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

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 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 auto-regulation 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.

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1 Recipient of a predoctoral fellowship from the Comunidad de Madrid.
2 Recipient of a predoctoral fellowship from Ministerio de Ciencia y Tecnología.
3 Recipient of a predoctoral fellowship from Fundación Teófilo Hernando. Present address: Instituto de Investigaciones Biomédicas “Alberto Sols,” CSIC-UAM, Arturo Duperier, 4, 28029 Madrid, Spain.
4 Researcher of the Ramón y Cajal Program. To whom correspondence should be addressed. Tel.: 34-91-5854448; Fax: 34-91-5854401; E-mail: mdiazguerra@ibib.uam.es.

5 The abbreviations used are: NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; CII/III, carbonyl oxy-valinyl-phenylalanine; CREB, cAMP response element-binding protein; DL-AP5, 2-amino-phosphonopentanoic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCUO, 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''-tetraacetic acid tetra(acetoxymethyl)ester; NSE, neuronal specific enolase; PBS, phosphate-buffered saline; RIPA, radioimmune precipitation assay; MOPS, 4-morpholinepropanesulfonic acid.

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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-phosphono- 
ap tic acid (DL-AP5) and ifenprodil were from Tocris-Cookson (Bris- 
tol, UK). NMDA, glutamate, glycine, cytosine β-D-arabinofuranos 
oide, actinomycin D, poly-I-L-lysine, 1-laminin, and 3-(4,5-dimethylthiazol-2- 
yl)-2,5-diphenyltetrazolium (MTT) were all from Sigma. Carbobenzo- 
xy-valinyl-phenylalaninal (calpain inhibitor III, herein after referred to 
as Cilill) and 1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacet 
ic acid tetra(acetoxymethyl)ester (BAPTA-AM) were from Calbiochem (Darmstadt, Germany).

Primary Neuronal Culture—Primary culture of embryonic rat neu 
rons was essentially as described (28) with some modifications. The 
plates were treated with poly-I-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 mechan 
ically dissociated in culture medium (Eagle’s minimum medium supplemented with 28.5 mM NaHCO3, 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 × 105 cells/cm2 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 follow 
ring concentrations of reagents were used for pretreatments or treat 
ments, as indicated under “Results”: 100 μM NMDA, 10 μM glycine, 100 
μM glutamate, 200 μM DL-AP5, 10 μM ifenprodil, 10 μM Cilill, 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 gl 
tamate 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 spectrophotometrically 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 rela 
tive to the untreated ones.

Immunoblot Analysis—The cultures were lysed in RIPA buffer (10 
mm Na2HPO4, pH 7.2, 150 mM NaCl, 1% sodium deoxicolat, 1% Non 
idel P-40, 0.1% SDS) containing protease inhibitors (1 mM phenyth 
ysulfonyl fluoride, 0.2 mM 1,10-phenanthroline, 10 mM pepstatin A, 
10 μg/ml leupeptin, 10 μg/ml aproatin, and 10 mM benzamidcine). 
Brain samples were homogenized in RIPA buffer containing 1% SDS, 1 
mM dithiothreitol, and protease inhibitors as above. Protein concentra 
tions 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 mem 
branes (Pall, Life Sciences).

Immunodetection of proteins was performed by standard proce 
dures. NR1 expression was detected with an anti-NMDAR NR1 mono 
clonal 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 Biotecnology (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.

Immunochemistry—Primary cultures were grown on coverslips 
treated with poly-l-lysine and 1-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 solu 
tion 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 conjugat 
ged 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 
invited Axiovert S100 TV microscope (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 [35S]methi 
onine + cysteine, washed with cold PBS, and lysed in RIPA buffer as 
before. When indicated, NMDA and glycine were present during 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 glyceralde 
hyde 3-phosphate dehydrogenase (GAPDH) cDNA.

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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 Instantimagor, 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 Molenoy 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, 140000 Sybr Green, and 3 mM MgCl₂. 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′-TCTCCAAGAGCCCTTCTGTG), and the reverse primer spanned nucleotides 1541–1561 (5′-AGTTCAACATCCGAAAAG GTGA). 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′-AC GACTGGGACTACGGCTG), and the reverse primer spanned nucleotides 1344–1364 (5′-CTTCTCTGCGCTCATTAGC), amplifying a 134-bp region of the NR2A cDNA.

Cell Transfection and Gene Reporter Assay—The plasmids pRL5.4 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 efficiency of transfection was estimated to be around 15%. After transfection, the cells were maintained in culture for 40 h before experimen tal treatments.

When indicated, the cells were pretreated for 2 h before stimulation with CiIII or with one of the Ca²⁺ 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 Ca²⁺ 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 halothane 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. 1A). Compared with untreated cells, there was a marked decrease in NR1 immuno-reactivity 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.
Immunofluorescence of primary cultures confirmed these results (Fig. 1B). In untreated cells (Fig. 1B, 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. 1B, panel b), both in cell bodies and dendrites.

To exclude the possibility that this 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. 1C), and the results from three independent experiments were quantified (Fig. 1D). 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. 1C). However, after 8 h there was no statistically significant decrease in the combined levels of truncated and full-length NR2 proteins (Fig. 1D). 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. 1D).

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. 2A). Glutamate ($100 \mu M$ in combination with $10 \mu M$ 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 ($10 \mu M$), 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. 2B), 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 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 densitometric 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. *, $p < 0.05$;**, $p < 0.01$. 
### Down-regulation of NR1 by NMDAR Activation

**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.

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. 3D). Levels of β-actin mRNA were unaffected. Consistent with the protein expression data shown in Fig. 2C, the reduction in NR1 mRNA expression after 24 h of continuous exposure to NMDA (Fig. 3D) was not affected by arrest of NMDA signaling with DL-AP5 added to the medium after a 2-h exposure to NMDA (Fig. 3E). 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. 4A) and real-time PCR (Fig. 4B). 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 markedly decreased by 4 h of treatment and was further reduced by 8 h; DL-AP5 completely prevented this decrease (Fig. 4A, top panel). In contrast, the levels of mRNA for the housekeeping gene GAPDH were not modified by NMDA (Fig. 4A, bottom panel).

Because we detected some neuronal death in response to NMDA stimulation (Fig. 1D), it was important to normalize NR1 mRNA expression to a neuronal mRNA that is not significantly modified by NMDA. We used NMDA for this, because de novo synthesis of this subunit was not affected by NMDA treatment (Fig. 3C). This mRNA was expressed at very low levels relative to NR1 but was not modified by NMDA stimulation (Fig. 4A, middle panel). Therefore, for NR1 mRNA quantitation we performed real-time PCR of both mRNAs and normalized NR1 mRNA amounts to those of N2R2A (Fig. 4B). 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. 4C, top panel). In these experiments, the results were normalized to rRNA because unlike mRNA its expression is not modified by actinomycin D treatment (Fig. 4C, 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. 4C). 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.
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. 5A). 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 ifenprodil, demonstrating that NR2B subunits are required (Fig. 5B). In contrast, a reduction in promoter activity of 57% (p < 0.01) was still observed in neurons pretreated with CiIII, 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. 2C, brief exposure to high concentrations of NMDA was sufficient to irreversibly inhibit the NR1 promoter activity measured at later times (Fig. 5C). For this experiment we used pNRL356, a plasmid containing only 356 bp of the NR1 promoter coupled to the firefly luciferase gene (29). When this experiment we used pNRL5.4, a plasmid containing 5.4 kb of the NR1 promoter coupled to the Renilla luciferase gene and was not modified by NMDA stimulation (data not shown).

Using this same experimental procedure, we demonstrated that NMDA-induced down-regulation of NR1 transcription was triggered...
Down-regulation of NR1 by NMDAR Activation

FIGURE 5. NR1 promoter activity in neurons treated with NMDA. A, neurons were transiently co-transfected with the reporter plasmid pHRIS4.4, which contains the NR1 promoter coupled to firefly 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 (10 μM) as indicated. Some cells were pretreated with CiIII (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 pHR1356, 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 treated 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 Ca2+ influx via the NMDAR (Fig. 5C). Chelation of extracellular free Ca2+ 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 Ca2+ such as that produced by NMDAR overactivation. These results thus demonstrate that an influx of calcium ions triggered by brief over-stimulation 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. 6A (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. 6A, 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. 6A, panel b). In marked contrast, NR1 immunoreactivity was significantly reduced in the infarcted tissue (Fig. 6A, 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. 6B, 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. 6B, 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. 6C). 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. 6C, 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. 2A) induces the spe-
Down-regulation of NR1 by NMDAR Activation

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. 5B) or for the decrease in the steady-state levels of NR1 protein (Fig. 3B). 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. 2A and 5B) 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. 2C), rapid fragmentation of NR2B subunits by calpain would not interfere with NR1 down-regulation. Nor is fragmentation of NR2B required for the inhibition of NR1 transcription (Fig. 5B) or for the decrease in the steady-state levels of NR1 protein (Fig. 3B). 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).

NR2B is expressed in hippocampal and cortical neurons early in development, when NMDARs are mostly nonsynaptic (14, 37). NR2B 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 neurotrophic factor (41).
Down-regulation of NR1 by NMDAR Activation

**FIGURE 7.** Model of NMDAR regulation in excitotoxicity and ischemia. Overactivation of NMDARs containing NR2B subunits by the co-agonists glutamate (Glut) 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 blockade 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).

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. 6C), 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 neuronal 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. 5B), 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.

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Down-regulation of NR1 by NMDAR Activation

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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
Sergio Gascón, Rubén Deogracias, Mónica Sobrado, José M. Roda, Jaime Renart, Ángeles Rodríguez-Peña and Margarita Díaz-Guerra

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