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Transcription of the NR1 Subunit of the *N*-Methyl-D-aspartate Receptor Is Down-regulated by Excitotoxic Stimulation and Cerebral Ischemia*

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The *N*-methyl-D-aspartate (NMDA) type of glutamate receptor (NMDAR) plays central roles in normal and pathological neuronal functioning. We have examined the regulation of the NR1 subunit of the NMDAR in response to excessive activation of this receptor in *in vitro* and *in vivo* models of excitotoxicity. NR1 protein expression in cultured cortical neurons was specifically reduced by stimulation with 100 μM NMDA or glutamate. NMDA decreased NR1 protein amounts by 71% after 8 h. Low NMDA concentrations (≤10 μM) had no effect. NR1 down-regulation was inhibited by the general NMDAR antagonist DL-AP5 and also by ifenprodil, which specifically antagonizes NMDARs containing NR2B subunits. Arrest of NMDAR signaling with DL-AP5 after brief exposure to NMDA did not prevent subsequent NR1 decrease. Down-regulation of NR1 did not involve calpain cleavage but resulted from a decrease in *de novo* synthesis consequence of reduced mRNA amounts. In contrast, NMDA did not alter the expression of NR2A mRNA or newly synthesized protein. In neurons transiently transfected with an NR1 promoter/luciferase reporter construct, promoter activity was reduced by 68% after 2 h of stimulation with NMDA, and its inhibition required extracellular calcium. A similar mechanism of autoregulation of the receptor probably operates during cerebral ischemia, because NR1 mRNA and protein were strongly decreased at early stages of blood reperfusion in the infarcted brains of rats subjected to occlusion of the middle cerebral artery. Because NR1 is the obligatory subunit of NMDARs, this regulatory mechanism will be fundamental to NMDAR functioning.

The *N*-methyl-D-aspartate (NMDA)[5] type of glutamate receptor (NMDAR) plays key roles in neuronal plasticity, learning, and memory in the central nervous system, most of which are related to its high permeability to Ca^{2+} (1). However, excessive activation of NMDARs induces excitotoxic cell death and contributes to neuronal degeneration in hypoxia, ischemia, and several neurodegenerative pathologies (2).

Functional NMDARs are hetero-oligomeric proteins composed of an obligatory NR1 subunit (3–7) and NR2 subunits, denoted A–D (3, 4, 8, 9). It is these NR2 subunits that confer functional variability to the receptor. In the post-synaptic membrane, NMDARs form large and dynamic signaling complexes by association with additional proteins (10), although there are also extrasynaptic NMDARs, which trigger different responses (11).

NMDARs are subjected to multiple levels of regulation, affecting subunit expression, subcellular location, and the assembly of functional receptors and their signaling complexes (12–17). The NR1 gene is expressed in virtually all neurons, whereas NR2 transcripts display developmental and regional patterns (5, 18). The NR1 gene is transcriptionally up-regulated during neuronal differentiation, mostly by promoter de-repression (19), although positive mechanisms are also required. Post-transcriptional mechanisms also contribute to NR1 regulation in brain development, and two pools of mRNA, with different translational activities, have been described (20). In addition, an important level of control is exerted at the level of protein turnover and trafficking. There is a close coordination in neurons between the assembly of functional heteromeric receptors and the fate of the individual subunits, as exemplified by the existence of two pools of NR1 protein that are differently assembled to the NR2 subunits and that have distinct turnover rates (21–23).

This dynamic regulation of NMDAR expression and function is highly sensitive to activation of the receptors by their ligands; activation-dependent targeting and trafficking of NMDARs to and from synapses is fundamental to synaptic maturation and plasticity and is driven by mechanisms that accurately regulate receptor number (12, 24). We are interested in how NMDAR expression and function are affected by overactivation of this receptor by excitotoxic brain insults. In cultured neurons and during forebrain ischemia, excitotoxicity induces C-terminal cleavage of the NR2A and NR2B subunits (25–27). This cleavage is mediated by the Ca^{2+}-dependent protease calpain and might represent a negative feedback mechanism to down-regulate NMDAR function. Such a possibility is supported by the significant reduction in whole cell NMDAR-mediated currents induced by prolonged stimulation of acutely isolated or cultured cortical neurons with glutamate or NMDA (27). However, the role of NR1 subunits in the response to NMDAR overactivation has remained to be established.

In this study, we have examined the effect of NMDAR overstimulation on NR1 expression *in vitro* and *in vivo*. We show that brief exposure of cortical neurons to excitotoxic concentrations of NMDA provokes a rapid, specific, and irreversible inhibition of NR1 transcription via a mechanism that requires the activation of receptors containing the

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[5] The abbreviations used are: NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; CIII, carbobenzoxy-valinyl-phenylalaninal; CREB, cAMP response element-binding protein; DL-AP5, 2-amino-phosphonopentanoic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCAO, middle cerebral artery occlusion; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; BAPTA-AM, 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester; NSE, neuronal specific enolase; PBS, phosphate-buffered saline; RIPA, radioimmune precipitation assay; MOPS, 4-morpholinepropanesulfonic acid.

NR2B subunit. Strong decreases in NR1 mRNA and protein were also observed during the reperfusion of infarcted rat brains. Given that the NR1 subunit is an essential component of the NMDAR, any modification of its expression will likely have a significant impact on receptor function. Our results thus reveal a new and important mechanism of autoregulation of the receptor by its agonist.

MATERIALS AND METHODS

Chemicals—The NMDAR antagonists 2-amino-phosphonopentanoic acid (DL-AP5) and ifenprodil were from Tocris-Cookson (Bristol, UK). NMDA, glutamate, glycine, cytosine β-D-arabinofuranoside, actinomycin D, poly-L-lysine, L-laminin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were all from Sigma. Carbobenzoxy-valinyl-phenylalaninal (calpain inhibitor III, herein after referred to as CiIII) and 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl)ester (BAPTA-AM) were from Calbiochem (Darmstadt, Germany).

Primary Neuronal Culture—Primary culture of embryonic rat neurons was essentially as described (28) with some modifications. The plates were treated with poly-L-lysine (100 μg/ml) and laminin (4 μg/ml) overnight at 37 °C before seeding. Cerebral cortices from 18-day-old rat embryos (Wistar) were dissected and mechanically dissociated in culture medium (Eagle's minimum medium supplemented with 28.5 mM $NaHCO_3$, 22.2 mM glucose, 0.1 mM glutamine, 5% fetal bovine serum, and 5% donor horse serum). The cells were seeded at a density of 0.3×10^5 cells/cm^2 in the same medium. To inhibit growth of glial cells, cytosine β-D-arabinofuranoside (10 μM) was added to the culture at day 7 and maintained until the end of experiments.

Experimental treatments were begun after 14 days in culture, at which time NR2A and NR2B subunits are both expressed. The following concentrations of reagents were used for pretreatments or treatments, as indicated under "Results": 100 μM NMDA, 10 μM glycine, 100 μM glutamate, 200 μM DL-AP5, 10 μM ifenprodil, 10 μM CiIII, 40 μM BAPTA-AM, 2 mM EGTA, and 2.5 μg/ml actinomycin D. Excitotoxicity was always induced by combined treatment with either NMDA or glutamate and the co-agonist glycine.

Assessment of Neuronal Injury—We used the MTT reduction assay to measure cell viability. MTT (0.5 mg/ml) was added to the medium, and after 4 h at 37 °C the formazan salts formed were solubilized in 5 mM HCl containing 5% SDS and were spectophotometrically quantified at 570 nm. The contribution to the absorbance of glial cells in the mixed cultures was established by exposing sister cultures to 400 μM NMDA and 10 μM glycine for 24 h before MTT assay. These conditions induce nearly complete neuronal death but no glial damage. Once these values were subtracted, we calculated the viability of stimulated neurons relative to the untreated ones.

Immunoblot Analysis—The cultures were lysed in RIPA buffer (10 mM Na_2HPO_4, pH 7.2, 150 mM NaCl, 1% sodium deoxicolate, 1% Nonidet P-40, 0.1% SDS) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.2 mM 1,10-phenanthroline, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 mM benzamidine). Brain samples were homogenized in RIPA buffer containing 1% SDS, 1 mM dithiothreitol, and protease inhibitors as above. Protein concentrations were determined with the BCA reagent from Pierce. Equal amounts of protein (25–50 μg) were separated by polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Pall; Life Sciences).

Immunodetection of proteins was performed by standard procedures. NR1 expression was detected with an anti-NMDAR NR1 monoclonal antibody (Pharmingen, San Diego, CA). Polyclonal antibodies were used to detect neuronal specific enolase (NSE) (ICN Biomedicals) and conserved regions in the N terminus (Pharmingen) and C terminus (Chemicon, Temacula, CA) of NMDAR subunits NR2A and 2B. Protein loading was monitored by comparison with the staining with an anti-β-actin monoclonal antibody (Sigma). Goat secondary antibodies coupled to horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA). Immunocomplexes were detected with the Bioluminescence kit from PerkinElmer Life Sciences. Densitometric analysis of bands was performed with NIH Image analysis software.

Immunofluorescence—Primary cultures were grown on coverslips treated with poly-L-lysine and L-laminin as before. After stimulation with NMDA and glycine as indicated, they were fixed for 2 min at 4 °C in 4% (w/v) paraformaldehyde in phosphate-buffered saline, pH 7.4 (PBS), washed with PBS, and permeabilized for 2 min at −20 °C in methanol. Nonspecific sites were blocked for 30 min at room temperature in 10% (v/v) horse serum, 0.1% (v/v) Triton X-100 in PBS, and the same solution was used for antibody dilution.

The cells were incubated with the monoclonal antibody for NR1 for 1 h at room temperature and, after washes, with an Alexa-488 conjugated secondary antibody for a further hour. Nuclear DNA was labeled with 2 μM TO-PRO 3 iodide (Molecular Probes) added to the secondary antibody solution. The coverslips were mounted in Fluoromount-G (SouthernBiotech), and immunoreactivity was detected by examination under a Radiance 2000 confocal microscope (Bio-Rad) coupled to an inverted Axiovert S100 TV microcope (Zeiss) fitted with a 63× Plan-Apochromat oil immersion objective. Overlaying the NR1 and TO-PRO 3 images produced the two-color merged image.

Metabolic Labeling and Immunoprecipitation—The cells were starved for 3 h in Dulbecco's modified Eagle's medium without methionine or cysteine (Biowhittaker) and containing 200 μM DL-AP5. They were then labeled for 4 h with 150 μCi/ml of [^{35}S]methionine + cysteine, washed with cold PBS, and lysed in RIPA buffer as before. When indicated, NMDA and glycine were present during starvation and labeling.

The trichloroacetic acid-precipitable counts in the extracts were measured, and equivalent counts were incubated with the following antibodies: anti-NR1 (2.5 μg); anti-NR2A/B (C-ter) (0.5 μg); or a rabbit polyclonal specific for calnexin (0.5 μl) (StressGen Biotechnologies, Victoria, Canada). The immunocomplexes were precipitated with 100 μl of 10% Protein A Sepharose (Sigma), and the beads were washed four times with RIPA buffer before solubilization in sample buffer.

Northern Blot Analysis—Total RNA was prepared using TriReagent (Sigma) according to the manufacturer's instructions. Approximately 10 μg of total RNA were fractionated on 1% agarose gels prepared in 20 mM MOPS buffer, pH 7.2, 0.6% formaldehyde. After transfer to nylon membranes, the RNA was hybridized to DNA probes corresponding to nucleotides 34–378 of rat β-actin cDNA or nucleotides 344–1280 of the NR1–1a splice variant, labeled by standard procedures.

RNase Protection Assay—Total RNA was treated with RNase-free DNase I (10 units, Promega) to eliminate any contaminating genomic DNA and then extracted and precipitated. RNA was quantified by spectrophotometry and checked by 1% agarose gel electrophoresis.

Antisense riboprobes were prepared by *in vitro* transcription with T3 or T7 RNA polymerase in the presence of [α-^{32}P]CTP, by use of the MAXIscript *in vitro* transcription kit (Ambion). The probe used for NR1 spans nucleotides 1250–1556 of the rat NR1 cDNA and corresponds to exons 7–9; that used for NR2A spans nucleotides 2442–2683 of the rat NR2A cDNA. As a loading control, we synthesized an antisense cRNA probe spanning nucleotides 196–357 of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA.

RNase protection assays were performed with the RPAII ribonuclease protection assay kit (Ambion). Total RNA from brain cortices or cultured neurons (15–20 μg) was hybridized to NR1 or NR2A probes together with GAPDH cRNA. Protected fragments were separated on 6% polyacrylamide sequencing gels, which were dried and exposed to x-ray films. Quantitation was performed with a Packard Instantimager, and the values were normalized to those obtained for GAPDH.

Quantitative Real Time PCR—Total RNA (2 μg) was transcribed in reverse by extension of random hexamers with Moloney murine leukemia virus reverse transcriptase (Promega). PCRs (20 μl) contained 0.5 μl of cDNA, 0.05 units of Taq polymerase (Biotools), 0.2 mM dNTPs, 0.2 μM amplification primers, 1:40000 Sybr Green, and 3 mM $MgCl_2$. The PCR was performed in a Rotor-Gene 2000 thermocycler (Corbett Research, Sidney, Australia). Denaturation at 95 °C for 2 min was followed by 35 cycles of 95 °C for 20 s, 62 °C for 20 s, and 72 °C for 30 s.

The NR1 forward primer spanned nucleotides 1473–1493 of the cDNA (5′-TCCACCAAGAGCCCTTCGTG), and the reverse primer spanned nucleotides 1541–1561 (5′-AGTTCAACAATCCGAAAAGCTGA). The 108-bp region amplified by this primer set is common to all NR1 isoforms. NR1 transcript amplification was normalized against NR2A; the forward primer spanned nucleotides 1250–1270 (5′-ACGACTGGGACTACAGCCTG), and the reverse primer spanned nucleotides 1344–1364 (5′-CTTCTCTGCCTGCCCATAGC), amplifying a 134-bp region of the NR2A cDNA.

Cell Transfection and Gene Reporter Assay—The plasmids pNRL5.4 (19) and 356 (29) were kindly provided by Dr. Guang Bai, and contain, respectively, 5.4-kb and 356-bp sequences of the rat NR1 promoter cloned upstream of the firefly luciferase reporter gene. Plasmid pRL-SV40 (Promega) contains the SV40 early enhancer/promoter region in control of the constitutive expression of *Renilla* luciferase.

Neurons cultured for 12 days were transfected plasmid DNA incorporated into Lipofectamine 2000 liposomes (Invitrogen). DNA-liposomes complexes were prepared in neurobasal medium (Invitrogen), with the NR1 promoter plasmids in a 5:1 molar excess over pRL-SV40. Two hours after addition to cells, the liposomes were removed, and the neurons were fed with conditioned medium. In control experiments, the efficiency of transfection was estimated to be around a 15%. After transfection, the cells were maintained in culture for 40 h before experimental treatments.

When indicated, the cells were pretreated for 2 h before stimulation with CiIII or with one of the Ca^{2+} chelators BAPTA-AM or EGTA. The cells were stimulated for the indicated times with NMDA/glycine, with or without DL-AP5 or ifenprodil. In the experiments with the Ca^{2+} chelators, after stimulation with NMDA/glycine for 1 h, the cells were washed, fed with conditioned medium plus DL-AP5, and analyzed 24 h later. Reporter gene activities were determined by the dual luciferase reporter assay system from Promega; firefly luciferase activity was normalized to the *Renilla* values obtained in the same sample.

Animal Model of Cerebral Ischemia—All of the animal procedures were performed in compliance with European Community law 86/609/EEC and were approved by the ethics committee of the Consejo Superior de Investigaciones Científicas. Male Sprague-Dawley rats (275–300 g) were anesthetized by intraperitoneal injection with a solution of diazepam (5 mg/kg), ketamine hydrochloride (Ketolar, 62.5 mg/kg), and atropine (0.25 mg/kg). The femoral artery was cannulated for continuous monitoring of arterial pressure and blood sampling. Analysis of blood pH, gases, and glucose was performed before and 15 min after occlusion and 10 min after reperfusion. Body and brain temperatures were respectively maintained at 37 ± 0.5 °C and 36 ± 0.5 °C during the whole procedure.

The surgical procedure was a variant of that described by Chen *et al.* (30) and Liu *et al.* (31). A small craniectomy was made over the trunk of the right middle cerebral artery and above the rhinal fissure, and the artery was transitorily ligated with a 9–0 suture just before its bifurcation into the frontal and parietal branches. Complete interruption of blood flow was confirmed by observation under an operating microscope. Then both common carotid arteries were also occluded, and all three arteries were kept this way for 60 min before reperfusion. After blood reperfusion for the indicated times, the animals were sacrificed by an inhaled overdose of halotane and decapitated. Sham-operated animals were subjected to anesthesia and surgical procedure but the occlusion of the arteries was omitted.

For the protein extracts, the brain was sectioned into 2-mm-thick slices and stained with a 2% solution of triphenyltetrazolium chloride. The unstained area of the cerebral cortex (right hemisphere), defined as infarcted tissue, was dissected; the corresponding contralateral region in the left hemisphere was also dissected for comparison. To prepare RNA, dissection of the infarcted and contralateral cortical tissue was performed directly in whole brain, and the samples were immediately frozen in liquid nitrogen until further use. For immunohistochemistry, 24 h after blood reperfusion, the rats were deeply anesthetized as before and perfused intracardially with cold 4% paraformaldehyde in PBS. The brains were removed immediately and post-fixed in the same fixative at 4 °C for an additional 24 h. They were then cryoprotected by serial immersion for at least 6 h in increasing concentrations of sucrose (10, 15, and 20%) in PBS at 4 °C. Frozen coronal sections (25 μm thick) were then prepared in a cryostat (Leica, Heidelberg, Germany) and processed for immunohistochemistry.

Immunohistochemistry—The infarcted tissue in the neocortex was identified by Nissl (0.1% (w/v) cresyl violet) staining of slide-mounted coronal sections. Adjacent sections were then processed for immunofluorescence. Briefly, the sections were permeabilized and blocked by treatment with 5% (v/v) sheep serum and 0.3% (v/v) Triton X-100 in PBS for 1 h at room temperature. This same solution was used for antibody dilution; washes were performed in 0.3% (v/v) Triton X-100 in PBS. The sections were incubated overnight at 4 °C with the NR1 monoclonal antibody (1:100). After several washes, the sections were incubated at room temperature for 60 min with the Alexa Fluor 568-conjugated anti-mouse IgG secondary antibody (1:400) (Molecular Probes, Eugene, OR). After further washes, the sections were counterstained for 1 h at room temperature with 2 μM TO-PRO 3 iodide (Molecular Probes) before mounting in Fluoromount-G solution (SouthernBiotech). Parallel controls without primary antibody showed very low levels of nonspecific staining. Confocal images were acquired as described above.

RESULTS

Excitotoxic Activation of the NMDAR Down-regulates Expression of the NMDAR NR1 Subunit in Vitro—In this investigation, we have characterized the effect that activation of the NMDAR has on the expression of NR1, a critical subunit of this glutamate receptor. Primary cultured rat cortical neurons of 14 days *in vitro* were incubated with NMDA (100 μM) and the co-agonist glycine (10 μM), and steady-state NR1 protein levels were determined by immunoblot analysis with a monoclonal antibody directed to an extracellular domain of this protein (Fig. 1*A*). Compared with untreated cells, there was a marked decrease in NR1 immunoreactivity after 8 h of agonist treatment. This effect was further accentuated by longer treatment, with NR1 protein nearly undetectable after 48 h. NMDA had no effect on the expression levels of the unrelated protein β-actin at any time tested.

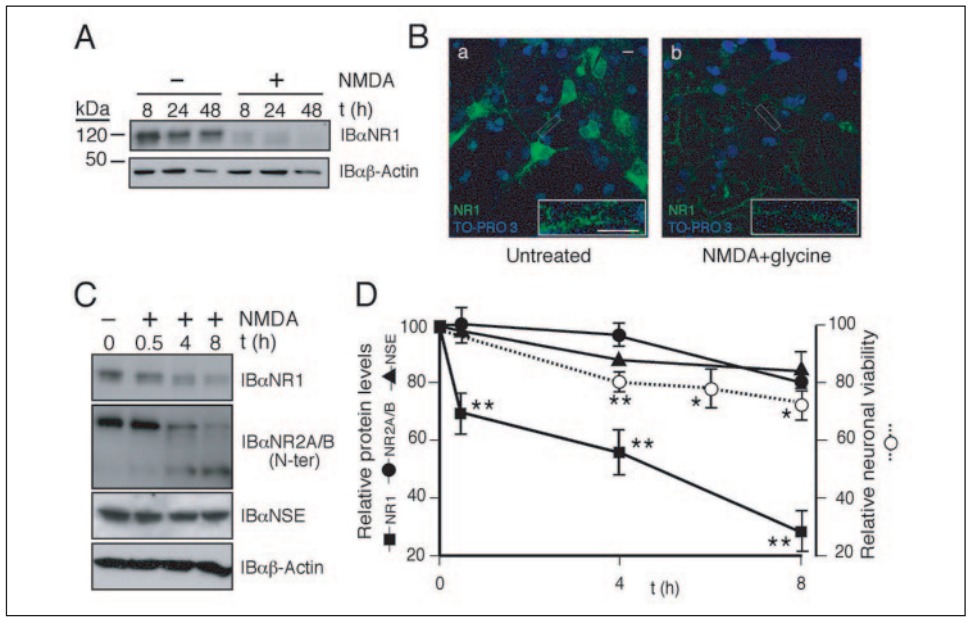

FIGURE 1. **Specific decrease of NR1 protein expression in cortical neurons stimulated with NMDA.** *A*, primary cultures of rat cortical neurons (14 days *in vitro*) were incubated with NMDA (100 μM) and glycine (10 μM) for 8, 24, and 48 h, and immunoblot (*IB*) analysis was used to determine expression levels of the NR1 subunit and β-actin. Time-matched untreated cells were used as controls. *B*, primary cultures were stimulated with NMDA and glycine for 8 h or left untreated. Confocal immunofluorescence analysis was used to detect NR1 (*green*) and cell nuclei, which were revealed by co-staining with TO-PRO 3 (*blue*). The confocal microscopy images correspond to a single section, and details of the cell dendrites are shown in the *insets*. The results are representative of three independent experiments. The *scale bars* represent 10 μm. *C*, neurons were stimulated with NMDA and glycine for 30 min, 4 h, or 8 h. Untreated cells were used as the control. The immunoblots show the expression levels of NR1, NR2A/B (N-ter), NSE, and β-actin. *D*, quantitation of the decline of NR1 protein expression and of neuronal viability with time of NMDA/glycine treatment. Expression levels of NR1 (*filled squares*), NR2A/B (115-kDa fragment and full-length combined: *filled circles*), and NSE (*filled triangles*) were measured by densitrometric analysis of immunoblots with NIH Image software. Protein levels are expressed as the percentage of the value in untreated cells. Neuronal viability was measured by MTT assay and is similarly expressed relative to untreated cells (*open circles*). The contribution of glial cells to MTT assay was excluded (see "Materials and Methods"). The data are the means ± S.D. of three independent experiments. Statistical differences between treated and untreated cells were assessed by the Student's unpaired *t* test. *, $p < 0.05$; **, $p < 0.01$.

Immunofluorescence of primary cultures confirmed these results (Fig. 1*B*). In untreated cells (Fig. 1*B*, *panel a*), we observed a characteristic immunoreactivity for this protein in the cell soma and in clusters at puncta on dendrites (*inset*). Staining of nuclear DNA with TO-PRO 3 iodide revealed the presence in the primary cultures of glial cells, which do not express NMDAR proteins. Stimulation with NMDA and glycine for 8 h induced a marked decrease in the intensity of staining (Fig. 1*B*, *panel b*), both in cell bodies and dendrites.

To exclude the possibility that the observed decrease was a consequence of neuronal cell death, we examined the expression of NR1 and other neuron-specific proteins at earlier times of treatment and measured neuronal viability by MTT assay. Immunoblot analysis was performed on neurons treated with NMDA from 30 min to 8 h (Fig. 1*C*), and the results from three independent experiments were quantified (Fig. 1*D*). Thirty minutes of NMDA stimulation were enough to significantly reduce NR1 band density by 29% compared with untreated cells ($p < 0.01$). By 4 and 8 h of treatment, NR1 band densities were, respectively, 43 and 71% below that from control cells ($p < 0.01$). In accordance with published results (25, 26), the NR2A and B subunits were cleaved, and a 115-kDa fragment recognized by an antibody specific for the N-terminal region of NR2A and NR2B appeared (Fig. 1*C*). However, after 8 h there was no statistically significant decrease in the combined levels of truncated and full-length NR2 proteins (Fig. 1*D*). There was similarly no significant decrease in the expression of NSE and β-actin. It thus seems that the NMDA-induced decrease in NR1 protein is not part of a general effect of neuronal death resulting from NMDAR activation, and this is further supported by the MTT assay, which estimated excitotoxic death to be only 25% after 8 h of NMDA treatment (Fig. 1*D*). However, the neurons in our cultures were committed to die, as shown by the progressive increase in neuronal death observed at later times of NMDA treatment (data not shown).

NR1 Down-regulation by Brief Overstimulation of NMDARs Requires NR2B Subunits and Is Irreversible—To investigate the receptor components required for NR1 down-regulation, we first examined the effects of different NMDAR agonists and antagonists (Fig. 2*A*). Glutamate (100 μM in combination with 10 μM of the co-agonist glycine) decreased NR1 expression similarly to NMDA, and the specific competitive NMDAR antagonist DL-AP5 prevented NMDA-mediated NR1 down-regulation. These findings confirmed that NR1 down-regulation was agonist-specific and mediated by direct activation of the NMDARs. But NR1 down-regulation was also prevented by ifenprodil (10 μM), which is a selective inhibitor of the NR2B subunits (32). Because neurons at this time in culture (14 days *in vitro*) express both NR2A and NR2B, we conclude that NR1 regulation specifically requires activation of NMDARs containing NR2B subunits. The amount of NR1 protein was not significantly modified in neurons treated only with DL-AP5 or ifenprodil compared with the untreated cells (data not shown), suggesting that basal activity of the NMDAR does not induce the down-regulatory process.

Therefore, we next analyzed the concentrations of NMDA required for regulation of NR1 expression. When cortical neurons were incubated for 6 h with different concentrations of NMDA (0.1–100 μM) (Fig. 2*B*), decreased NR1 expression was observed only at concentrations higher than 10 μM, suggesting that NR1 down-regulation requires excitotoxic stimulation of the NMDAR. A nonlinear response to NMDA concentration has been previously described in the neuronal injury induced by long term treatment with this agonist (33).

In agreement with these results, a chronic but nonexcitotoxic increase in the NMDAR activity of the primary neuronal cultures did not down-regulate the expression of the NR1 subunit. We increased NMDAR activity by blocking inhibitory synaptic inputs for 48 h with the γ-aminobutyric acid, type A receptor antagonist bicuculline (40 μM). As shown before for hippocampus cells (34), this treatment did not signif-

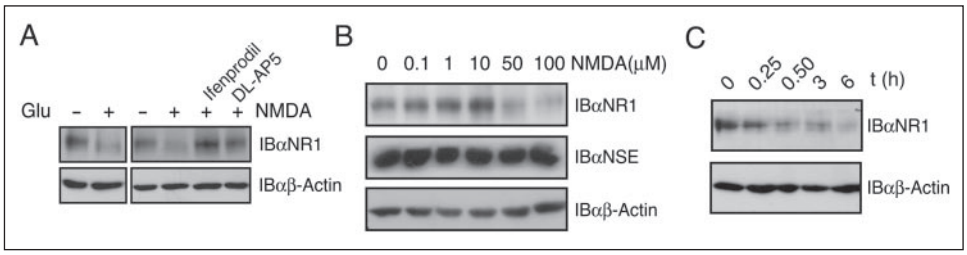

FIGURE 2. **Irreversible down-regulation of NR1 by excitotoxic stimulation of NMDARs containing NR2B subunits.** *A*, the effect of different NMDAR agonists and antagonists on NR1 expression. Primary cultures were incubated for 12 h with glutamate (*Glu*) (100 μM) or NMDA (100 μM), each in combination with the co-agonist glycine (10 μM). Where indicated, the cells were also treated with the antagonists DL-AP5 (200 μM) or ifenprodil (10 μM). NR1 and β-actin protein expression levels were determined by immunoblot (*IB*). *B*, neurons were stimulated for 6 h with glycine (10 μM) and a range of concentrations of NMDA from 0.1 to 100 μM. Expression levels of NR1, NSE, and β-actin were determined by immunoblot. *C*, cultures were incubated with NMDA/glycine for the indicated times before the addition of DL-AP5 (200 μM) and then left to complete 24 h. Untreated cells were used as the control. The expression levels of NR1 and β-actin were determined by immunoblot.

icantly change the total amounts of NR1 in the cortical neurons (data not shown). However, high concentrations of NMDA were able to down-regulate NR1 expression irreversibly after only brief overstimulation of the NMDAR (Fig. 2*C*). When neurons were stimulated with NMDA for 15 min, and the signaling was then arrested by protecting receptor sites with DL-AP5, the protein levels of NR1 detected by immunoblot 24 h later were reduced by 25% compared with non-NMDA-treated cells. Arrest of signaling after 30 min of treatment produced a 49% decrease in NR1, which is similar to the decrease observed after 5 h of continuous stimulation (Fig. 1*D*). We therefore conclude that the down-regulation of NR1 is triggered during a short critical period after overstimulation of NMDARs, after which it cannot be blocked or reversed by antagonists.

Down-regulation of NR1 Protein Expression Is Independent of Calpain and Is Mediated by a Decrease in NR1 mRNA—Activation of the NMDAR in cultured hippocampal or cortical neurons leads to a rapid calpain-specific proteolysis of the C termini of the NR2A and NR2B subunits (25–27). We used two approaches to investigate whether this activity is also responsible of the decrease in NR1 induced by NMDA. We first analyzed whether the reduction in full-length NR1 was accompanied by the appearance of truncated forms of this protein. We performed immunoblot analysis of NR1 in neurons treated with NMDA for 8 h (Fig. 3*A*). The monoclonal antibody specific for NR1 recognizes an extracellular domain, so calpain cleavage of the cytosolic C-terminal domain would be expected to yield detectable N-terminal fragments. Contrary to this, the decrease in levels of full-length protein occurred without the appearance of N-terminal fragments, suggesting that calpain is not involved in the mechanism of NR1 down-regulation. To confirm this, we analyzed the effect on NR1 down-regulation of pretreatment with the CiIII. Calpain activity was not required for NR1 down-regulation, detected after 12 h of NMDA treatment, whereas it was required for the down-regulation of full-length NR2A/B (Fig. 3*B*) observed with an antibody directed to the C terminus and unable to detect the calpain products. Experiments performed with specific inhibitors also excluded the participation of the lysosomal or proteasomal degradative pathways in the down-regulation of NR1 induced by NMDA (data not shown). Considering these data and the relatively slow kinetics of NR1 reduction (Fig. 1*D*), we next addressed the possibility that overstimulation of the NMDAR inhibits *de novo* synthesis of NR1 protein. Untreated and NMDA-treated cortical neurons were metabolically labeled with [^{35}S]cysteine + methionine, and an equal number of counts immunoprecipitated using specific antibodies (Fig. 3*C*). We observed similar levels of synthesis of the neuronal proteins calnexin or NR2A/B in NMDA-treated or untreated cells, whereas this treatment blocked *de novo* synthesis of NR1 protein.

One explanation for this effect might be a reduction by NMDAR overactivation of the expression of one or more of the NR1 transcripts. Northern blot analysis with a specific DNA probe able to detect all alternatively spliced forms of this messenger revealed that agonist stimulation for 8 h was sufficient to strongly reduce the steady-state levels of NR1 mRNA (Fig. 3*D*); levels of β-actin mRNA were unaffected. Consistent with the protein expression data shown in Fig. 2*C*, the reduction in NR1 mRNA expression after 24 h of continuous exposure to NMDA (Fig. 3*D*) was not affected by arrest of NMDA signaling with DL-AP5 added to the medium after a 2-h exposure to NMDA (Fig. 3*E*). This experiment thus reveals a new mechanism of NMDAR down-regulation in which brief overactivation of the receptor specifically and irreversibly reduces the expression levels of NR1 mRNA.

Overstimulation with NMDA Decreases Neuronal Expression of NR1 mRNA by the Specific Inhibition of NR1 Transcription—To better characterize the time course of NR1 mRNA down-regulation during the first hours of NMDA treatment, when neuronal death is low, we determined its expression by RNase protection assay (Fig. 4*A*) and real time PCR (Fig. 4*B*). The NR1 probe and the specific oligonucleotides used in these experiments were designed to detect all isoforms of this mRNA.

The amount of the NR1 protected fragment was notably decreased by 4 h of treatment and was further reduced by 8 h; DL-AP5 completely prevented this decrease (Fig. 4*A*, *top panel*). In contrast, the levels of mRNA for the housekeeping gene GAPDH were not modified by NMDA (Fig. 4*A*, *bottom panel*).

Because we detected some neuronal death in response to NMDA stimulation (Fig. 1*D*), it was important to normalize NR1 mRNA expression to a neuronal mRNA that is not significantly modified by NMDA. We used NR2A for this, because *de novo* synthesis of this subunit was not affected by NMDA treatment (Fig. 3*C*). This mRNA was expressed at very low levels relative to NR1 but was not modified by NMDA stimulation (Fig. 4*A*, *middle panel*). Therefore, for NR1 mRNA quantitation we performed real time PCR of both mRNAs and normalized NR1 mRNA amounts to those of NR2A (Fig. 4*B*). Exposure to NMDA for 2 h decreased the expression of NR1 mRNA by 41% ($p < 0.05$) and by 58 and 77% after 4 and 8 h, respectively ($p < 0.01$).

A possible reason for the marked down-regulation of NR1 mRNA might be an NMDA-induced increase in the turnover of this mRNA. To test this possibility, we performed RNase protection assays to compare the kinetics of decay of NR1 mRNA in cultures treated for different times with transcriptional inhibitor actinomycin D, alone or together with NMDA (Fig. 4*C*, *top panel*). In these experiments, the results were normalized to rRNA because unlike mRNA its expression is not modified by actinomycin D treatment (Fig. 4*C*, *middle panel*). The half-life of NR1 mRNA was the same in cells treated with actinomycin D alone or in combination with NMDA (represented in the *lower panel* of Fig. 4*C*). This suggests that both compounds are affecting the same step of NR1 regulation and excludes the possibility that NMDA increases the turnover of NR1 mRNA.

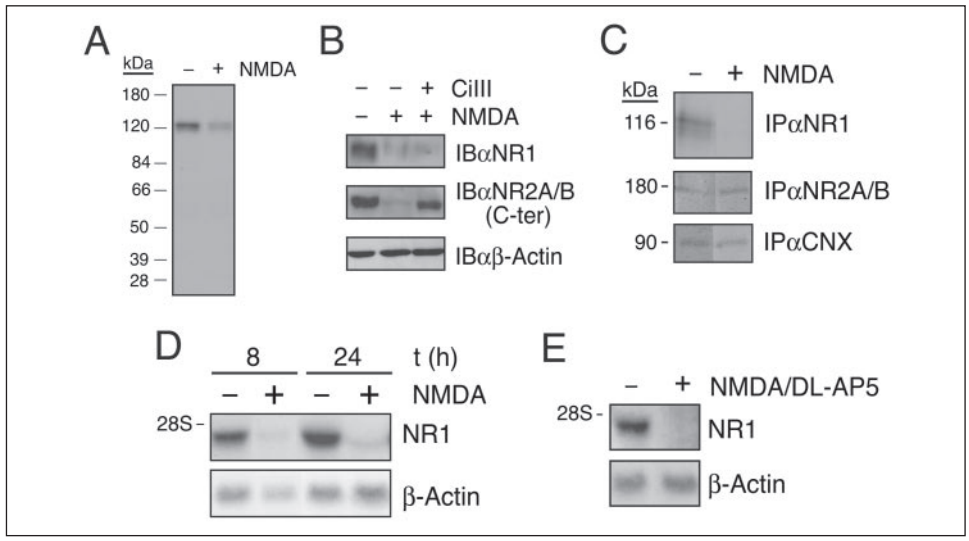

FIGURE 3. **Effect of activation of the NMDAR on NR1 mRNA and protein synthesis.** *A*, primary cultures of neurons were incubated with NMDA/glycine for 8 h as indicated, and NR1 was detected by immunoblot (*IB*). The NR1 antibody only recognizes the full-length protein, and no proteolytic fragments could be detected, even after film overexposure. *B*, neurons were preincubated for 2 h with 10 μM CiII before a 12-h treatment with NMDA/glycine. CiII remained in the culture medium for the duration of the experiment. Immunoblots were performed as before for NR1 and β-actin and also with an antibody to a conserved region in the C terminus of subunits NR2A and B. *C*, analysis of *de novo* synthesis of receptor subunits in neurons treated with NMDA. Neuronal cultures were stimulated with NMDA/glycine and, after 3 h, were labeled during a further 4 h with [^{35}S]methionine + cysteine (150 μCi/ml). Untreated cells were used for the control. Protein extracts were immunoprecipitated with antibodies specific for the NR1 subunit, the NR2A/B subunits (C-ter), or calnexin (*CNX*). Autoradiography of the immunoprecipitated proteins is shown. *D*, Northern blot analysis of NR1 mRNA. Total RNA was prepared from cells treated with NMDA/glycine for the indicated times and from time-paired untreated cells. The DNA probe used corresponds to 936 nucleotides spanning exons 1–7 of NR1 isoform 1a and therefore is able to hybridize all the alternatively spliced forms of this messenger. We detected β-actin mRNA as a control. *E*, Northern blot analysis of NR1 mRNA from neurons treated with NMDA/glycine for 2 h before the addition of DL-AP5 (200 μM) and incubation up to 24 h.

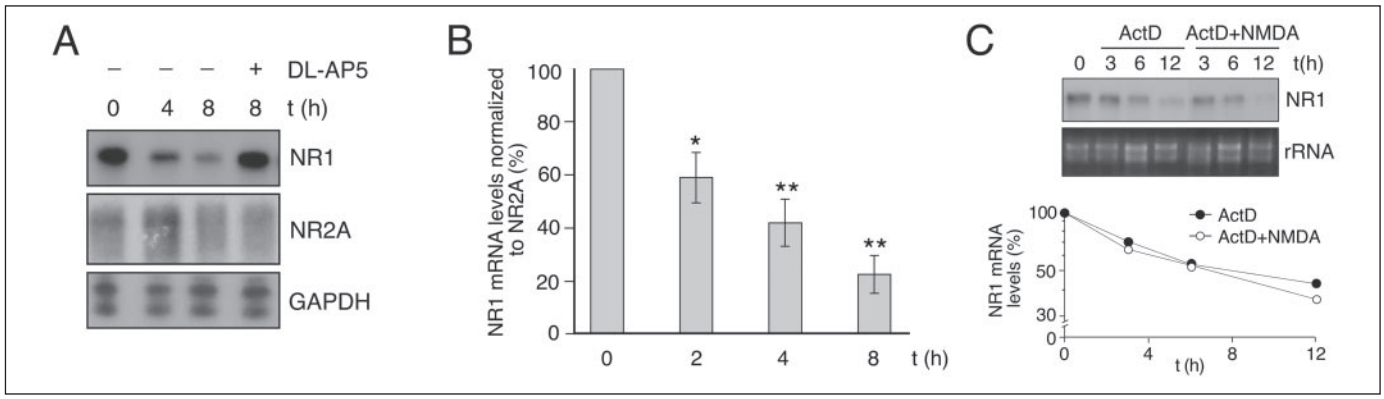

FIGURE 4. **Time course and specificity of the down-regulation of NR1 mRNA.** *A*, time course of NR1 mRNA decay. Total RNA was extracted from cultured neurons treated with NMDA/glycine for 4 and 8 h and from untreated controls. In another control, the cells were stimulated for 8 h with NMDA/glycine in the presence of DL-AP5. RNase protection assay was performed on 20 μg of total RNA with RNA probes for NR1, NR2A, and GAPDH. *B*, quantitation of the decay of NR1 mRNA. Levels of NR1 mRNA measured by quantitative real time PCR were normalized to those of NR2A in the same samples. The values are expressed as the percentage of those found in untreated cells. The data shown are the means ± S.D. of three independent experiments, and statistical significance was evaluated by Student's unpaired *t* test. *, $p < 0.05$; **, $p < 0.01$. *C*, time course of NR1 mRNA decay in cells treated with actinomycin D (*ActD*) alone or in combination with NMDA. The cells were pretreated with ActD (2.5 μg/ml) for 1 h, and where indicated NMDA/glycine was added. The times refer to the length of incubation with NMDA/glycine. ActD was present for the duration of the experiment. RNase protection assay was performed as before, and rRNA was used as a loading control. The plots show the quantitation of NR1 mRNA in neurons treated with ActD alone (*filled circles*) and ActD plus NMDA/glycine (*open circles*). NR1 mRNA amounts were normalized to the rRNA present in the same samples, and the values are expressed as percentages of those in untreated cells.

We examined the effect of NMDA on NR1 transcription in gene promoter/reporter assays (Fig. 5). Cortical neurons were transiently transfected with pNRL5.4 (19), a plasmid containing 5.4 kb of the NR1 promoter coupled to the firefly luciferase gene. For normalization, the cells were co-transfected with pRL-SV40 plasmid, which constitutively expresses *Renilla* luciferase and was not modified by NMDA stimulation (data not shown).

Transfected neurons were treated with NMDA for different times, and luciferase activities were measured in the cell lysates (Fig. 5*A*). The activity of the NR1 promoter was reduced by 34% relative to unstimulated cells after treatment for 1 h ($p < 0.05$) and by 68% ($p < 0.001$) after 2 h. NMDA treatment for 6 h decreased NR1 promoter activity by 79% ($p < 0.001$), and this was completely prevented by DL-AP5 and by ifenprodil, demonstrating that NR2B subunits are required (Fig. 5*B*). In contrast, a reduction in promoter activity of 57% ($p < 0.01$) was still observed in neurons pretreated with CiII, indicating that the activity of calpain is not required for the inhibition of NR1 promoter activity.

In accordance with the protein expression data shown in Fig. 2*C*, brief exposure to high concentrations of NMDA was sufficient to irreversibly inhibit the NR1 promoter activity measured at later times (Fig. 5*C*). For this experiment we used pNRL356, a plasmid containing only 356 bp of the NR1 promoter coupled to the firefly luciferase gene (29). When transfected neurons were stimulated with NMDA for 1 h, followed by protection with DL-AP5, the luciferase activity measured 24 h later was reduced by 67%.

Using this same experimental procedure, we demonstrated that NMDA-induced down-regulation of NR1 transcription was triggered

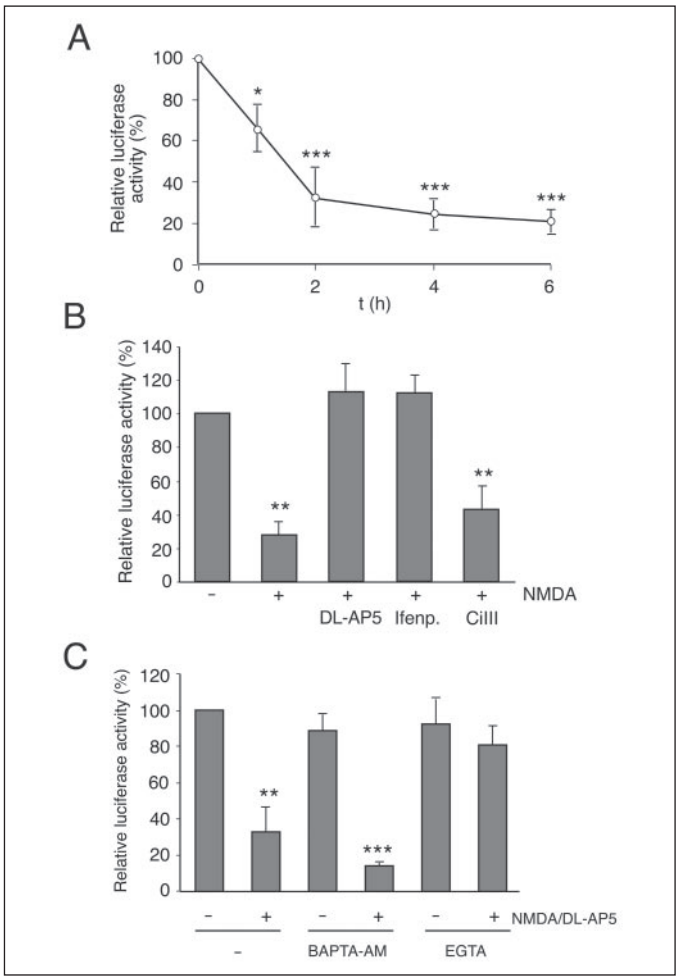

FIGURE 5. **NR1 promoter activity in neurons treated with NMDA.** *A*, neurons were transiently co-transfected with the reporter plasmid pNRL5.4, which contains the NR1 promoter coupled to the firefly luciferase gene, together with pRL-SV40, which constitutively expresses *Renilla* luciferase. Forty hours after transfection, the cells were treated with NMDA/glycine for the indicated times. The firefly luciferase activity was normalized to *Renilla* values obtained in the same samples. Relative luciferase activity is expressed as a percentage of that in untreated cells. The data shown are the means ± S.D. of three independent experiments, and the statistical significance of differences between treated and untreated cells was evaluated by Student's unpaired *t* test. *, $p < 0.05$; ***, $p < 0.001$. *B*, neuronal cultures transiently transfected as before were stimulated for 6 h with NMDA/glycine alone or together with the antagonists DL-AP5 (200 μM) or ifenprodil (*ifenp*, 10 μM) as indicated. Some cells were pretreated with CIII (10 μM) for 2 h before stimulation with NMDA/glycine. Luciferase activity was normalized and expressed as before. **, $p < 0.01$. *C*, neuronal cultures were transiently transfected with pNRL356, which contains the proximal 356 base pairs of the NR1 promoter coupled to firefly luciferase and with pRL-SV40. Forty hours later, the cells were pretreated with BAPTA-AM (40 μM) or EGTA (2 mM) for 2 h before treatment with NMDA/glycine for 1 h, still in the presence of the calcium chelators. The cells were then washed and fed with conditioned medium plus DL-AP5 (200 μM). Expression of luciferase was determined 24 h later. The results were normalized and expressed as before. **, $p < 0.01$; ***, $p < 0.001$.

by Ca^{2+} influx via the NMDAR (Fig. 5*C*). Chelation of extracellular free Ca^{2+} by preincubation for 2 h with EGTA (2 mM) completely prevented the decrease in NR1 promoter activity induced by NMDA treatment. In contrast, loading cells with the intracellular calcium chelator BAPTA-AM (40 μM) had no effect. Probably, the capacity of BAPTA-AM is insufficient to chelate a local or post-synaptic increase in Ca^{2+} such as that produced by NMDAR overactivation. These results thus demonstrate that an influx of calcium ions triggered by brief overstimulation of NMDARs containing NR2B subunits leads to the specific transcriptional inhibition of the NR1 gene.

NR1 Expression Is Down-regulated in an Animal Model of Cerebral Ischemia—Excitotoxicity induced by overactivation of NMDARs is responsible for the neuronal degeneration observed in diverse pathologies, including cerebral ischemia (2). Considering the results we obtained *in vitro* in the cortical neuron model of excitotoxicity, we sought to establish whether a similar process occurs *in vivo* in an animal model of cerebral ischemia. We characterized the expression of the NR1 subunit in the cortices of rats subjected to transient focal cerebral ischemia induced by 1 h of occlusion of the middle cerebral artery (MCAO) followed by reperfusion from 0 to 48 h. This is a highly reliable model in which the characteristic changes of ischemic necrosis are limited to the cortex and the subcortical structures and basal ganglia are spared (30). Large infarcts are reproducibly produced in the right middle cerebral artery territory after 24 h of reperfusion, as shown in Fig. 6*A* (*upper panel*), where cortical tissue poorly stained by Nissl is clearly visible in coronal sections of the brain.

Expression of NR1 was first determined by immunohistochemistry of contiguous sections, and the specific staining was compared between equivalent areas of the infarcted region and the contralateral hemisphere (Fig. 6*A*, *panels a* and *b*). As expected, we observed numerous NR1-positive neurons in the neocortex of the contralateral area, with immunoreactivity mainly associated with the cell soma and the dendrites (Fig. 6*A*, *panel b*). In marked contrast, NR1 immunoreactivity was significantly reduced in the infarcted tissue (Fig. 6*A*, *panel a*).

To better characterize the time course and specificity of NR1 down-regulation in the animal model, NR1 protein expression in the infarcted area was compared with that in the corresponding region of the contralateral hemisphere and in sham-operated animals (Fig. 6*B*, *top panel*). The amount of NR1 protein detected in the infarcted region decreased progressively with the time of reperfusion; the levels were moderately decreased after 2 h and were almost undetectable after 24 h. NR1 expression was unchanged in brain extracts from animals sacrificed immediately after the occlusion period or from sham-operated rats. Immunoblot analysis of NSE and β-actin (Fig. 6*B*, *middle* and *bottom panels*, respectively) demonstrated that down-regulation of NR1 is not a general effect of the ischemic process.

We next used RNase protection assay to investigate whether the decay in NR1 was due to a decrease in the levels of its coding mRNA in the infarcted area (Fig. 6*C*). Total RNA was prepared from the ischemic region and from the corresponding area of the contralateral hemisphere of animals subjected to MCAO and reperfused for 1, 2, or 4 h. NR1 mRNA expression in the ipsilateral hemisphere, normalized to GAPDH, was expressed as the percentage of that detected in the contralateral one; for sham-operated animals, the right and left hemispheres were compared. Levels of NR1 mRNA in the ipsilateral hemispheres of animals subjected to 2 h of reperfusion were reduced by 41% ($p < 0.01$), whereas the reduction was 47% by 4 h ($p < 0.01$). The variation in NR1 mRNA expression between the brain hemispheres of sham operated animals, or of operated animals reperfused for only 1 h, was not statistically significant. The decrease is specific for NR1 and was not observed for NR2A mRNA (Fig. 6*C*, *inset*), in agreement with the post-translational mechanism of down-regulation previously proposed for NR2A/B in transient forebrain ischemia (25). In conclusion, our experiments show that cerebral ischemia results in the negative regulation of NR1 mRNA at early reperfusion times, with consequent down-regulation of this obligatory NMDAR subunit.

DISCUSSION

We have identified a new mechanism of down-regulation of the NMDAR in neurons, schematically represented in Fig. 7, which is induced by excessive receptor activation. Because this mechanism affects the expression of NR1, the essential subunit of the NMDAR, it will have a great impact on the functionality of all NMDAR variants. Excessive activation of the NMDAR by co-agonists NMDA and glycine (Fig. 2*A*) induces the spe-

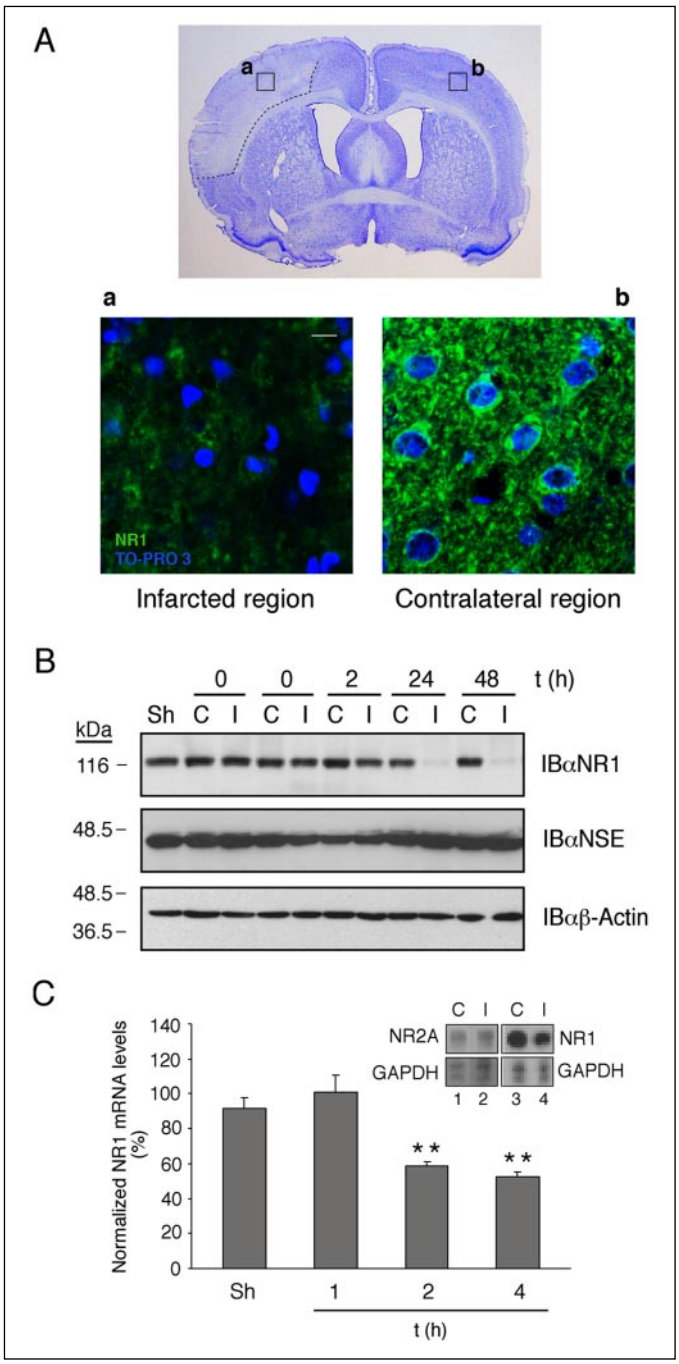

FIGURE 6. **Down-regulation of NR1 mRNA and protein in transient brain ischemia.** *A*, coronal section of a rat brain from an animal subjected to 1 h of MCAO treatment followed by 24 h of blood reperfusion. Nissl staining revealed an area of infarcted tissue in the neocortex of the right hemisphere, highlighted by the *dashed line*. Immunohistochemistry of adjacent sections was performed to detect expression of NR1 (*green*). Representative results of those obtained in the infarcted region (*box a*) and the corresponding area of the contralateral hemisphere (*box b*) are shown. TO-PRO 3 was used to stain the cell nuclei (*blue*). Confocal microscopy images correspond to a single section, and the *scale bars* represent 10 μm. *B*, Protein extracts were prepared from the infarcted region of the cortex (*I*) and the corresponding area in the contralateral hemisphere (*C*) of rats subjected to MCAO followed by reperfusion for the indicated times. Control extracts were obtained from the brains of sham-operated animals (*Sh*). Equals amounts of protein (50 μg) were analyzed by immunoblot with antibodies for NR1, NSE, and β-actin. *C*, RNase protection assay was performed as before on total RNA (15 μg) from the cortices of rats subjected to MCAO followed by reperfusion for 1, 2, or 4 h. Amounts of NR1 mRNA were normalized to the amount of GAPDH mRNA in the same sample. The results obtained for the infarcted region were then compared with values found in the corresponding area of the contralateral hemisphere, assigned a value of 100%. We also compared the right and left hemispheres of sham-operated animals. The data show the means ± S.E. of the amount of NR1 mRNA remaining in each animal group ($n = 3$). Statistical significance of the difference between the contralateral and ipsilateral hemispheres was evaluated by Student's unpaired *t* test. **, $p < 0.01$. The *inset* shows a representative result from one animal subjected to 4 h of reperfusion. RNase protection was performed with RNA probes for NR2A and GAPDH mRNAs (*lanes 1* and *2*) or to NR1 and GAPDH (*lanes 3* and *4*).

cific and rapid transcriptional inhibition of the NR1 promoter (Fig. 5*A*) in a process strictly dependent on Ca^{2+} influx via this receptor (Fig. 5*C*). Inhibition of NR1 transcription results in a progressive decrease in NR1 mRNA (Fig. 4*B*) and protein (Fig. 1*D*). The decay profile of NR1 that we observe is compatible with the biphasic pattern previously described for this protein. This pattern results from the existence of two populations of NR1 with half-lives of 2 and 34 h, which respectively correspond to unassembled NR1 and NR1 assembled with NR2 subunits in NMDARs at the cell surface (23). Enhanced endocytosis/degradation is most likely not involved in the down-regulation of NR1 induced by NMDA, because the use of specific inhibitors does not alter the process. Our results also suggest that the agonist does not modify the normal turnover of the NR1 protein already present in the neuron at the time of NMDA overstimulation. The decrease in NR1 mRNA and protein is not caused by neuronal cell death, because the activity of the NR1 promoter is already reduced by 68% 2 h after NMDA stimulation (Fig. 5*A*), at which time the decrease in neuronal viability is lower than 18% (Fig. 1*D*).

The inhibition of *de novo* NR1 synthesis induced by NMDA will probably lead to the retention of newly synthesized NR2 proteins in the endoplasmic reticulum, as occurs in NR1 knock-out mice (35). Whether or not NR2 processing is affected in this way, the result of NMDA overstimulation would be a progressive decrease in NMDAR activity, because the functionality of this receptor is strictly dependent on the expression of NR1/NR2 hetero-oligomers at the cell surface (4).

Fig. 7 also highlights the fact that the mechanism of NR1 regulation is different from the one previously characterized for NR2A/B (25). Neuronal calpain, activated very early by calcium entry through the NMDAR (36), efficiently cleaves NR2A/B, producing an N-terminal fragment of unknown function that likely remains in the cell membrane (25). This is in marked contrast to the down-regulation of NR1, where calpain activity is not required for the inhibition of NR1 transcription (Fig. 5*B*) or for the decrease in the steady-state levels of NR1 protein (Fig. 3*B*). Calpain-dependent down-regulation of NR2A/B might represent a second negative feedback mechanism to down-regulate NMDAR function; calpain inhibitors have been reported to prevent a significant reduction in whole cell NMDAR-mediated currents in acutely isolated or cultured cortical neurons treated with NMDA or glutamate (27).

Down-regulation of NR1 expression specifically requires activation of NMDARs containing NR2B subunits (Figs. 2*A* and 5*B*) and is therefore associated with signaling pathways coupled to NR1/NR2B or NR1/NR2A/NR2B receptors. Because brief receptor stimulation is sufficient to irreversibly reduce levels of NR1 (Fig. 2*C*), rapid fragmentation of NR2B subunits by calpain would not interfere with NR1 down-regulation. Nor is fragmentation of NR2B required for NR1 down-regulation, which was not prevented by calpain inhibition (Figs. 3*B* and 5*B*).

NR2B is expressed in hippocampal and cortical neurons early in development, when NMDARs are mostly nonsynaptic (14, 37). NR2A appears later (5) and is mainly incorporated into synapses of mature neurons, whereas NR2B predominates at extrasynaptic sites (14, 37–39). The localization of the NMDARs affects their biophysical properties (40) and biological responses. The activation of synaptic receptors initiates changes in synaptic efficacy and promotes survival coupled to induction of the activity of CREB and gene expression of brain-derived neurotrophic factor. In contrast, stimulation of extrasynaptic NMDARs activates a general and dominant CREB shut-off pathway that blocks induction of brain-derived neu-

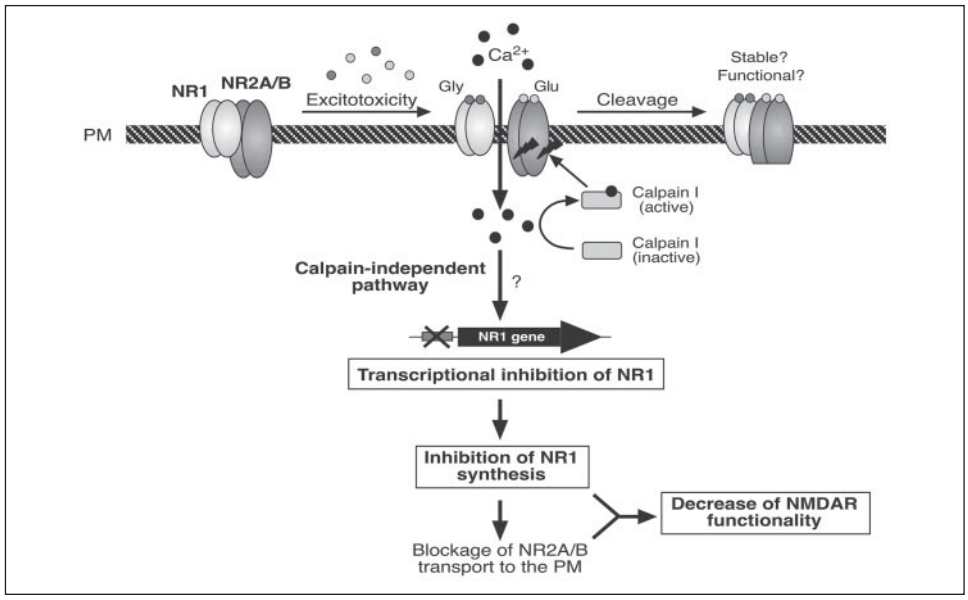

FIGURE 7. **Model of NMDAR regulation in excitotoxicity and ischemia.** Overactivation of NMDARs containing NR2B subunits by the co-agonists glutamate (*Glu*) and glycine (*Gly*) leads to Ca^{2+} overload in post-synaptic neurons, which results in the specific and rapid inhibition of the transcription of the NR1 gene. The mechanism responsible for transcriptional blockade is still unknown, although we have determined that is not dependent on calpain activation but does require calcium influx. The progressive decrease produced in the steady-state levels of the NR1 subunit has important implications for NMDAR functionality because NR1 is an obligatory subunit of this receptor (3). The inhibition of *de novo* NR1 synthesis will probably have an additional effect on receptor activity, through a possible blockage of transport of the NR2A and NR2B subunits to the plasma membrane (*PM*) (35). In parallel to these effects, the Ca^{2+}-dependent protease calpain is activated and efficiently cleaves NR2A/B subunits to produce N-terminal fragments of unknown function that are thought to remain in the PM (25).

rotrophic factor expression and is coupled to cell death pathways (11). The shut-off of CREB has been also described in stroke conditions (Ref. 41 and references therein). The requirement of NR2B subunits and of excitotoxic concentrations of agonists for NR1 down-regulation thus suggests a fundamental role of the extrasynaptic NMDARs overactivation in this regulatory process.

Interestingly, three cAMP regulatory elements have been characterized in the rat NR1 promoter, and these have been suggested to be critical for its expression (42). The inhibition of NR1 transcription might therefore be explained by the shut-off of CREB activity produced by NMDA stimulation of extrasynaptic receptors. In support of this hypothesis, the NR2B-specific antagonist ifenprodil, which prevents NR1 transcriptional down-regulation (Fig. 5*B*), also blocks the decay in CREB phosphorylation mediated by extrasynaptic receptors (11).

Another possible explanation of our results would be the interaction of a repressor element 1 in the NR1 promoter with REST/NRSF (repressor element 1-silencing transcription factor/neuron-restriction silencer factor). This element is a determinant for NR1 up-regulation during neuronal differentiation, a process concomitant with the decrease in levels of REST/NRSF and its interaction with repressor element 1 (19). Interestingly, ischemic insults de-repress expression of this silencing factor in those neurons committed to die, resulting in the suppression of the promoter activity and expression of the GluR2 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type of glutamate receptor (43). It will be very interesting to establish whether this repressor might be also responsible for the suppression of NR1 transcription found in excitotoxic conditions.

The results obtained *in vivo* with the animal model of transient cerebral ischemia (Fig. 6) suggest that this mechanism of NR1 autoregulation operates similarly in the adult brain. This is not unexpected because excitotoxic activation of NMDARs is a key event in neuronal degeneration and death produced during hypoxia, ischemia, and several neurodegenerative pathologies (2). Transcriptional control plays a role in the pathophysiology of the post-ischemic brain, and there are multiple examples of genes down- and up-regulated in the cortex and striatum of rats subjected to transient focal ischemia (44). The down-regulation of NR1 mRNA, starting 2 h after reperfusion (Fig. 6*C*), may have an important role in delayed neuronal death, as suggested for GluR2 (43); NR1 subunits are hardly detectable by 24 h of reperfusion (Fig. 6, *A* and *B*), and there will therefore be no functional synaptic receptors available for signaling to survival pathways. However, the possibility that NR1 regulation is part of an adaptative response of neurons to high concentrations of glutamate cannot be excluded.

Recently, in a mouse model of traumatic brain injury, a long lasting loss of NMDAR function has been described following short lived hyperactivation (<1 h) (45). This decrease in functionality might contribute to the cognitive and neurological impairment produced in patients of traumatic or ischemic brain injury and might also explain the failure of clinical trials with NMDAR antagonists (45). Our results, obtained using a different model of brain injury, suggest that the decrease in NMDAR functionality elicited by hyperactivation may be a general response of neurons to damage and would be mediated in part by regulation of NR1 gene expression.

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REFERENCES

1. Mori, H., and Mishina, M. (1995) *Neuropharmacology* **34,** 1219–1237
2. Choi, D. W. (1988) *Neuron* **1,** 623–634
3. Meguro, H., Mori, H., Araki, K., Kushiya, E., Kutsuwada, T., Yamazaki, M., Kumanishi, T., Arakawa, M., Sakimura, K., and Mishina, M. (1992) *Nature* **357,** 70–74
4. Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakmann, B., and Seeburg, P. H. (1992) *Science* **256,** 1217–1221
5. Monyer, H., Burnashev, N., Laurie, D. J., Sakmann, B., and Seeburg, P. H. (1994) *Neuron* **12,** 529–540
6. Hollmann, M., and Heinemann, S. (1994) *Annu. Rev. Neurosci.* **17,** 31–108
7. Westbrook, G. L. (1994) *Curr. Opin. Neurobiol.* **4,** 337–346
8. Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1991) *Nature* **354,** 31–37
9. Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., Akazawa, C., Shigemoto, R., Mizuno, N., and Masu, M. (1993) *J. Biol. Chem.* **268,** 2836–2843
10. Husi, H., Ward, M. A., Choudhary, J. S., Blackstock, W. P., and Grant, S. G. (2000) *Nat. Neurosci.* **3,** 661–669
11. Hardingham, G. E., Fukunaga, Y., and Bading, H. (2002) *Nat. Neurosci.* **5,** 405–414
12. Wenthold, R. J., Prybylowski, K., Standley, S., Sans, N., and Petralia, R. S. (2003) *Annu. Rev. Pharmacol. Toxicol.* **43,** 335–358
13. Tovar, K. R., and Westbrook, G. L. (2002) *Neuron* **34,** 255–264
14. Tovar, K. R., and Westbrook, G. L. (1999) *J. Neurosci.* **19,** 4180–4188
15. Rao, A., and Craig, A. M. (1997) *Neuron* **19,** 801–812
16. Prybylowski, K., Fu, Z., Losi, G., Hawkins, L. M., Luo, J., Chang, K., Wenthold, R. J., and Vicini, S. (2002) *J. Neurosci.* **22,** 8902–8910
17. Barria, A., and Malinow, R. (2002) *Neuron* **35,** 345–353

18. Buller, A. L., Larson, H. C., Schneider, B. E., Beaton, J. A., Morrisett, R. A., and Monaghan, D. T. (1994) *J. Neurosci.* **14,** 5471–5484
19. Bai, G., Zhuang, Z., Liu, A., Chai, Y., and Hoffman, P. W. (2003) *J. Neurochem.* **86,** 992–1005
20. Awobuluyi, M., Lipton, S. A., and Sucher, N. J. (2003) *J. Neurochem.* **87,** 1066–1075
21. Chazot, P. L., and Stephenson, F. A. (1997) *J. Neurochem.* **68,** 507–516
22. Hall, R. A., and Soderling, T. R. (1997) *J. Biol. Chem.* **272,** 4135–4140
23. Huh, K. H., and Wenthold, R. J. (1999) *J. Biol. Chem.* **274,** 151–157
24. Carroll, R. C., and Zukin, R. S. (2002) *Trends Neurosci.* **25,** 571–577
25. Simpkins, K. L., Guttmann, R. P., Dong, Y., Chen, Z., Sokol, S., Neumar, R. W., and Lynch, D. R. (2003) *J. Neurosci.* **23,** 11322–11331
26. Dong, Y. N., Waxman, E. A., and Lynch, D. R. (2004) *J. Neurosci.* **24,** 11035–11045
27. Wu, H. Y., Yuen, E. Y., Lu, Y. F., Matsushita, M., Matsui, H., Yan, Z., and Tomizawa, K. (2005) *J. Biol. Chem.* **280,** 21588–21593
28. Rose, K., Goldberg, M. P., and Choi, D. W. (1993) *Methods Toxicol.* **1A,** 46–60
29. Bai, G., and Kusiak, J. W. (1995) *J. Biol. Chem.* **270,** 7737–7744
30. Chen, S. T., Hsu, C. Y., Hogan, E. L., Maricq, H., and Balentine, J. D. (1986) *Stroke* **17,** 738–743
31. Liu, T. H., Beckman, J. S., Freeman, B. A., Hogan, E. L., and Hsu, C. Y. (1989) *Am. J. Physiol.* **256,** H589–H593
32. Williams, K. (1993) *Mol. Pharmacol* **44,** 851–859
33. Koh, J. Y., and Choi, D. W. (1988) *J. Neurosci.* **8,** 2153–2163
34. Mu, Y., Otsuka, T., Horton, A. C., Scott, D. B., and Ehlers, M. D. (2003) *Neuron* **40,** 581–594
35. Fukaya, M., Kato, A., Lovett, C., Tonegawa, S., and Watanabe, M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 4855–4860
36. Adamec, E., Beermann, M. L., and Nixon, R. A. (1998) *Brain Res. Mol. Brain Res.* **54,** 35–48
37. Li, J. H., Wang, Y. H., Wolfe, B. B., Krueger, K. E., Corsi, L., Stocca, G., and Vicini, S. (1998) *Eur. J. Neurosci.* **10,** 1704–1715
38. Stocca, G., and Vicini, S. (1998) *J. Physiol.* **507,** 13–24
39. Rumbaugh, G., and Vicini, S. (1999) *J. Neurosci.* **19,** 10603–10610
40. Li, B., Chen, N., Luo, T., Otsu, Y., Murphy, T. H., and Raymond, L. A. (2002) *Nat. Neurosci.* **5,** 833–834
41. Walton, M. R., and Dragunow, I. (2000) *Trends Neurosci.* **23,** 48–53
42. Lau, G. C., Saha, S., Faris, R., and Russek, S. J. (2004) *J. Neurochem.* **88,** 564–575
43. Calderone, A., Jover, T., Noh, K. M., Tanaka, H., Yokota, H., Lin, Y., Grooms, S. Y., Regis, R., Bennett, M. V., and Zukin, R. S. (2003) *J. Neurosci.* **23,** 2112–2121
44. Kim, J. B., Piao, C. S., Lee, K. W., Han, P. L., Ahn, J. I., Lee, Y. S., and Lee, J. K. (2004) *J. Neurochem.* **89,** 1271–1282
45. Biegon, A., Fry, P. A., Paden, C. M., Alexandrovich, A., Tsenter, J., and Shohami, E. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 5117–5122