on NR2 subunits in neurons. Neurons were exposed to NMDA receptor agonist stimulation over a 0–30 min time course and subjected to Western blot analysis with an N-terminal antibody that recognizes NR2A, NR2B (both 170 kDa), and NR2C (135 kDa) subunits. A representative Western blot (*A*) demonstrates the decrease in full-length NR2A/2B (170 kDa) ($p = 0.016$ for 30 vs 0 min; $n = 13$) as well as the simultaneous increase in levels of a 115 kDa species (0 vs 30 min = 0.0044; $n = 12$). This blot was overexposed to show more clearly both the breakdown product and the decrease of full-length NR2A/2B. The increase in the LMW species was inhibited by inclusion of 100 μM MK-801 in the treatment conditions ($p = 0.0135$ MK-801 vs 30 min; $n = 12$) (*C*). Inclusion of 100 μM MK-801 in the treatment conditions also prevented the decrease of full-length NR2A/2B (MK-801 vs 30 min = 0.0004; $n = 12$). NR2 immunoreactivity was quantitated as a percentage of the MK-801 control condition.

the LMW fragment were associated phenomena. Inclusion of 100 μM MK-801 in the treatment conditions prevented the decrease in full-length NR2A/2B (MK801 vs 30 min = 0.0004; $n = 12$) Fig. 2*B*) and the increase in levels of the LMW fragment ($p = 0.0135$ MK-801 vs 30 min; $n = 12$) (Fig. 2*C*). Given the size of the LMW fragment and the location of the N-terminal epitope (amino acids 25–130), this fragment must be derived from C-terminal cleavage of NR2A or NR2B, or both, matching the results in heterologous systems (Guttmann et al., 2001, 2002). No significant cell death using Trypan blue exclusion was noted under the 30 min glutamate and glycine condition compared with cells protected by MK-801, suggesting that the cleavage is not merely an effect of cell lysis (data not shown).

To determine the selectivity of NR2 subunit cleavage, samples were probed for NR1 and actin, which are both poor calpain substrates (Guttmann et al., 2001, 2002). Neither actin (Fig. 3*A*) nor NR1 (Fig. 3*B*) was degraded. These data suggest that at least one NR2 subunit is rapidly cleaved in the C-terminal region in hippocampal neurons after NMDA receptor stimulation and that this is not a generalized effect on all proteins. Because the N-terminal antibody recognizes both NR2A and NR2B (which have similar molecular weights), we sought to identify which NR2 subunit was cleaved in this process. An NR2B-specific antibody demonstrated a 45% decrease of full-length NR2B immunoreactivity ($p = 0.0021$, 30 min vs MK-801; $p = 0.0020$, 30 vs 0 min; $n = 7$) (Fig. 3*C*,*D*) with the same time course but greater in amount than the decrease of full-length NR2A/2B immunoreactivity (22%) (Fig. 2). In contrast, no change was seen in NR2A levels using a subunit-selective antibody to the C terminus of NR2A (Fig. 3*E*). This identifies NR2B as the major subunit cleaved after NMDA receptor activation. Decreases in levels of NR2B immunoreactivity and an increase in the levels of the LMW fragment were also produced by incubation with lower levels of glutamatergic stimulation (as low as 1 μM glutamate) and matched the cleavage of spectrin by calpain in a glutamate dose–response curve (Fig. 3*F*).

No shorter fragment was observed using the NR2B-selective antibodies, suggesting either that the product of NR2B cleavage is not stable in neurons or that the cleavage of NR2B occurs within the epitope of the 2B-selective antibody (amino acids 984–1104), roughly the expected site of cleavage on the basis of the LMW species in Figure 2. We sought to confirm that the cleavage was within the epitope of the NR2B antibody by comparing the molecular weight of the LMW fragment with truncated forms of

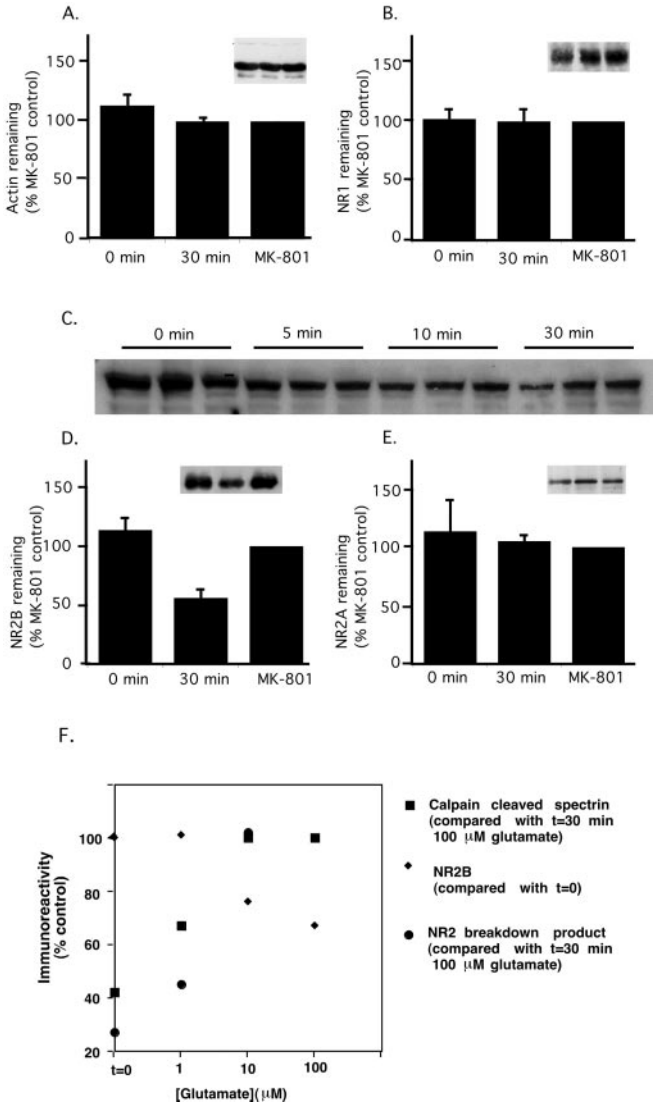

Figure 3. Selective cleavage of NR2B subunits. To determine which subunits were degraded after activation of calpain by the NMDA receptor, samples treated with 100 μM glutamate and glycine in the presence or absence of 100 μM MK-801 were immunoblotted with NR1 (*B*), NR2A (*E*), and NR2B (*C*, *D*) selective C-terminal antibodies as well as with an anti-actin (*A*) antibody. Representative Western blots (shown as insets for 0 min, 30 min, and MK-801 control in *A*, *B*, *D*, *E*) and bar graphs for each are shown. Actin showed no loss of immunoreactivity under the treatment conditions (*A*) nor did NR1 (*B*). NR2B showed a 45% decrease of immunoreactivity in the 30 min condition compared with 0 min and samples including MK-801 (*C*, *D*) ($p = 0.0021$ vs MK-801; $p = 0.0020$ vs 0 min; $n = 7$). NR2A (*E*) showed no decrease of immunoreactivity in the treatment condition. Decrease of NR2B subunit immunoreactivity and the increase of the LMW form was also produced by lower levels of agonist and matched the production of calpain-cleaved spectrin in glutamate dose–response curves (*F*).

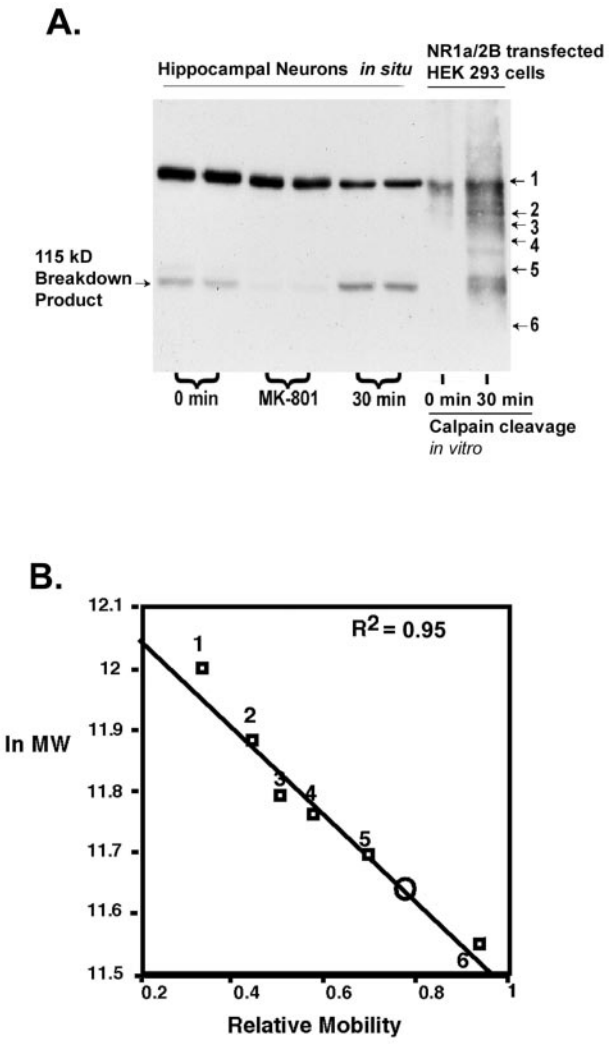

Figure 4. Characterization of the size of the NR2A/2B breakdown fragment. Neurons were treated with agonists as described in Materials and Methods and compared with NR2B cleaved *in vitro* with calpain (from receptors produced in HEK293 cells) using Western blotting with the N-terminal antibody. The size of the LMW breakdown product in neurons matched that of the smallest calpain-generated fragment of NR2B produced by *in vitro* cleavage. The LMW fragment was also compared on parallel blots with full-length NR2A and five C-terminally truncated forms of NR2A or NR2B of known size (*A*, 1–6): 1 = full-length NR2A (170 kDa); 2 = NR2A1279 (144 kDa); 3 = NR2B1170 (133 kDa); 4 = NR2Bdel1034–1370 (127 kDa); 5 = NR2A1051 (120 kDa); 6 = NR2A895 (104 kDa). The mobility plot (*B*) suggests that the LMW form (○) has an MW of 114 kDa or ~1030 amino acids. It is also of note that the LMW fragment can be present under basal conditions to some degree (*A*, first 2 lanes) in a manner that is inhibited by inclusion of MK-801.

NR2 subunits produced in transfected HEK293 cells (Fig. 4*A*). When we compared the LMW form with NR2A subunits truncated at amino acids 895 (104 kDa), 1051 (120 kDa), 1279 (144 kDa), and NR2B lacking amino acids 1038–1370 (127 kDa), or truncated at amino acid 1170 (133 kDa), the LMW form was ~1030 amino acids (Fig. 4*B*). This confirms that the LMW fragment is likely not to be recognized by the NR2B selective antibody. We also compared the molecular weight of the LMW fragment with that produced by cleavage of NR2B by calpain *in vitro* (Fig. 4*A*,*B*). Although NR2B was cleaved by calpain *in vitro* at several sites in the C-terminal region, the size of the smallest product was identical to that produced in hippocampal neurons, suggesting that calpain is the protease giving rise to this fragment. NR1/2B receptors cleaved by calpain *in vitro* retained the ability to bind ^{125}I-MK801 (99.7 ± 20.1% of control), suggesting that, as found in other reports, receptors containing NR2B subunits of this size should be active (Liao et al., 2001).

We also sought to confirm directly that calpain was the protease involved in NR2 subunit degradation in cultured hippocampal neurons. CalI3 ($p = 0.048$ by ANOVA; $n = 7$; $p = 0.0021$ CalI3 vs 30 min) but not inhibitors of caspases or cathepsin ($p > 0.1$; $n = 5$–6 for both) prevented the change in levels of full-length NR2B (Fig. 5*A*). Similarly, using the N-terminal antibody (A6475), degradation of total NR2A/2B was inhibited by CalI3 ($p = 0.028$ by ANOVA; $n = 10$; $p = 0.018$ CalI3 vs 30 min) but not inhibitors of caspases or cathepsin ($p > 0.1$; $n = 9$–10 for

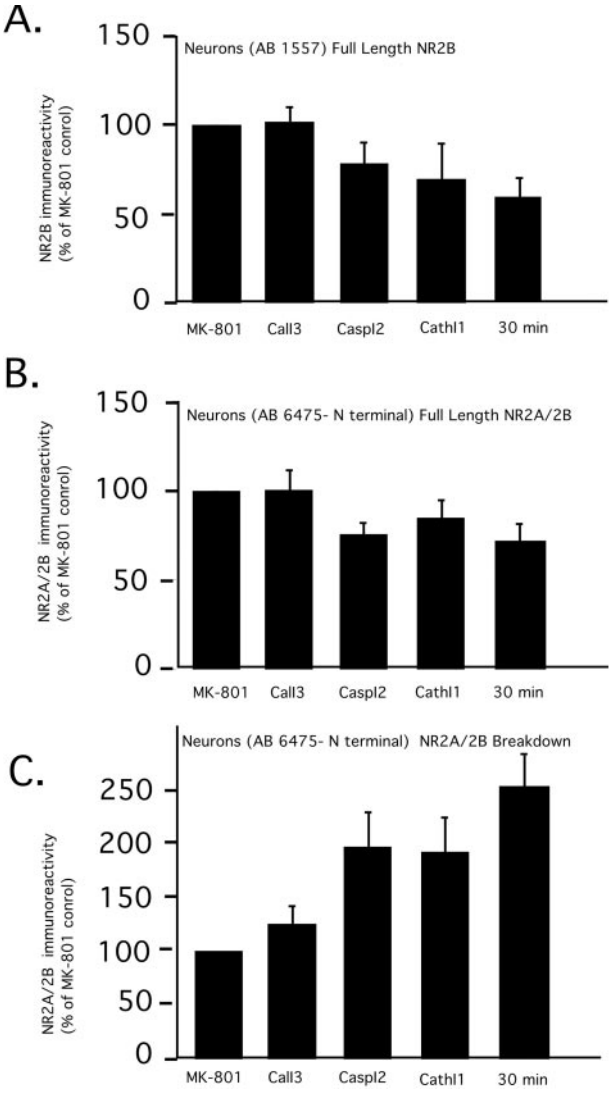

Figure 5. Identification of the protease involved in NR2B subunit cleavage. Neurons treated with 100 μM glutamate and glycine for 30 min in the presence of calpain inhibitor III (CalI3), caspase inhibitor II (CaspI2), or cathepsin inhibitor I (CathI1). The inhibition of calpain ($n = 7$; $p = 0.002$; CalI3 vs 30 min) but not caspases or cathepsin ($p > 0.1$; $n = 5–6$ for both) prevented the decrease of full-length NR2B (*A*). Likewise, using the N-terminal antibody, degradation of total NR2A/2B was significantly inhibited by the calpain inhibitor ($n = 10$; $p = 0.02$; CalI3 vs 30 min) (*B*) as was the increased appearance of the LMW fragment (*C*) ($n = 9$; $p = 0.004$ CalI3 vs 30 min). Levels of the LMW form were normalized to the breakdown product in the MK-801 control such that increases in levels appear as increases over the control level of 100%.

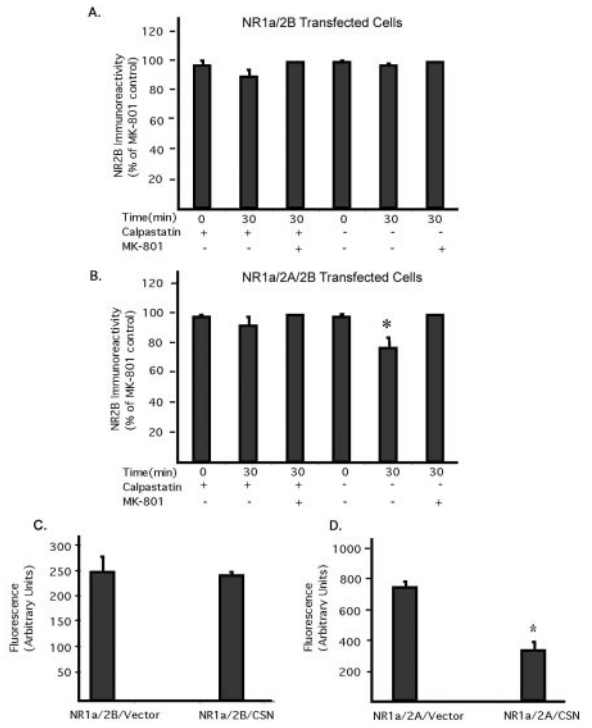

Figure 6. Cleavage of NR2B in heterologous systems. NR1a/2B-transfected HEK293 cells that were cotransfected with calpastatin (CSN) or control vector showed no change in NR2B immunoreactivity after 30 min agonist treatment with or without inclusion of MK-801 (*A*). Cells transfected with NR1a/2A/2B, however, showed decreased NR2B immunoreactivity after 30 min of agonist treatment ($p = 0.024$; $n = 5$) that was inhibited by cotransfection with calpastatin and by inclusion of MK-801 in the treatment conditions (*B*). Calpain activity was measured in NR1a/2A- or NR1a/2B-transfected cells that were cotransfected with calpastatin or control vector. Cells were treated by the stimulation of NMDA receptors with 100 μM of both glutamate and glycine for 40 min, and the amount of fluorescence was quantitated. Agonist treatment of NR1a/2B-transfected cells compared with calpastatin-cotransfected cells resulted in no significant difference in fluorescence (*C*), whereas treatment of NR1a/2A-transfected cells resulted in increased cleavage of the substrate Suc-LLVY-AMC as shown by increased fluorescence (*$p = 0.004$; $n = 3$) (*D*).

both) (Fig. 5*B*). We observed less of an appearance of the LMW fragment of NR2A/2B with the CalI3 ($p = 0.0022$ by ANOVA; $n = 9$; $p = 0.004$ CalI3 vs 30 min) (Fig. 5*C*). Caspase and cathepsin inhibition did not significantly reverse production of the LMW fragment ($p > 0.10$; $n = 7–8$). These data show that calpain is the major protease leading to the decrease in levels of full-length NR2B and total NR2A/2B and the increases in the truncated form of NR2.

Cleavage of NR2B in heterologous systems

Cleavage of NR2B is consistent with *in vitro* data, but only NR2A has been shown to be degraded *in situ* in heterologous systems (Bi et al., 1998; Guttmann et al., 2001, 2002). Thus, we tested whether NR2B also might be cleaved by calpain in other cellular models. When HEK293 cells were transfected with NR1/2B and treated with glutamate and glycine, NR2B was not degraded (Fig. 6*A*). In contrast, in NR1/2A/2B-transfected cells, NR2B was degraded ($p = 0.0237$; $n = 5$) as demonstrated by a decrease of full-length NR2B immunoreactivity using the NR2B-specific antibody (Fig. 6*B*). This effect was inhibited by cotransfection of calpastatin, the specific endogenous calpain inhibitor. This appeared to reflect the failure of NR2B-containing receptors to activate calpain after agonist application because no cleavage of the calpain substrate Succ-LLVY-AMC was found in NR1/2B-transfected cells, whereas NR1/2A transfection led readily to Succ-LLVY-AMC degradation ($p = 0.0053$; $n = 3$) (Fig. 6*C*,*D*). This suggests that in transfected cell models, as in cultured neurons, NR2B can be cleaved by calpain, but only if the NR2A subunit is present to facilitate calpain activation.

Localization of cleaved NR2B

We also sought to establish the intracellular or extracellular localization of the LMW fragment produced by NR2A/2B cleavage. When hippocampal neurons were treated with glutamate and glycine, and cell surface components were labeled with NHS-biotin, the total cellular lysate contained increasing levels of the 115 kDa breakdown product with time, whereas full-length levels declined in the total lysate (Fig. 7*A*). Seventy to eighty percent of

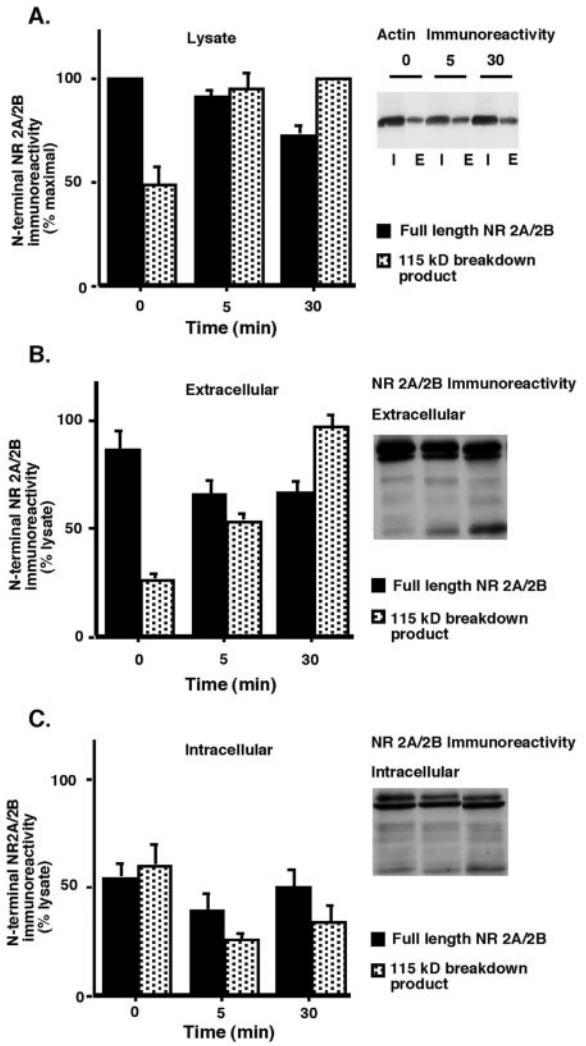

Figure 7. Localization of cleaved NR2B. Hippocampal neuron cultures were preincubated with 100 μM glutamate and glycine for 0, 5, or 30 min ($n = 4$). Cells were then labeled with NHS-biotin under non-lytic conditions, and the extracellular and intracellular fragments were separated. Thirty micrograms of total protein were separated by SDS-PAGE and immunoblotted with an N-terminal antibody for NR2. The amount of immunoreactivity in the lysate was quantified as a percentage of the amount of full-length NR2A/2B found at the 0 min time point or the amount of the LMW fragment found in the 30 min time point. For extracellular and intracellular fractions, the immunoreactivity was quantified as a percentage of the amount in the total lysate at that time point. Over time, full-length NR2A/2B in both the total lysate (*A*) and extracellular (*B*) fractions decreased, and the level of the LMW fragment in the extracellular fraction increased with time (*B*). So that we may assume preservation of membrane integrity in biotinylation of these cultures, samples were probed with an anti-actin antibody to confirm that actin remains primarily in the intracellular fraction; 70–80% of the actin was found in the intracellular fraction (I) at 0, 5, and 30 min as shown by a representative blot (*A*). The intracellular full-length NR2A/2B fraction does not significantly change during treatment (*C*). Likewise, the small amount of the LMW fragment found in the intracellular fraction remains relatively constant; however, the percentage of LMW fragment in the intracellular fraction decreased over time because the amount of the LMW fragment increased with time in the total lysate fraction. In these experiments the amount of full-length extracellular NR2A/2B decreased from 88 ± 4 to 51 ± 5% of the total extracellular NR2A/2B over the 30 min incubation. Because each fraction is normalized to the level in the lysate, the total of intracellular and extracellular fractions can be slightly different from 100%, reflecting small nonlinearities in quantification across substantially different optical density values.

actin (a marker of intracellular protein) was found in the intracellular fraction (Fig. 7*A*) at all times after agonist activation, demonstrating that the treatment conditions do not cause cell lysis. In the same experiments, the increase in the LMW fragment was found in the biotinylated fraction, characteristic of a cell surface location ($p = 0.0002$). The LMW fragment increased in the cell surface fraction within 5 min of agonist application (Fig. 7*B*). In contrast, only a small amount of LMW fragment was present in the intracellular fraction, and its level remained constant over the 30 min time course (Fig. 7*C*). Full-length NR2A/2B declined over the time course, decreasing from the extracellular fraction ($p = 0.045$). Approximately 61% of the full-length NR2 immunoreactivity was found in the extracellular fraction under basal conditions. The extracellular level of full-length NR2A/2B declined from 88 ± 4 to 51 ± 5% of the total extracellular NR2A/2B in these experiments, suggesting that 37% of the NR2 immunoreactivity was cleaved in these experiments. These data suggest that activation of the NMDA receptor in neurons leads to cleavage of the NMDA receptor by calpain to produce large potentially active fragments that remain on the cell surface.

Cleavage of NR2 in models of ischemia

Because calpain appears to cleave NR2B rapidly in cell culture models of excitotoxicity, we then sought to determine whether such events occur in animal models of human excitotoxic diseases such as ischemia. We examined the possibility of calpain cleavage of NR2A/2B subunits in hippocampus after transient ischemia. In this model, which uses a transient bilateral carotid occlusion, calpain is activated rapidly and in a delayed manner 24–48 hr after initial ischemia (Neumar et al., 2001). When we examined levels of NR2 in ischemic and control hippocampus 48 hr after transient ischemia using Western blotting with the N-terminal antibody, this antibody demonstrated increased levels of a low molecular weight form of NR2 in ischemic tissue (Fig. 8*A*,*C*) ($p = 0.002$), matching that produced by calpain in cell culture, as well as an increase in the ratio of LMW form to full-length NR2A/2B after ischemia (Fig. 8*B*) ($p = 0.002$). Thus, cleavage of the NMDA receptor by calpain appears to occur in excitotoxic events of the brain as well as in cell culture models.

Discussion

The present study demonstrates NMDA receptor activation-induced cleavage of the NR2 subunit by calpain in neurons after glutamatergic stimulation. The major cleaved subunit in cultured hippocampal neurons appears to be NR2B, with no detectable cleavage of NR2A and no cleavage of NR1. In addition, the cleavage of NR2B creates a fragment that remains on the extracellular surface after its production. This fragment should be active on the basis of predictions from its molecular weight and the lack of effect of calpain cleavage on ^{125}I-MK-801 binding to NR1/2B receptors (Liao et al., 2001). This suggests that novel, active cleavage products of the NR2B subunit are created during excitotoxic conditions. Cleavage of the C-terminal region by calpain would remove sites critical for NMDA receptor modulation by protein kinase C (S1303 and S1323), tyrosine kinase (many sites), and calcium-calmodulin-dependent kinase (S1303) (Omkumar et al., 1996; Liao et al., 2001). Because this region also contains the binding site for postsynaptic density-95 (PSD-95), calpain-cleaved NR2B subunits would no longer be attached to PSD-95, an association thought to be involved in NR2B turnover (Roche et al., 2001). Thus, calpain cleavage would create NR2B subunits that are no longer under the control of modulatory elements, similar to effects of calpain noted previously on protein kinase C in long-term potentiation (Hrabetova and Sacktor, 1996). These receptors could still be modulated by sites in coassembled subunits (NR1, NR2A), however, and still may be attached to

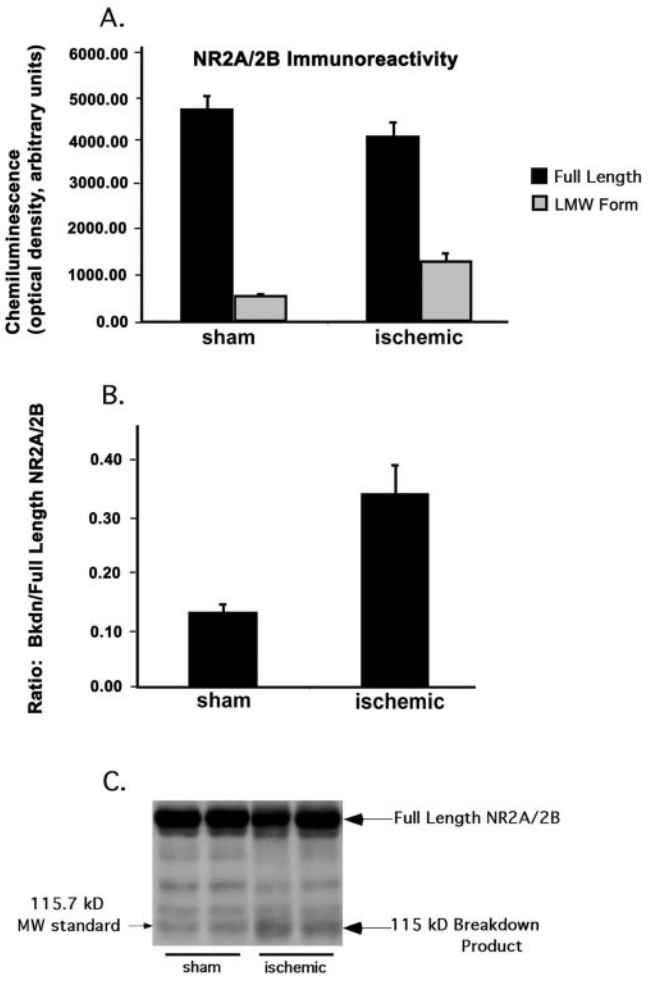

Figure 8. Cleavage of NR2 After transient ischemia. The hippocampus of control and ischemic rats was examined by Western blotting with an N-terminal antibody to NR2. Samples were obtained 48 hr after either 10 min transient forebrain ischemia or sham operation. In ischemic samples, this antibody demonstrated greater levels of a low molecular weight form of NR2 (*A*), matching that produced by calpain in neuronal culture ($p = 0.002$). There was also an increase in the ratio of the LMW form to full-length NR2A/2B in the ischemic samples (*B*) ($n = 7$) ($p = 0.002$). A representative Western blot (*C*) comparing NR2A/2B full-length and breakdown product in synaptosomal fractions of sham and ischemic hippocampus 48 hr after ischemia shows the presence of an LMW fragment in ischemic samples.

PSD-95 by coassembled NR2A. This would create heteromeric receptors with potentially complex modulation and turnover properties (Sheng et al., 1994).

The present results differ from our results in transfected cells in several ways. First, in NR1a/2A-transfected cells, NR2A subunits are readily cleaved after stimulation with glutamate and glycine (Guttmann et al., 2001, 2002), whereas NR2B cleavage in HEK293 cells appears to require cotransfection with NR2A. In contrast, activation of NR2A-containing receptors appears important for calpain activation, but NR2A itself is not cleaved in our neuronal model (Guttmann et al., 2001, 2002). This suggests that other neuronal factors prevent NR2A from being readily cleaved. Such factors may include differing levels of phosphorylation between NR2A and NR2B, because phosphorylation alters calpain cleavage of NR2 subunits *in vitro* (Bi et al., 1998a,b). NR2A subunits are also preferentially associated with synapses in neuronal culture, and synaptic receptors may be less accessible to proteolytic degradation (Stocca and Vicini, 1998; Tovar and Westbrook, 1999; Li et al., 2002); however, the role of NR2A in activation of calpain (implying a synaptic activation of calpain) and the presence of cleaved NR2B in the synaptosomal fraction of ischemic animals suggest that simple synaptic versus extrasynaptic localization cannot explain the present data. The present data are overall more consistent with cleavage of synaptic NR2B, possibly when coassembled with NR1 and NR2A. Additional experiments may explore these possibilities. Interestingly, inhibition of the proteosome over longer time periods in conjunction with synaptic activation of NMDA receptors preferentially leads to preservation of NR2B with little change in NR2A (Ehlers, 2003). This suggests the presence of mechanisms stabilizing NR2A from multiple proteases.

In addition, cleavage of NR2 subunits in transfected cells is a degradative process because calpain-produced cleavage products of NR2A were not observed, whereas calpain-generated products of NR2 are found in neurons (Guttmann et al., 2002). Neurons may make stabilizing "factors" (e.g., kinases) that allow persistence of truncated forms of the NR2 subunit, or they could have more tightly controlled proteolytic activity for receptor degradation subsequent to calpain cleavage. Alternatively, the stable fragment in neurons is a component of NR2B rather than NR2A and thus may be inherently more stable than the cleavage products of NR2A predicted from heterologous systems. Finally, the endocytic mechanisms and internalization processes that cause rapid NR2 destruction in HEK293 cells may be substantially slower in neurons. Neurons, and particularly dendrites (where NMDA receptors are located), contain structural features that may impede rapid turnover of proteins in response to a dramatic stimulus. Clathrin-mediated endocytosis is restricted to specialized locations in dendrites (Blanpied et al., 2002). If NR2 receptors are internalized through endosomes, then the relative lack of endosomes in single dendrites would prevent rapid internalization of large quantities of receptor, particularly under excitotoxic conditions in which protein degradation by calpain would be increased and cytoskeletal damage is present (Cooney et al., 2002; Buddle et al., 2003). Experiments directly testing these possibilities may determine the exact reason for differences between neurons and HEK293 cells.

One unexpected finding of the present study was that NR2A-containing receptors appear to activate calpain more readily than NR2B-containing receptors in transfected cells and possibly in neurons. This may reflect the greater rise in calcium and greater opening probability of NR2A-containing receptors compared with NR2B-containing receptors in transfected cells (Grant et al., 1998; Chen et al., 1999). When neuronal calpain activity has been measured, it is typically highest early in development, with subsequent decline. This does not clearly match the developmental pattern of NR2 subunits, with NR2B representing the major prenatal subunit, and increasing levels of NR2A over the first few weeks of life in the rodent. Because measurements of calpain activity are frequently made on the basis of cleavage of distinct protein substrates, the temporal course of calpain development may reflect both enzymatic and substrate considerations. Our data showing a greater role of NR2A in calpain activation are consistent with this possibility.

In most experimental paradigms, calpain cleavage of NMDA receptor subunits in neurons has been noted after prolonged agonist stimulation, and no N-terminal fragments have been characterized (Bi et al., 1998a,b). In the present study, we have identified a stable N-terminal fragment and demonstrated rapid cleavage of the NR2B subunit (<5 min). Although conditions used here are excitotoxic, the present changes are too rapid to represent purely cell death responses, and the cells are not lysed

by this treatment as indicated by the lack of actin labeling by NHS-biotin. In addition, the changes that we note also begin to occur at lower levels of glutamatergic stimulation (1 μM) than used in fully excitotoxic conditions, and the LMW fragment appears to a small degree in basal levels of untreated cultures (Stout et al., 1998; Hashimoto et al., 2002). Previously, calpain has been demonstrated to mediate synaptic remodeling and restructuring in the period after glutamatergic stimulation (Faddis et al., 1997). The present results suggest that calpain-mediated cleavage of the NR2B subunit may be involved in early events of excitotoxicity and neuronal remodeling after glutamatergic stimulation, including that noted in ongoing synaptic activity.

Finally, we demonstrate that the calpain-generated fragments of the NR2 subunit are also generated *in vivo* after transient forebrain ischemia. Such receptors could potentially contribute to postischemic cytotoxicity and delayed neuronal death. NMDA receptors with novel properties have been reported previously in animal models of excitotoxicity (Lieberman and Mody, 1999). More detailed analysis of these paradigms is needed to determine whether the calpain-generated forms of NMDA receptor noted here represent these unusual physiologic forms.

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