Molecular Psychiatry (2008) 13, 99–114

www.nature.com/mp

ORIGINAL ARTICLE

Excitotoxicity and focal cerebral ischemia induce truncation of the NR2A and NR2B subunits of the NMDA receptor and cleavage of the scaffolding protein PSD-95

S Gascón[1], M Sobrado[2,3], JM Roda[2], A Rodríguez-Peña[1] and M Díaz-Guerra[1]

[1]*Instituto de Investigaciones Biomédicas 'Alberto Sols', CSIC-UAM, Arturo Duperier, Madrid, Spain and* [2]*Unidad de Investigación Cerebrovascular, Hospital La Paz, Madrid, Spain*

The *N*-methyl-D-aspartate receptor (NMDAR) is central to physiological and pathological functioning of neurons. Although promising results are beginning to be obtained in the treatment of dementias, clinical trials with NMDAR antagonists for stroke, trauma and neurodegenerative disorders, such as Hungtinton's disease, have failed before. In order to design effective therapies to prevent excitotoxic neuronal death, it is critical to characterize the consequences of excessive NMDAR activation on its expression and function. Previous data have reported partial downregulation of the NR1 and NR2B receptor subunits in response to excitotoxicity and cerebral ischemia. However, the effect of NMDAR overactivation on NR2A, a subunit fundamental to synaptic transmission and neuronal survival, is still elusive. In this study, we report the rapid and extensive proteolytic processing of NR2A, together with the scaffolding protein postsynaptic density-95 (PSD-95), induced by excitotoxic stimulation of cortical neurons *in vitro* and by transient focal cerebral ischemia. Processing of the C terminus of NR2A is irreversibly induced by brief agonist exposure of NR2B-containing receptors, and requires calcium influx and the activity of calpain, also responsible for PSD-95 cleavage. The outcome is a truncated NR2A subunit that is stable and capable to interact with NR1 at the surface of neurons, but lacking the structural domains required for association with scaffolding, downstream signaling and cytoskeletal proteins. Therefore, a rapid and significant uncoupling of synaptic NMDARs from downstream survival pathways is expected to occur during ischemia. This novel mechanism induced by excitotoxicity helps to explain the failure of most therapies based on NMDAR antagonists.
Molecular Psychiatry (2008) **13,** 99–114; doi:10.1038/sj.mp.4002017; published online 8 May 2007

Keywords: Ischemia; excitotoxicity; NMDAR; calpain; PSD-95; cortical neurons

Introduction

The *N*-methyl-D-aspartate type of glutamate receptor (NMDAR) plays key roles in neuronal plasticity, learning and memory in the central nervous system, fundamentally due to its high Ca^{2+} permeability.[1] However, inappropriate activation of the NMDAR is involved in the etiology of several human diseases. Particularly, overstimulation of the NMDARs by agonists can induce excitotoxic cell death and contribute to neuronal degeneration in different disorders, including acute insults such as hypoxia, ischemia, trauma and epilepsy, or chronic neurodegenerative pathologies such as Alzheimer's disease, Parkinson's disease, Huntington's disease, HIV-associated dementia or amyotrophic lateral sclerosis.[2,3] On the other hand, NMDAR hypofunction is also implicated in other pathologies such as schizophrenia.[4]

The receptors are hetero-oligomeric proteins formed by obligatory NR1 subunits[5,6] interacting with NR2A-D, conferring functional variability.[7,8] The major NR2 subunits in adult neocortex and hippocampus are NR2A and NR2B. Although they are not exclusive locations,[9] NR2A is predominantly confined to synapses of mature neurons[5] while NR2B is distributed mainly extrasynaptically.[10–12] The synaptic NMDARs form large and dynamic signaling complexes in the postsynaptic membrane,[13] mainly by interaction of their intracellular NR2 C terminus with additional proteins such as postsynaptic density-95 (PSD-95), which functions as a scaffolding and organizer protein of the PSD. Synaptic NMDAR activity is extremely important for neuronal survival,[14,15] while the extrasynaptic NMDARs are coupled to cell-death pathways.[14] Activation of the synaptic NMDARs promotes survival through the induction of the activity of cAMP response element-binding

Correspondence: Dr M Díaz-Guerra, Instituto de Investigaciones Biomédicas 'Alberto Sols', CSIC-UAM, Arturo Duperier, 4, 28029-Madrid, Spain.
E-mail: mdiazguerra@iib.uam.es
[3]Current address: Instituto de Investigaciones Biomédicas 'Alberto Sols', CSIC-UAM, Arturo Duperier, 4, 28029-Madrid, Spain.
Received 3 October 2006; revised 22 February 2007; accepted 19 March 2007; published online 8 May 2007

protein (CREB) and CREB-mediated expression of prosurvival genes. In contrast, stimulation of extrasynaptic NMDARs activates a general and dominant CREB shut-off pathway that blocks CREB-regulated gene transcription.[14]

Excitotoxicity is a fundamental target for neuroprotective strategies because of its central role in acute and neurodegenerative disorders.[3] Although promising results have been recently reported for uncompetitive NMDAR antagonists in treatment of several forms of dementia,[16] clinical trials with other NMDARs antagonists have failed to be effective on stroke, traumatic brain injury and some neurodegenerative disorders such as Huntington's disease.[17] To design effective neuroprotective therapies, it is critical to understand the reasons for those failures fully. Here, we investigate the hypothesis that NMDAR overactivation might be inducing a remarkable downregulation of the function of synaptic receptors in neurons, helping us to understand how the use of receptor antagonists would further worsen their possibilities of survival.

We have selected an animal model of transient focal cerebral ischemia producing in rats the type of infarct most frequently found in humans. In this model, we have previously established the transcriptional suppression of the NR1 gene in neurons undergoing excitotoxicity.[18] Limited cleavage of NR2B dependent on calpain,[19] an important effector of Ca^{2+} overload during excitotoxicity,[20] was also reported. Nevertheless, according to previous results, NR2A was not regulated by agonist stimulation of mature hippocampal[19,21] or cortical neurons,[22] although a partial decrease of this subunit could be observed at earlier times in culture.[21–23] It was suggested that NR2A was protected from cleavage during neuronal maturation by increased association with PSD-95.[22] Therefore, in order to test our hypothesis, it was essential to characterize unequivocally the response of NR2A, fundamental subunit of synaptic NMDARs, to excitotoxic stimulation of adult neurons. In this work, we demonstrate and describe in depth the extensive processing by calpain of the C-terminal regions of both NR2A and NR2B subunits taking place in mature cortical neurons treated with NMDA, and during transient focal cerebral ischemia in rats. Furthermore, we also identify PSD-95 as an additional calpain substrate in excitotoxic conditions. These results unveil a negative feedback mechanism, which rapidly decreases the function of synaptic and extrasynaptic NMDARs in the adult brain under pathological conditions. This novel mechanism is fundamental to our understanding of the neuronal degeneration taking place during excitotoxicity, since it affects NR2A, an NMDAR subunit critical for synaptic transmission and neuronal survival. Additionally, these data strongly suggest that treatment with NMDAR antagonists of neurons surviving an ischemic insult, and likely having a decreased synaptic NMDAR function, would be detrimental for cell survival.

Materials and methods

Chemicals

NMDAR antagonists 2-amino-phosphonopentanoic acid (DL-AP5) and ifenprodil were obtained from Tocris-Cookson (Bristol, UK). *N*-methyl-D-aspartate (NMDA), glycine, cytosine β-D-arabinofuranoside (AraC), poly-L-lysine, L-laminin, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (calpain inhibitor I, herein ALLN), *N*-acetyl-L-leucyl-L-leucyl-L-methioninal (calpain inhibitor II, herein ALLM) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were obtained from Sigma Co. (St Louis, MO, USA). Carbobenzoxy-valinyl-phenylalaninal (calpain inhibitor III, herein Ci III) and z-VAD-FMK (caspase inhibitor I) were obtained from Calbiochem. The BCA (bicinchoninic acid assay) reagent was obtained from Pierce (Rockford, IL, USA).

Plasmids and viruses

Plasmid yellow fluorescent protein (YFP)-NR2B, generously provided by Dr J Luo (Zhejiang University School of Medicine, Hangzhou, China), encodes the NR2B subunit containing the YFP protein inserted between the fifth and sixth codons after the predicted sequence for the signal peptide.[24] Plasmids pME18S-HA-NR2A and pME18S-HA-NR2B were generous gifts from Dr T Yamamoto (University of Tokyo, Tokyo, Japan) and contain, respectively, the rat NR2A and the mouse NR2B cDNAs with hemagglutinin epitopes in the N terminus between amino-acid residues 51 and 52 for NR2A[25] and between residues 66 and 67 for NR2B.[26] The Syn-GFP lentivirus plasmid was used as a vector for NR2A and NR2B sub-cloning and to produce virus. This plasmid contains a neuronal-specific synapsin promoter regulating the expression of the green fluorescent protein (GFP) gene (Gascón *et al.*, unpublished results). We replaced the GFP gene of Syn-GFP with an EcoRI-SpeI fragment of pME18S-HA-NR2A to produce Syn-HA-NR2A. For NR2B sub-cloning, a NotI site was first introduced upstream of the initiation codon in pME18S-HA-NR2B and pYFP-NR2B by performing mutagenesis with the oligonucleotides 5′-CATGCTCT CTCCCTTAAT**GC**G**G**CCGCCTAGAGGTTTGGCG-3′ and 5′-CGCCAAACCTCTA G**G**CG**GC**CGCATTAAGGGAG AGAGCATG-3′. The mutated nucleotides are highlighted in bold and the *Not*I site is underlined. We then replaced the GFP gene of Syn-GFP with *Not*I-*Eco*RI fragments containing the N-terminal tagged NR2B subunits to generate the plasmids Syn-HA-NR2B and Syn-YFP-NR2B. Lentiviral vectors were produced by co-transfection of HEK293 cells according to Lois *et al.*[27]

Primary neuronal culture

Primary culture of embryonic rat neurons was performed basically as described[28] with some modifications. 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 minimum medium supplemented with 28.5 mM $NaHCO_3$, 22.2 mM glucose, 0.1 mM glutamine, 5% FBS and 5% donor horse serum). Cells were seeded at a density of 0.3×10^6 cells/cm^2 in the same media. To inhibit growth of glial cells, AraC (10 μM) was added to the culture at day 7 and kept until experiment completion. Unless otherwise indicated, cells cultured for 14 days were treated with the following concentrations of reactives: 100 μM NMDA, 10 μM glycine, 200 μM DL-AP5, 10 μM ifenprodil, 5 μM ALLN, 5 μM ALLM, 100 μM zVAD, 2 mM ethylene glycol tetraacetic acid (EGTA) or 10 μM Ci III. The NR2A and NR2B subunits are both expressed at this time in culture.[11]

Lentivirus infection of primary cultures
Primary cultures grown as described previously for 7 days *in vitro* (DIV) were infected with lentivirus directly added to the growing media. Infection proceeded for 7 days before treatment with NMDA and glycine at 14 DIV, as indicated. Conventional virus titration is not possible in the mixed cultures since expression of the recombinant proteins is neuron-specific, as shown below in Supplementary Figure 3. Therefore, we established in advance the amount of virus suspension required to obtain complete infection of the neuronal population in the culture. This was accomplished by double immunofluorescence of the infected cultures with a neuronal-specific antibody and a second antibody for the recombinant protein.

Animal model of cerebral ischemia
All animal procedures were performed in compliance with European Community law 86/609/EEC and were approved by the Consejo Superior de Investigaciones Científicas committee. Male Sprague–Dawley adult rats (275–300 g) were anesthetized with a solution of diazepam (5 mg/kg), ketamine hydrochloride (Ketolar, 62.5 mg/kg) and atropine (0.25 mg/kg), injected intraperitoneally. The femoral artery was cannulated for continuous monitoring of arterial pressure and blood sampling. Analysis of pH, gases and glucose in the blood was performed before and 15 min after occlusion as well as 10 min after reperfusion. Body and brain temperature were maintained at 37 ± 0.5 and 36 ± 0.5°C, respectively, during the whole procedure. The physiological variables studied were not significantly different between groups of animals before, during or after middle cerebral artery occlusion (MCAO) (data not shown). The surgical procedure was a variant of that described by Chen *et al*.[29] and Liu *et al*.[30] A small craniectomy was made over the trunk of the MCA 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 the blood flow was confirmed under an operating microscope. Then, both common carotid arteries were also occluded and all three arteries were kept in this state for 60 min. After blood reperfusion for the indicated times, animals were killed by an inhaled overdose of halothane, and were decapitated. Sham-operated animals were subjected to anesthesia and the surgical procedure, but the occlusion of the arteries was omitted. For protein extracts, the brain was sectioned into 2-mm-thick slices and stained with a 2% solution of triphenyltetrazolium chloride (TTC) (Merck Bioscience, Darmstadt, Germany). The unstained area of the cerebral cortex (right hemisphere), defined as infarcted tissue, was dissected, as well as the corresponding region in the left hemisphere. For immunohistochemistry, 24 h after blood reperfusion, rats were deeply anesthetized as described above and perfused intracardially with cold 4% paraformaldehyde in Phosphate-buffered saline (PBS, pH 7.4). The brains were removed immediately and postfixed in the same fixative at 4°C for an additional 6 h. Then, they were cryoprotected by serial immersion for at least 6 h in increasing concentrations of sucrose (10, 15 and 20%) in PBS at 4°C. After that, coronal frozen sections (25 μm thick) were prepared using a cryostat (Leica, Heidelberg, Germany) and processed for immunohistochemistry.

Assessment of neuronal injury in the primary cultures
We used the MTT reduction assay to measure cell viability by adding MTT (0.5 mg/ml) to the media. The formazan salts formed after 4 h at 37°C were solubilized in 5% sodium dodecyl sulfate (SDS), 5 mM HCl and spectrophotometrically quantified at 570 nm. The contribution of glial cells in the mixed cultures to absorbance was established by exposing sister cultures to 400 μM NMDA, 10 μM glycine for 24 h, conditions which induce nearly complete neuronal death and no glial damage. After subtracting this value, we calculated the viability of stimulated neurons relative to the untreated ones.

Immunoblot analysis
Cultures were lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM Na_2HPO_4, pH 7.2, 150 mM NaCl, 1% sodium deoxycolate, 1% NP-40, 0.1% SDS) containing protease inhibitors (1 mM PMSF, 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 (DTT) and protease inhibitors as before. Protein determination was done using the BCA reagent, and equal amounts of protein (25–50 μg) were fractioned by polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Pall, Life Sciences, East Hills, NY, USA). Immunodetection of proteins was performed by standard procedures using monoclonal antibodies specific for the NR1 subunit (Pharmingen, San Diego, CA, USA), PSD-95 (Chemicon, Temacula, CA, USA), hemagglutinin epitope (HA) (Covenance Research Products, Berkeley, CA, USA), β-actin (Sigma Co.,

St Louis, MO, USA) and non-erythroid spectrin (Chemicon), as well as rabbit polyclonal antibodies that recognize neuron-specific enolase (NSE) (ICN Biomedicals, Costa Mesa, CA, USA), HA (Bethyl Laboratories, Montgomery, TX, USA) or conserved regions in the N terminus (Pharmingen) or the C terminus (Chemicon) of NR2A and 2B subunits. We also used goat polyclonal antibodies specific for the C terminus of the NR2A subunit and goat secondary antibodies coupled to horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunocomplexes were detected with the Bioluminescence kit from Perkin-Elmer Life Sciences (Boston, MA, USA). Densitometric analysis of bands was performed with NIH Image analysis software.

Immunoprecipitation

Approximately 300 μg of protein extracts prepared in RIPA buffer as described above were incubated with 2.5 μg of the NR1 antibody. After 1 h at 4°C, immunocomplexes were precipitated with 100 μl of 10% protein A Sepharose for 1 h at the same temperature. Beads were washed four times with RIPA buffer before solubilization in sample buffer and gel fractionation as described above.

Immunofluorescence

Primary cultures were grown on coverslips treated with poly-L-lysine and L-laminin as described above. After stimulation with NMDA and glycine as indicated, they were fixed in 4% (w/v) paraformaldehyde in PBS for 2 min at 4°C and washed with PBS. Blocking was then performed by incubation in 10% (v/v) horse serum at room temperature for 30 min. When indicated, cells were permeabilized and blocked for 30 min at room temperature in 10% (v/v) horse serum, 0.1% (v/v) Triton X-100 in PBS. Dilution of the antibodies was performed in 10% (v/v) horse serum and cells were incubated for 1 h at room temperature as indicated. We used monoclonal antibodies for neuronal nuclei protein (NeuN) (Abcam, Cambridge, UK) or HA (Covance, Berkeley, CA, USA) and the rabbit sera for NR2A/B (N- or C-terminal specific), GFP (Invitrogen, Carlsbad, CA, USA) or glial fibrillary acidic protein (GFAP) (Chemicon). Immunoreactivity was detected with secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 647 or Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA), as indicated, before mounting with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). Confocal images were acquired using a Radiance 2000 confocal (BioRad, Hercules, CA, USA) coupled to an inverted Axiovert S100 TV microscope (Zeiss, Thornwood, NY, USA) with a $\times 63$ Plan-Apochromat oil immersion objective. Overlaying images produced the two-color merged image.

Immunohistochemistry

Identification of the infarcted tissue in the neocortex was performed by Nissl (0.1% (w/v) cresyl violet) staining of slide-mounted coronal sections. Adjacent sections were then processed for immunohistochemistry. Briefly, sections were permeabilized and blocked by treatment with 10% (v/v) sheep serum, 0.4% (v/v) Triton X-100 in Tris-buffered saline (TBS) for 3 h at room temperature. Antibody dilution was performed in 4% (v/v) sheep serum, 0.2% (v/v) Triton X-100 prepared in TBS. Sections were incubated overnight at 4°C with the primary antibodies diluted as described. After several washes in TBS, the sections were incubated for 2 h at room temperature with the secondary antibodies: Alexa Fluor 568-conjugated anti-mouse IgG, Alexa Fluor 594-conjugated anti-rabbit and Alexa Fluor 488-conjugated anti-goat IgG from Molecular Probes, all diluted at 1:200. After washing, sections were counterstained for 30 min at room temperature with 2 μM TO-PRO 3 iodide (Molecular Probes) before mounting in Fluoromount-G solution (SouthernBiotech). Controls performed in parallel without primary antibodies showed very low levels of non-specific staining. Confocal images were acquired as described above.

Results

Specific decrease of NR2A, NR2B and PSD-95 in focal cerebral ischemia

Considering the central role of the NR2A subunit in synaptic transmission and neuronal survival, it was critical to establish whether levels of this NMDAR subunit were regulated *in vivo* in mature neurons undergoing excitotoxic cell death. We selected an animal model of cerebral ischemia, a pathology where neuronal degeneration is mainly due to excitotoxicity induced by overactivation of NMDARs. In addition, we selected transient focal ischemia because this is the type of cerebral infarct most frequently found in humans. Therefore, transient focal cerebral ischemia was induced in rats by a 1 h occlusion of the MCA followed by reperfusion for variable lengths of time (0–48 h).[29,30] In this model, the characteristic changes of ischemic necrosis were limited to the parietal and sensory-motor cortex and large infarcts were reproducibly generated in the right MCA territory after 24 h of reperfusion. Staining of coronal sections with Nissl showed a clearly hypochromatic area in the ipsilateral neocortex indicative of neuronal injury (Figure 1a, central panel). In the studies presented here, we have compared equivalent regions in the neocortex of the contralateral (Figure 1a, box B) and ipsilateral hemispheres (Figure 1a, box A) of animals subjected to MCAO and 24 h of reperfusion. In addition to the loss of cytoplasmic Nissl staining throughout the lesion, cells changed from a rounded appearance in the control cortex (Figure 1a, panel B) to a triangular morphology with a reduced size of cell body and processes in the infarcted region (Figure 1a, panel A). Also, we observed a characteristic nuclear condensation in some cells of the injured area. All these changes are indicative of ischemia as progressively leading to neuronal loss in the infarcted area.

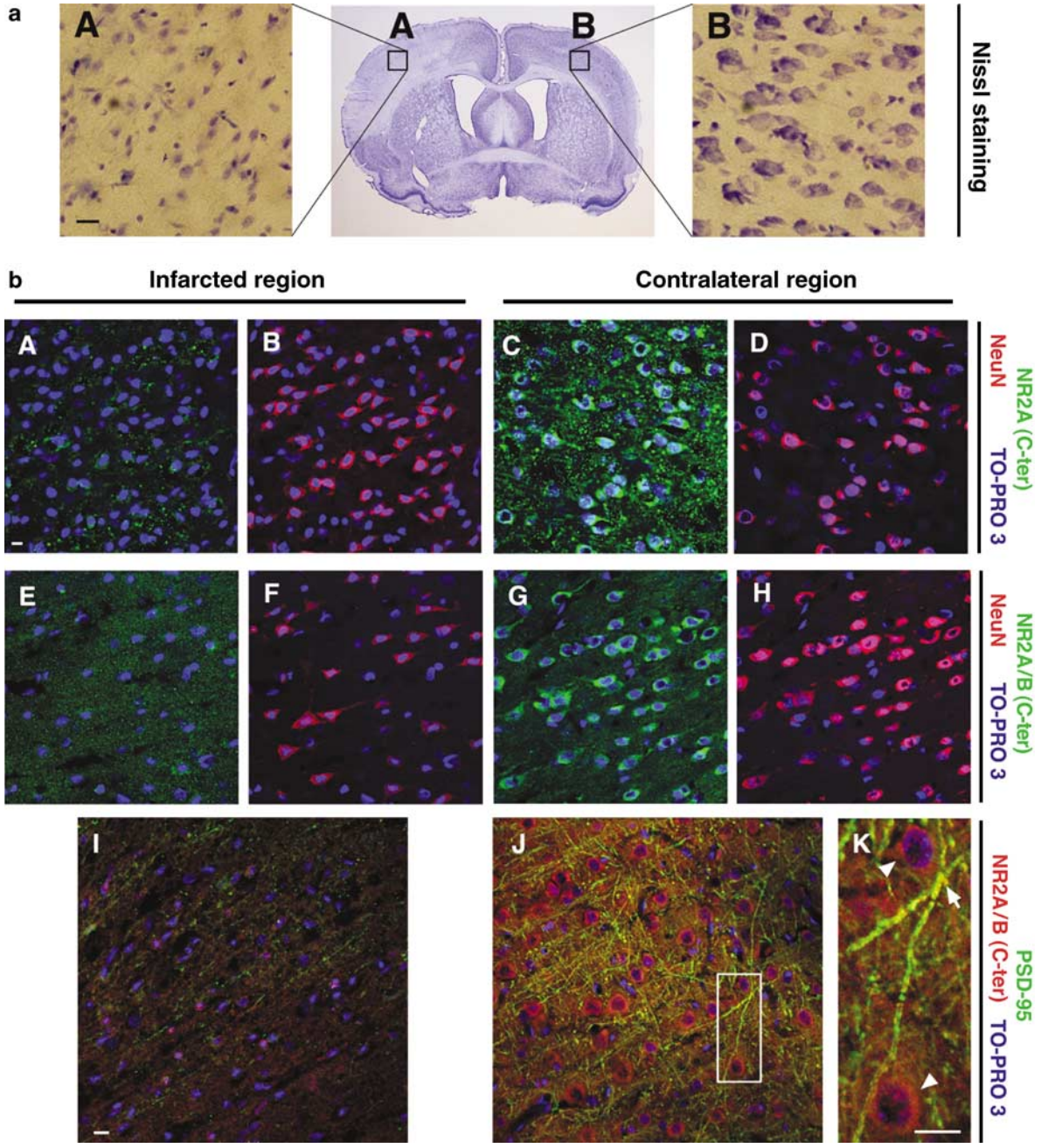

Figure 1 Specific decrease in levels of NR2A, NR2B and protein postsynaptic density-95 (PSD-95) in focal transient brain ischemia. (**a**) Coronal sections of the brain from an animal subjected to 1 h middle cerebral artery occlusion (MCAO) followed by 24 h of blood reperfusion. Nissl staining revealing a hypochromatic area in the ipsilateral neocortex (shown in detail in panel A) indicative of neuronal injury, compared with the equivalent region of the contralateral hemisphere (panel B). (**b**) Double immunohistochemistry of adjacent sections showing representative results obtained in the contralateral hemisphere (right panels) and the infarcted region (left panels). Expression of NR2A (C-ter) (green) (panels A and C) and neuronal nuclei protein (NeuN, red) (panels B and D), or NR2A/B (C-ter) (green) (panels E and G) and NeuN (red) (panels F and H) was detected. Panels I and J: expression of PSD-95 (green) and NR2A/B (C-ter) (red). A detail showing NR2A/B expression in the soma of neurons (arrowheads) and coexpression with PSD-95 in the membrane of neurites (arrow) is presented in panel K. TO-PRO 3 was used to stain the cell nuclei (blue). Confocal microscopy images correspond to single sections, and the scale bars represent 10 μm.

In order to analyze the possible regulation of the NR2A subunits during cerebral ischemia, double immunohistochemistry of sections adjacent to those stained with Nissl was performed using antibodies directed against an intracellular C-terminal domain of this protein (Figure 1b, panels A and C). Immunoreactivity was observed in the soma and dendrites of numerous neurons in the cortex of the contralateral

hemisphere (panel C), while the staining dramatically decreased in the ischemic region (panel A). Moreover, immunohistochemistry of NeuN, a specific marker of post-mitotic neurons, showed that this decrease occurred in neurons in the infarcted cortex surviving ischemia and expressing NeuN (Figure 1b, compare panels A and B), demonstrating that the reduction of NR2A was specific and not a general effect of the ischemic process. Similar results were obtained with an antibody directed against conserved regions in the C termini of both the NR2A and NR2B subunits (Figure 1b, panels G and E). These results, therefore, demonstrate a dramatic decrease of both NR2A and NR2B subunits associated with transient focal cerebral ischemia.

Next, we decided to characterize the stability during cerebral ischemia of PSD-95, a major postsynaptic protein interacting with the C-terminal domains of the NR2 subunits[31] and suggested to protect NR2A from calpain-cleavage in mature neurons.[22] Double immunohistochemistry using a PSD-95 antibody showed the staining of numerous neurites in the cortex of the contralateral hemisphere (Figure 1b, panel J), partially colocalizing with the NR2 subunits (Figure 1b, panel K). However, a strong reduction in PSD-95 levels was clearly observed in the infarcted region in parallel to the decrease in NR2 subunits (Figure 1b, panel I). In conclusion, these results demonstrate a specific decrease of NR2A subunits, concurrent with NR2B and PSD-95, that is induced in adult neurons by excitotoxic processes taking place *in vivo*.

Specific C-terminal processing of NR2A and NR2B subunits in cultured neurons induced by NMDA treatment

To approach in detail the mechanism of NR2A regulation induced by excitotoxicity, we have used an *in vitro* model consisting of primary cultures of rat cortical neurons subjected to overactivation of NMDARs. We selected cortical neurons because these were the cells mainly affected by ischemia in the animal model. Neurons cultured for 14 DIV were incubated with high concentrations of NMDA (100 μM) and the co-agonist glycine (10 μM), conditions that stimulate synaptic and extrasynaptic NMDARs. The levels of the NR2A subunit at different times of agonist treatment were determined by immunoblot analysis with the antibody specific for the C terminus of this subunit. First of all, the results showed that NR2A was also regulated *in vitro* by excitotoxicity (Figure 2a, upper panel). A detailed kinetic analysis of the decrease of NR2A was performed by quantitation of the results obtained in three independent experiments (Figure 2b). Thirty minutes of NMDA stimulation were enough to significantly reduce this subunit by 45% ($P<0.01$), although by 4 and 8 h of treatment, the decrease was about 80% relative to the untreated cells ($P<0.01$). Analysis of the levels of NR2A and NR2B with the antibody for the C terminus (Figure 2a) showed a progressive decrease in the full-length proteins with similar kinetics to NR2A (data not shown), indicative of both NR2 subunits being regulated in a time-dependent way by NMDA.

Levels of other proteins such as β-actin (data not shown) or NSE (Figure 2a and b) were not significantly modified for the duration of NMDA treatment, suggesting that the significant decrease in full-length NR2A is not a consequence of neuronal cell death. Measurement of neuronal viability by MTT assay confirmed this hypothesis since excitotoxic death was estimated to be 25% at 8 h of NMDA treatment (Figure 2b), well below the reduction observed in NR2A levels at this time (74%). Therefore, the decrease in the NR2 subunits is not a general effect of neuronal death due to long-term activation of NMDARs. However, although viability is still high in the time-window analyzed here, these neurons are committed to die and a progressive increase in neuronal death is observed at later times of NMDA treatment (data not shown).

To characterize further the decrease in NR2 levels, we used an antibody recognizing sequences conserved in the N-termini of NR2A and NR2B subunits. We demonstrated the accumulation of a 115-kDa fragment along with NMDA treatment, concomitant to the decrease in the full-length subunits (Figure 2a). This result strongly suggests that the mechanism of NR2A regulation during excitotoxicity is proteolytic processing of the C-terminal region, as previously shown for NR2B.[19] When NMDA treatment is prolonged enough, the fragmentation of the NR2A and NR2B subunits progresses to near completion, rendering truncated subunits which are quite stable in the neuron.

These results were confirmed by immunofluorescence of permeabilized neurons with the antibodies directed to the C- or N-terminal regions of the NR2 subunits (Figure 2c). In the untreated cells, we observed the characteristic immunoreactivity for the NMDAR, with staining of the cell soma and clusters at puncta on dendrites (Figure 2c, panels A and C). Stimulation with NMDA and glycine for 3 h induced a significant decrease in the staining of the C-terminal-specific antibody, both in the cell bodies and dendrites of neurons otherwise immunoreactive for NeuN (Figure 2c, panel B). Similar results were obtained with antibodies directed against the C terminus of NR2A (data not shown). In contrast, with the antibody for the N terminus, we found comparable levels of NR2A and NR2B subunits in treated and untreated neurons (Figure 2c, panels C and D), although distinctive dendritic focal swelling and varicosities were observed in cells undergoing excitotoxicity (Figure 2c, panel D).[32] In conclusion, these experiments confirm the significant processing of the C-terminal region of both NR2A and NR2B in cortical neurons treated with high concentrations of NMDA, and the stability of the N-terminal fragments of these subunits.

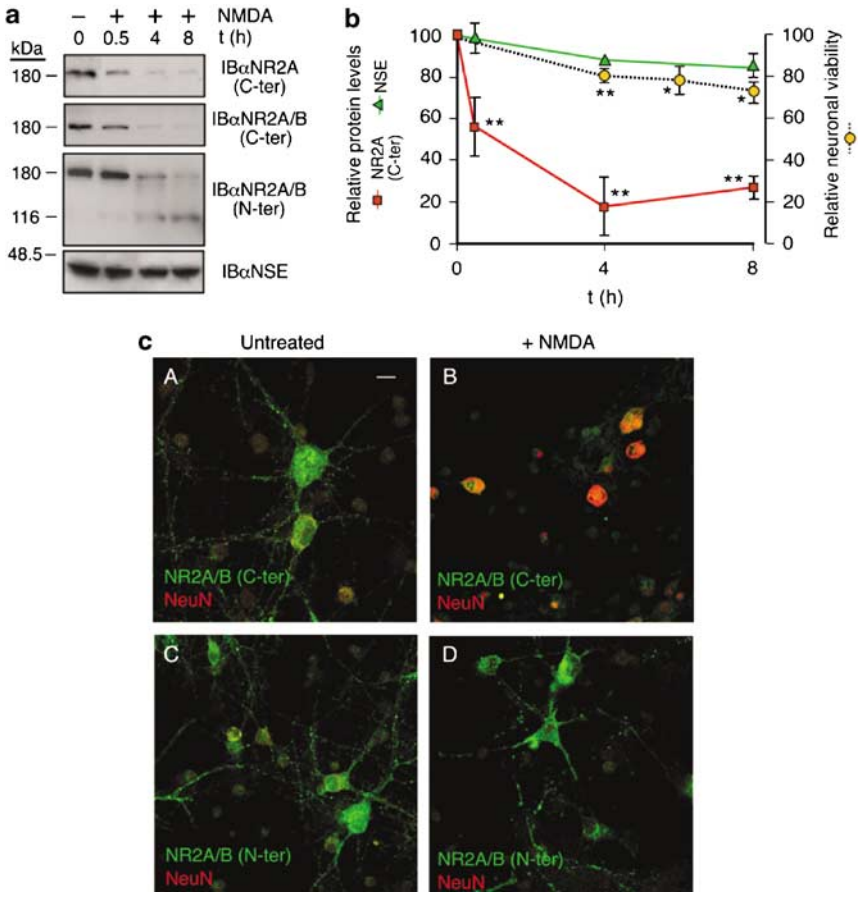

Figure 2 Specific decrease of NR2A and NR2B levels in a cellular model of excitotoxicity. (**a**) Time-course of NR2 decrease. Primary cultures of rat cortical neurons (14 days *in vitro* (DIV)) were incubated with *N*-methyl-D-aspartate (NMDA, 100 μM) and glycine (10 μM) for 30 min, 4 and 8 h, or left untreated. Immunoblotting was performed using antibodies for NR2A (C-ter), NR2A/B (C-ter or N-ter) or neuron-specific enolase (NSE). (**b**) Quantitation of the decay of NR2A and neuronal viability with time of NMDA/glycine treatment. Levels of NR2A (red squares) and NSE (green triangles) were established by densitometric analysis of immunoblots using NIH Image software. Values obtained at different times were compared with those found in untreated cells, arbitrarily given a 100% value. Viability of neurons in the mixed cultures is shown relative to the untreated cells (yellow circles). Average of three independent experiments with standard deviations is given. Student's *t*-test was used to assess the differences between treated and untreated cells (*$P<0.05$, **$P<0.01$). (**c**) Primary cultures were stimulated with NMDA and glycine as described for 3 h or left untreated. Double immunofluorescence analysis was performed to study levels of the NR2 subunits (green) and neuronal nuclei protein (NeuN, red), used to label the postmitotic neurons of the mixed cultures. The NR2 antibodies were specific for conserved regions in the C terminus (A and B) or N terminus (C and D) of subunits NR2A and 2B. Confocal microscopy images correspond to a single section. Results are representative of three independent experiments. The scale bars represent 10 μm.

Cleavage of NR2A and NR2B is irreversibly induced by brief overstimulation of NMDARs containing NR2B subunits and requires calcium influx

To define whether the mechanism of NR2 regulation by NMDA is associated with excitotoxic stimulation of the NMDAR or could also be observed with low concentrations of agonists, we analyzed the cleavage of NR2A and NR2B using concentrations of NMDA lower than 100 μM (Figure 3a). Cortical neurons were incubated for 6 h with glycine (10 μM) and different concentrations of NMDA (0.1–100 μM). We observed a slight decrease in NR2A levels starting at 10 μM (17%, $P<0.05$), although higher NMDA concentrations were required for a dramatic reduction (82% for 100 μM NMDA, $P<0.001$), suggesting that NR2A processing is associated with excitotoxic stimulation of NMDARs. A nonlinear response to NMDA concentration was previously described in the neuronal loss induced by long-term treatment with this agonist.[33] Similar concentration dependence was also observed with antibodies directed against the C-terminal or N-terminal regions of NR2A and NR2B (Figure 3a, middle panels). Therefore, in this model, both NR2A and NR2B require concentrations of NMDA in the excitotoxic range to be efficiently processed.

In order to rationally design therapies for excitotoxicity, and considering the critical role of NR2A cleavage in neuronal degeneration, we need to define if NMDAR antagonists can prevent this process after brief overstimulations. Thirty minutes of NMDA

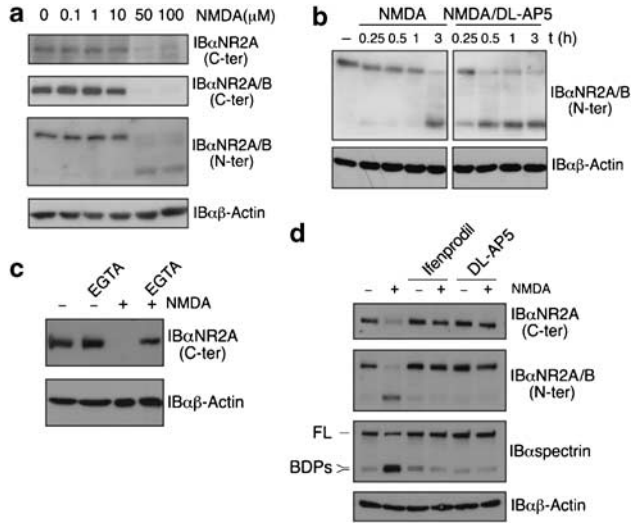

Figure 3 Irreversible decrease of NR2A and NR2B by excitotoxic stimulation of *N*-methyl-D-aspartate receptors (NMDARs) containing NR2B subunits requires calcium influx. (**a**) Primary cultures were stimulated with glycine (10 μM) and different concentrations of *N*-methyl-D-aspartate (NMDA) ranging from 0.1 to 100 μM for 6 h. Levels of NR2A (C-ter), NR2A/B (N-ter and C-ter) or β-actin were established as described. (**b**) Neurons were incubated with NMDA (100 μM) and glycine (10 μM) for the indicated times. Then, they were either collected or treated with the antagonist 2-amino-phosphonopentanoic acid (DL-AP5, 200 μM) for the times needed to add up to 24 h of stimulation. Control samples correspond to untreated cells. Levels of NR2A/B (N-ter) or β-actin were established by immunoblot as above. (**c**) Primary cultures of neurons were pre-incubated for 2 h with the Ca^{2+} chelator EGTA (2 mM) before addition of NMDA/glycine. One hour after agonist treatment, cells were washed and incubated in media containing DL-AP5 (200 μM) for the next 24 h. Immunoblotting was performed as described for NR2A (C-ter) and β-actin. (**d**) Analysis of the effect of different antagonists of the NMDAR. Primary cultures were incubated for 6 h with NMDA/glycine as indicated, with or without the antagonists DL-AP5 (200 μM) or ifenprodil (10 μM). Immunoblotting was used to establish the levels of NR2A (C-ter), NR2A/B (N-ter), spectrin or β-actin. The electrophoretical mobility of full-length (FL) brain spectrin or the breakdown products (BDPs) resulting from calpain activation is indicated. Results are representative of three independent experiments.

treatment followed by protection with the specific competitive antagonist DL-AP5 (200 μM) (Figure 3b, right panel) had the same effect on the NR2 levels measured 24 h later as observed after 3 h of continuous stimulation (Figure 3b, left panel). Fifteen minutes of treatment produced intermediate results. From these experiments, we can conclude that the regulation of NR2A and NR2B by ligand activation cannot be blocked or reverted by antagonists after a critical period of time.

Activation of NMDARs leads to Ca^{2+} influx due to the high permeability of the receptor to this ion.[1] To characterize if the mechanism of NR2 regulation by excitotoxicity is dependent on Ca^{2+} entry, we performed immunoblot analysis of cultures treated with NMDA in the presence of a Ca^{2+} chelator (Figure 3c). To prevent possible toxic effects of long treatments with chelators, we chose to perform a brief exposure to NMDA (1 h) followed by protection with DL-AP5 for 24 h. The cultures were pre-incubated with EGTA (2 mM) for 2 h before the NMDA addition, and were also maintained in EGTA during agonist treatment. Then, cells were washed and fed with media containing DL-AP5 as described. Buffering of extracellular Ca^{2+} by incubation with EGTA prevented the decrease in NR2A measured 24 h later (Figure 3c, upper panel). Thus, the decrease in NR2A levels induced by NMDA, observed to be $97\% \pm 0.01$, was reduced to only $23\% \pm 5$ when EGTA was also present ($P < 0.01$). Similar results were obtained with the antibody for the C-termini of the NR2A and NR2B subunits (data not shown). Therefore, this experiment demonstrates that the mechanism leading to a decrease of NR2 subunits is triggered by the entry of calcium into neurons as a consequence of even a brief NMDAR overstimulation.

Cell death pathways are coupled in neurons to the activation of extrasynaptic NMDARs,[14] where NR2B is the major NR2 subunit.[11] To test whether the regulation of NR2 induced by excitotoxicity had a specific requirement for this type of subunit, we used ifenprodil, a selective inhibitor of NR2B.[34] As shown by immunoblot analysis (Figure 3d), ifenprodil (10 μM) prevented the regulation of the NR2A and NR2B subunits induced by NMDA similarly to DL-AP5. Since neurons at this time in culture express NR2A and NR2B, we can conclude that regulation of NR2 subunits specifically requires overactivation of NR1/NR2B or NR1/NR2A/NR2B receptors.

Previous results demonstrating a requirement for Ca^{2+} (Figure 3c) and reports showing calpain as a major effector in excitotoxicity and ischemia[35–37] suggested that activation of this protease might be responsible for the NR2A cleavage induced by NMDA, as shown before for NR2B.[19] We first confirmed the activation of calpain in the *in vitro* model of excitotoxicity used in these experiments. Appearance of characteristic breakdown products from brain spectrin (BDPs) is widely used as a marker for the activation of this protease. Cleavage by calpain of this cytoskeletal protein (240 kDa) produces fragments of molecular weights 150 and 145 kDa, which are stable in the cell. Immunoblot analysis with antibodies specific for this protein (Figure 3d) demonstrated the fragmentation of the full-length spectrin (FL) to the characteristic BDPs occurring in cortical neurons treated with NMDA in parallel to NR2 cleavage. Significantly, treatment with ifenprodil or DL-AP5, which prevented regulation of the NR2 subunits by NMDA, also blocked the activation of calpain, suggesting that both processes were probably related. All together, the experiments in Figure 3 define the mechanism of cleavage of the NR2A and NR2B subunits as an irreversible process induced

even by brief overstimulation of NMDARs containing NR2B subunits. Since the agonist-induced cleavage of NR2A requires calcium influx and is concomitant to calpain activation, this protease is probably responsible for the proteolytic processing of this subunit.

NR2A subunit and PSD-95 are both calpain substrates in cortical neurons of different times in culture

To analyze the involvement of calpain in the regulation of NR2A, we characterized the levels of this subunit in neurons treated with NMDA for 6 h in the presence of different cell-permeable protease inhibitors (Supplementary Figure 1). Pre-incubation with neutral cysteine protease inhibitors ALLN (5 μM) and ALLM (5 μM), but not the pan-caspase inhibitor zVAD (10 μM), prevented the decrease in NR2A levels, supporting a possible requirement for calpain activity in the regulatory process. Then, we used the Ci III to directly demonstrate the involvement of this protease in NR2A regulation stimulated by excitotoxicity (Figure 4a). Pre-incubation of primary cultures with Ci III (10 μM) nearly prevented the proteolysis of spectrin and, therefore, the activation of calpain induced by 6 h of NMDA treatment. Interestingly, this calpain inhibitor also prevented the decrease in the levels of NR2A observed in excitotoxic conditions, from $75 \pm 19\%$ to only $15 \pm 8\%$ when Ci III was also present ($P < 0.01$). Therefore, the activity of calpain is required for NR2A proteolysis as shown before for NR2B.[19]

Since we previously observed a dramatic decrease in PSD-95 levels taking place during cerebral ischemia, we decided to characterize the stability of this protein in excitotoxic conditions produced *in vitro* (Figure 4a). Immunoblot analysis with specific antibodies showed a significant decrease in PSD-95 levels in neurons treated with NMDA for 6 h ($69 \pm 3\%$) which was reduced to a $24 \pm 18\%$ when Ci III was also present ($P < 0.01$). Therefore, these results demonstrate for the first time the efficient processing by calpain of PSD-95, together with the NR2 subunits, induced in cortical neurons by NMDAR overactivation.

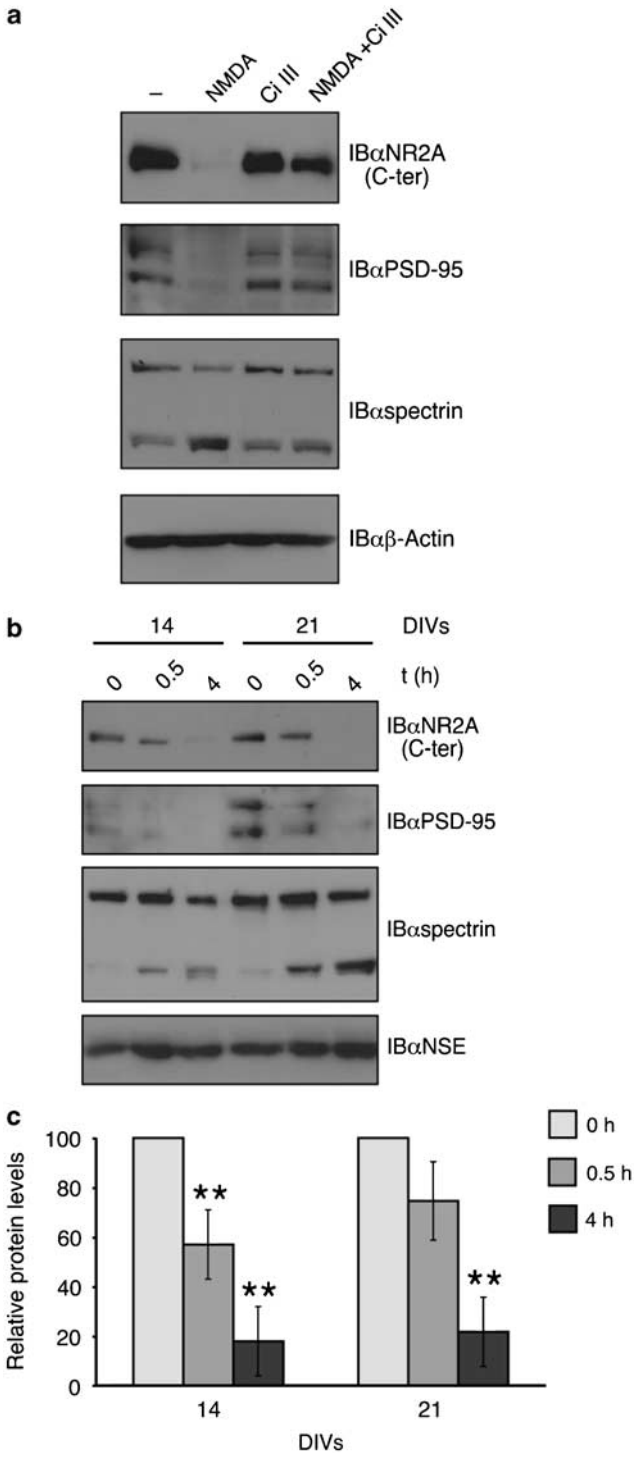

Figure 4 The NR2A subunit and protein postsynaptic density-95 (PSD-95) are calpain substrates during excitotoxicity, and cleavage is induced in cortical neurons of different times in culture. (**a**) Cultures of neurons were pre-incubated for 2 h with calpain inhibitor III (Ci III) (10 μM) before a 6 h treatment with *N*-methyl-D-aspartate (NMDA)/glycine as described. The inhibitors were also present for the duration of the experiment. Immunoblotting was performed for NR2A (C-ter), PSD-95, spectrin and β-actin as indicated. Results are representative of three independent experiments. (**b**) Cultures of cortical neurons of 14 or 21 days *in vitro* (DIV) were incubated as described with NMDA and glycine for 30 min, 4 h or left untreated. Immunoblot analysis was used to establish levels of NR2A subunit (C-ter), PSD-95, spectrin or NSE. (**c**) Quantitation of the decay of the NR2A subunit with time of NMDA/glycine treatment at DIV 14 and 21. Levels of NR2A were established by densitometric analysis of immunoblots using NIH Image software. Values obtained at different times were referred to those found in untreated cells, arbitrarily given a 100% value. Average of three independent experiments with standard deviations is given. Student's *t*-test was used to assess the differences between treated and untreated cells (**$P < 0.01$).

Previous reports have suggested that NR2A is not regulated by agonist stimulation in mature neurons[19,21] because it is protected from cleavage by increased association with PSD-95 along with neuronal maturation.[22] Therefore, we tested this hypothesis by comparing the regulation of NR2A and PSD-95 in cortical neurons of two different times in culture (14 and 21 DIV) during NMDAR overactivation. Neurons were incubated with NMDA for 30 min and 4 h, and levels of these proteins were established by immunoblot analysis with specific antibodies. In the case of NR2A, we present a representative result (Figure 4b, upper panel) and the statistical analysis of three independent experiments (Figure 4c). Consistent with previous reports,[38] levels of the NR2A subunit increased in the untreated neurons with time in culture. In addition, we observed a decrease in NR2A immunoreactivity upon agonist treatment, although it was not statistically significant in cultures of 21 DIV stimulated for only 30 min (Figure 4c). The levels of NR2A were decreased similarly in cultures of 14 or 21 DIV by longer NMDA treatments, with the amount of this subunit being approximately 20% of that observed in control cells after 4 h ($P<0.01$). Meanwhile, the levels of NSE were not affected by this treatment (Figure 4b, lower panel).

For PSD-95, we first confirmed data showing a dramatic increase in levels of this protein with time in culture.[39] By immunoblot analysis, we verified that levels of PSD-95 were low in neurons cultured for 7 DIV, and progressively increased at 14 and 21 DIV, as described (Supplementary Figure 2). Then, we compared cleavage of PSD-95 to calpain activation detected by spectrin fragmentation (Figure 4b). Very early after NMDA stimulation of cultures of 14 DIV, we observed activation of calpain as well as a strong decrease in the levels of PSD-95. Although the starting levels of PSD-95 were approximately four times higher in neurons cultured for 21 DIV compared with 14 DIV (Supplementary Figure 2), a strong decrease in PSD-95 could also be observed after 30 min of agonist treatment, with this protein being hardly detectable by 4 h. In conclusion, the results presented here show that a consistent reduction in the levels of NR2A and PSD-95 is induced by NMDA treatment in neurons of different times in culture.

Analysis of the cellular localization and interactions of the truncated NR2 subunits produced by NMDA treatment

The results presented so far suggested that the N-terminal fragments of NR2A and NR2B produced by calpain-cleavage were stable in neurons treated with NMDA. However, a detailed characterization of each one of the truncated NMDAR subunits was hampered by the availability of antibodies directed against nonconserved amino acids in their N-terminal regions which would distinguish NR2A and NR2B. Consequently, we decided to express selectively in neurons recombinant NR2 subunits with N-terminal tags (HA) or fusions proteins (YFP), which would allow us to distinguish endogenous and recombinant NR2 proteins and to separately identify each recombinant subunit. To optimize the fidelity and reliability of the processes induced by NMDA treatment on the recombinant subunits, we chose to perform these experiments in primary cultures of neurons. Therefore, we generated lentiviral vectors expressing HA-NR2A, HA-NR2B and YFP-NR2B by sub-cloning into Syn-GFP, a plasmid that contains the neuronal-specific synapsin promoter driving the expression of GFP (Gascón *et al.*, unpublished results). Infection of primary cultures with lentivirus syn-GFP illustrated the specificity of synapsin promoter to direct efficient expression of the recombinant proteins exclusively in neuronal cells (Supplementary Figure 3a). GFP fluorescence was excluded from the astrocytes present in the cultures, detected as GFAP positive cells. We performed analysis of the expression and localization of the recombinant NR2 proteins relative to the endogenous subunits by immunofluorescence of cultures doubly infected with viruses carrying syn-HA-NR2A and syn-YFP-NR2B, using antibodies against the C terminus of the NR2A subunit, HA or GFP (Supplementary Figure 3b). The results demonstrated nearly complete infection of the neurons in the primary culture (panel A) and a general colocalization of the recombinant NR2 proteins with each other (panel B) and with the endogenous subunits (panel A). We compared infected and uninfected cells by immunoblot analysis with NR2-specific antibodies and estimated that the expression levels of the recombinant proteins were nearly a 50% of those due to the endogenous subunits (data not shown). Finally, by immunofluorescence of nonpermeabilized cells with HA antibodies, we demonstrated the localization at the plasma membrane of the NR2 recombinant subunits expressed in cortical neurons (Supplementary Figure 3c). Cultures were infected with viruses carrying syn-HA-NR2A or syn-HA-NR2B together with syn-GFP, a virus used to facilitate the localization of the infected cells by direct fluorescence. We observed the characteristic pattern for the NMDAR with staining at the membrane of the cell soma and clusters at puncta on dendrites (see enlargements in panels B and D, respectively, for HA-NR2A and HA-NR2B).

Once we had confirmed the fidelity of the expression system, we characterized the stability and localization of the recombinant NR2A subunits in neurons undergoing excitotoxicity. In addition, we also considered it important to re-examine previous data for the truncated NR2B[19] because under our conditions cleavage of this subunit is almost complete. Cultures infected with syn-HA-NR2A (Figure 5a) or syn-HA-NR2B (Figure 5b) were stimulated with NMDA and glycine for 1 and 3 h, or left untreated. Then, an analysis of the recombinant NR2 subunits was performed by immunoblot and immunofluorescence of nonpermeabilized cells using a mouse antibody directed against the HA epitope. Importantly, the untreated cells expressed a recombinant

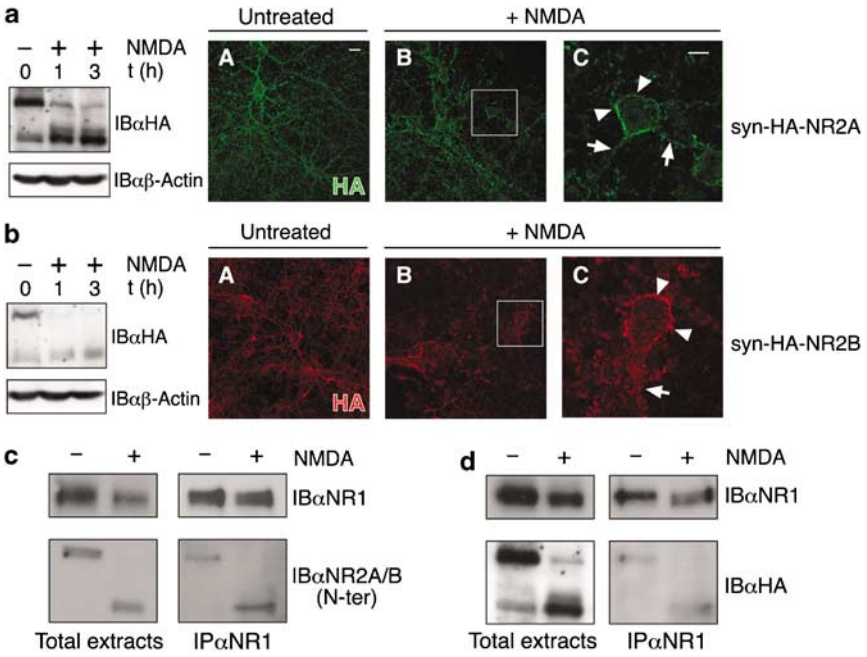

Figure 5 The N-terminal fragments of NR2 produced by calpain cleavage are stable in the membrane where they interact with NR1. (**a**) Primary cultures infected as described with syn-HA-NR2A were stimulated at 14 days *in vitro* (DIV) with *N*-methyl-D-aspartate (NMDA) and glycine for 1 or 3 h or left untreated. Immunoblot analysis with mouse hemagglutinin epitope (HA)-specific antibodies was performed to analyze the levels of the recombinant NR2A subunit (left panel). Levels of β-actin were also established as a control. Immunofluorescence analysis of nonpermeabilized cells was performed with HA-specific antibodies in untreated neurons (panel A) or neurons treated for 3 h with NMDA and glycine (panels B and C). The enlarged image shows staining of the membrane in the cell soma (arrowheads) and clusters on dendrites (arrows) (panel C). Confocal microscopy images correspond to a single section. Results are representative of three independent experiments. The scale bars represent 10 μm. (**b**) Primary cultures were infected with syn-HA-NR2B and processed as described. (**c**) Interaction of the N-terminal fragments of the endogenous NR2 subunits with NR1 in NMDA-treated neurons. Total protein extracts prepared from cultures incubated with NMDA for 3 h or left untreated were immunoprecipitated with antibodies specific for the NR1 subunit. We performed immunoblot analysis of the immunoprecipitated proteins and total extracts using that same antibody (upper panels) or the one recognizing NR2A/B (N-ter) (lower panels). (**d**) Interaction of the N-terminal fragments of the recombinant NR2A subunits with NR1 in NMDA-treated neurons. Primary cultures were infected with syn-HA-NR2A as before. Total protein extracts prepared from cultures incubated with NMDA for 3 h or left untreated were immunoprecipitated with antibodies specific for the NR1 subunit. We performed immunoblot analysis of the immunoprecipitated proteins using that same antibody (upper panels) or a rabbit polyclonal antibody recognizing the HA epitope (lower panels).

HA-NR2A subunit of the expected size, which was readily processed after NMDA treatment to produce a 115-kDa fragment which was stable for the duration of the experiment (Figure 5a). In accordance with this, we did not observe significant differences in the levels of HA-NR2A present at the surface of treated and untreated cells. Except for some degeneration of the neurites (Figure 5a, compare panels A and B), the pattern of staining of the cell bodies was not modified by NMDA treatment (compare Supplementary Figure 3c, panel B, and Figure 5a, panel C). Results obtained for the recombinant HA-NR2B subunit were similar (Figure 5b), although we systematically observed lower levels of expression for this protein compared with HA-NR2A and a reduced stability upon NMDA stimulation. In conclusion, these experiments demonstrate the stability and the cell surface location of the truncated subunit NR2A and, in a lower extent, NR2B in neurons treated with high concentrations of NMDA.

Since the N-terminal fragments of NR2A and NR2B are in the membranes of overstimulated neurons, we next explored the possibility of their interaction with NR1 subunits. To this aim, we performed co-immunoprecipitation assays of non-infected cultures (Figure 5c), and also of cells infected with syn-HA-NR2A (Figure 5d) or syn-HA-NR2B (data not shown). We selected a time of treatment with NMDA of 3 h since NR1 downregulation was only partial at this time,[18] while significant proteolytic processing of full-length NR2 proteins had occurred (Figure 5c, left panels). Immunoprecipitation of those extracts with an NR1-specific antibody demonstrated the interaction of this subunit with full-length NR2 proteins in the untreated cells and with the truncated NR2 subunits in NMDA-stimulated neurons (Figure 7c, right panels). Once we had demonstrated the interaction of NR1 with the N-terminal fragments produced by cleavage of endogenous NR2 subunits, we wanted to establish if both NR2A and NR2B truncated proteins were

independently able to form heteromeric complexes with NR1. We performed similar co-immunoprecipitation experiments in the infected cells, although this time we used a rabbit polyclonal antibody directed against the HA epitope to analyze the recombinant HA-NR2 subunits co-immunoprecipitating with NR1. The results obtained in neurons expressing HA-NR2A (Figure 5d) were similar to those obtained for the endogenous NR2A subunits (Figure 5c), therefore demonstrating that NR1 interacts with the truncated NR2A subunit. In the case of HA-NR2B, the interaction with NR1 could not be established probably due to the low levels of truncated recombinant protein (data not shown). In conclusion, these experiments show that at least the 115-kDa truncated form of NR2A produced by calpain-cleavage after NMDAR overactivation is stable and also capable of forming hetero-oligomers with the NR1 subunit at the cell membrane.

Truncation of the NR2A and NR2B subunits and cleavage of PSD-95 in focal cerebral ischemia

The results obtained *in vivo* demonstrated the cleavage of both NR2 subunits and PSD-95 in association with transient focal cerebral ischemia. After the detailed analysis of the regulation of these proteins performed in a model of excitotoxicity *in vitro*, we sought to establish whether a similar mechanism might be working in the ischemic brain. We were also interested in characterizing the kinetics of the regulatory process, since it might be important to estimate its consequences *in vivo*. Therefore, we analyzed the brains of animals subjected to MCAO followed by different times of blood reperfusion ($n=3$ in each group). We prepared extracts from the infarcted area (I), and compared them with those obtained from the corresponding region of the contralateral hemisphere (C) or to sham-operated animals (Sh) (Figure 6). The levels of NR2 subunits were characterized by immunoblot analysis with different antibodies (Figure 6a). In this model, MCAO was sufficient to induce a significant decrease in NR2A, as demonstrated in animals killed immediately after 1 h of closure. The levels of this subunit were reduced by $59\pm10\%$ in the ipsilateral hemispheres of those animals compared with their contralateral regions ($P<0.001$). However, blood reperfusion further increased the extent of NR2A reduction, with this protein being hardly detectable by 24 h of reperfusion ($94\pm2\%$ decrease, $P<0.001$). Similar results were obtained with the NR2A/B antibody specific for the C terminus of these proteins, showing that there was nearly complete processing of both NR2A and NR2B subunits. Immunoblot analysis with the NR2A/B antibody specific for the N terminus not only confirmed the extent of the NR2 processing, demonstrated by the loss of the full-length proteins, but also showed the accumulation of 115-kDa truncated forms of these subunits that were stable even after 48 h of reperfusion. Analysis by immunoblot of NSE established that processing of NR2A and NR2B was

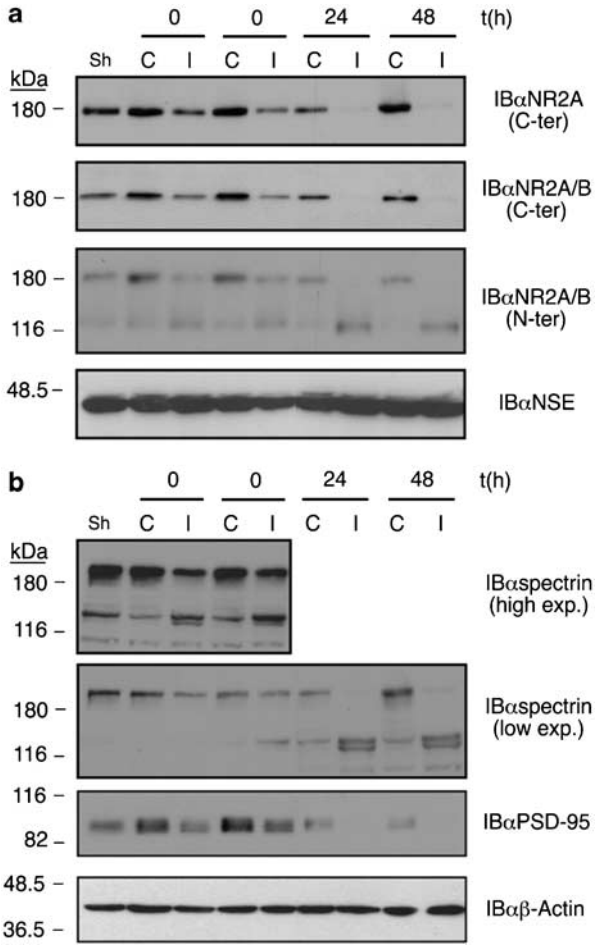

Figure 6 Truncation of NR2A and NR2B, and cleavage of protein postsynaptic density-95 (PSD-95) in transient brain ischemia. (**a**) Protein extracts were prepared from the infarcted region of the cortex (I) and the corresponding area in the contralateral hemisphere (C) of rats subjected to 1 h middle cerebral artery occlusion (MCAO) followed by reperfusion for the indicated times, and from sham-operated animals (Sh) ($n=3$, each group). Equal amounts of protein (50 μg) were analyzed by immunoblot with antibodies for NR2A (C-ter), NR2A/B (C-ter and N-ter) and neuron-specific enolase (NSE). (**b**) Protein extracts prepared as above were analyzed by immunoblot with antibodies for spectrin, PSD-95 and β-actin. In order to help detection of the breakdown products (BDPs), two different exposures of the spectrin immunoblot are shown. Results are representative of three independent experiments.

specific and not a general effect of the ischemic process. Therefore, these results demonstrate that the C-terminal regions of both NR2 proteins are processed very early in ischemia, resulting in truncated subunits that are not further processed or degraded for at least 48 h.

Considering that calpain is activated very early during the ischemic process[36,37] and the results previously obtained in the *in vitro* model of excitotoxicity, it is very likely that this protease is responsible for the proteolytic processing of the NR2A and NR2B subunits occurring in ischemia.

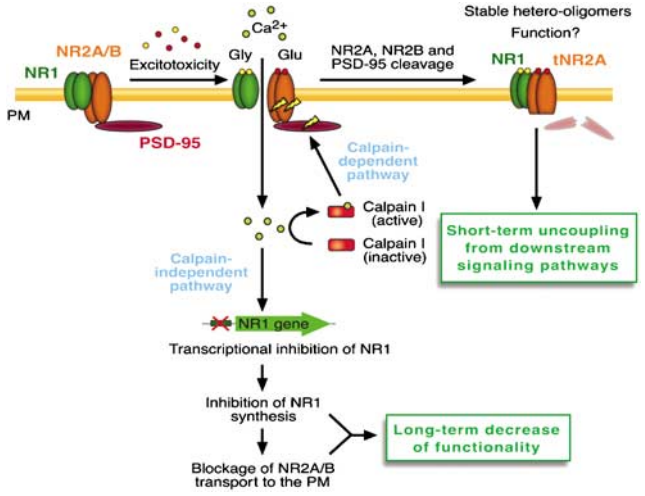

Figure 7 Model of *N*-methyl-D-aspartate receptors (NMDAR) regulation during excitotoxicity and ischemia. Excessive activation of NMDARs containing NR2B subunits leads to Ca^{2+} overload in postsynaptic neurons, which results in the activation of the Ca^{2+}-dependent protease calpain I. This protease cleaves, among other substrates, the C-terminal regions of the NR2A and NR2B subunits, protein postsynaptic density-95 (PSD-95) and the cytoskeletal brain protein spectrin. A complex formed by the NR1 subunit and N-terminal fragments of NR2A (tNR2A), devoid of sequences important for interactions with the intracellular signaling machinery and scaffolding proteins, is stable in overactivated neurons, although we do not know if it might have some novel function. The result of NR2 downregulation will be a short-term decrease of synaptic, and probably also extrasynaptic, NMDAR functionality. Calcium overload is also responsible for transcriptional blockage of the NMDAR obligatory subunit NR1,[18] which both directly and indirectly will constitute a long-term mechanism of downregulation of the NMDAR.

We confirmed calpain activation by the production of brain spectrin BDPs, paying special attention to the kinetics of the process (Figure 6b). We already observed calpain activity in the ipsilateral hemispheres of those animals killed immediately after release of the MCA, showed by a decrease of $34 \pm 6\%$ in levels of brain spectrin ($P < 0.001$), demonstrating the activation of this protease even during the occlusion time (Figure 6b, upper panel). However, calpain activity increased with the extent of reperfusion and hardly full-length spectrin could be detected after 24 h. It is noteworthy that the kinetics of calpain activation were not only parallel to the processing of the NR2 subunits but also to the decrease in PSD-95 observed at early times in the ischemic process (Figure 6b). The levels of this protein were reduced by $50 \pm 6\%$ in the ipsilateral hemispheres of animals killed immediately after 1 h of closure or by $90 \pm 2\%$ of animals killed after 24 h of reperfusion, compared with their corresponding contralateral regions ($P < 0.001$). Meanwhile, the amount of β-actin, used as a control for the specificity of the proteolytic process, was not modified by ischemia (Figure 6b, lower panel).

All together, the results presented here strongly suggest that calpain is responsible for a fast and efficient dismantling of the structural organization of the postsynaptic density (PSD) occurring in the infarcted region of animals subjected to cerebral ischemia. This is accomplished through the coordinated processing of the NR2A and NR2B subunits of the NMDAR and their associated proteins spectrin, which links the NMDAR to the actin cytoskeleton,[40] and PSD-95, a major scaffolding protein tethering the NMDAR to signaling proteins and the neuronal cytoskeleton.

Discussion

We have unveiled a new mechanism of autoregulation of the NMDAR induced in mature neurons by agonist overactivation, schematically represented in Figure 7. Since it affects NR2A, an essential subunit of synaptic NMDARs, it will have a great impact on synaptic transmission and survival, fundamental functions of this subunit in mature neurons. In a cellular model of excitotoxicity, activation of NMDARs induces a specific and rapid decrease in levels of the NR2A subunit, which is not due to neuronal loss. The regulation of NR2A during excitotoxicity is dependent on calcium influx and requires calpain activity. This protease cleaves very efficiently the C-terminal region in NR2A, producing a fragmented subunit lacking sequences important to form signaling complexes at the post-synaptic membrane.[13] The results presented here also confirm and extend previous observations concerning a similar mechanism of regulation for NR2B,[19] although by lengthening treatment with NMDA, we observe an extensive processing of this subunit. Since NR2B is predominantly outside synapses,[10–12] the function of the extrasynaptic NMDARs will be affected by NMDA in addition to that of the synaptic receptors. Furthermore, we have previously demonstrated a late-onset mechanism of NMDAR regulation operating on the obligatory NR1 subunit, consisting of transcriptional suppression of this gene under excitotoxic conditions.[18] Inhibition of NR1 synthesis, both directly and indirectly, through retention of newly synthesized NR2 proteins in the endoplasmic reticulum similarly to NR1 knockout mice,[41] will also result in a progressive decrease in the activity of synaptic and extrasynaptic NMDARs (Figure 7). Although NR1 regulation is independent of the activity of calpain, it is similar in several other aspects to the regulation of NR2 subunits. It is associated with signaling pathways coupled to NR1/NR2B or NR1/NR2A/NR2B receptors, requires calcium influx, and brief stimulation of NMDARs with excitotoxic concentrations of agonist is sufficient to irreversibly reduce the levels of this receptor subunit. Interestingly, a significant reduction in whole-cell NMDAR-mediated currents has been shown to take place in acutely isolated or cultured cortical neurons after agonist stimulation.[23]

The intracellular C-terminal domains of the NR2 subunits are required for NMDAR localization and function, as demonstrated in genetically modified mice expressing receptors devoid of those sequences.[42,43] Considering the dramatic processing of NR2 C-terminal regions during excitotoxicity, the uncoupling of the synaptic NMDARs from scaffolding proteins, downstream signaling pathways and the cytoskeleton is expected. Additionally, we reveal that PSD-95, a major postsynaptic scaffolding protein interacting with the NR2 subunits, is also very efficiently cleaved by calpain during excitotoxicity. Finally, it is well established that brain spectrin, a protein linking subunits NR1 and NR2 to the cytoskeleton, is likewise cleaved by calpain. All together, these data show that activation of calpain during excitotoxicity profoundly affects the structural organization of the PSD, and suggest that the dismantling of this structure will have a great impact on functions coupled to synaptic NMDARs. Given the importance of these receptors for neuronal survival, we propose that autoregulation of the NMDARs induced by excitotoxicity has an important role in neuronal degeneration. A requirement for NMDAR-mediated synaptic transmission in neuronal survival has been shown before. Blockade of the NMDAR induces widespread apoptosis and worsening of ongoing neurodegeneration in the developing and adult central nervous system.[44–46] Environmental enrichment, which stimulates synaptic activity, inhibits spontaneous apoptosis in the hippocampus and is neuroprotective.[47] Regarding the consequences of the decrease in function of extrasynaptic NMDARs, coupled to cell-death pathways,[14] a protective role might be suggested. However, we do not believe this to be really the case since a brief NMDAR overstimulation is sufficient to induce excitotoxicity.

We have characterized the destiny of the NR2 fragments produced by calpain under excitotoxic conditions. C-terminal NR2 fragments are not detected by NR2A/B antibodies specific for this region, suggesting that they are probably unstable. However, the stability of the NR2 N-terminal fragments produced under excitotoxic conditions is demonstrated by our results. Expression of recombinant NR2 subunits with N-terminal tagging sequences establishes that the NR2A-truncated protein is not only stable but still located in the plasma membrane of neurons treated with NMDA, where it interacts with NR1 subunits. We do not presently know if these truncated NMDAR, devoid of physiological function, might instead have novel functions in neurons. In the case of the NR2B-truncated subunit, we find that it is also partially stable and present in the membrane of neurons undergoing excitotoxicity, although the interaction with NR1 could not be established. Given the different properties and functions associated to NR2A and NR2B subunits, it will be interesting to further characterize in the future the differential stability found for the NR2 subunits during excitotoxicity.

The experiments performed *in vivo* with the animal model of transient focal cerebral ischemia show a similar mechanism of NR2A and NR2B autoregulation operating in the adult brain in a pathological situation. This result could be anticipated since the excitotoxic activation of NMDARs is a key event in neuronal degeneration and death produced in different disorders, including hypoxia and ischemia and several neurodegenerative pathologies.[3] The correlation between the cellular and animal models of excitotoxicity is noteworthy and, although regulation of NR2A by overactivation has remained elusive for some time, the results presented here obtained *in vivo* and *in vitro* unequivocally demonstrate cleavage of this protein in mature neurons. In the cellular model of excitotoxicity, the slightly slower kinetics of the decrease of NR2A observed in cortical cultures of 21 DIV compared with younger neurons might explain the observations of Dong *et al.*,[22] which show no significant NR2A cleavage after brief agonist stimulations (30 min). The higher levels of PSD-95 in neurons at 21 DIV might be initially slowing down the processing of the NR2A subunit, but this protein is also cleaved soon after the NMDA treatment.

In animals killed immediately after occlusion, a significant decrease in NR2A and NR2B levels is already observed even before blood reperfusion. The regulatory process is not only rapid but specific and due to C-terminal cleavage of these proteins in the ischemic cortex, where the truncated subunits are stable for at least 48 h after reperfusion. Since we have described before similar levels of NR2A mRNA in the contralateral and ipsilateral hemispheres of rats subjected to MCAO and 4 h of reperfusion,[18] a post-transcriptional mechanism of NR2 regulation is suggested. Calpains are important mediators of cellular toxicity and pathology[48] and are activated in processes of brain ischemia and excitotoxic degeneration, among others.[35–37] In order to understand the process of neuronal degeneration associated to these pathologies, it is important to characterize the substrates of this protease and the way calpain cleavage modifies their functions. One of them is the Na^+/Ca^{2+} exchanger (NCX),[49] the major plasma membrane Ca^{2+} extruding system in neurons. Inactivation of this protein by calpain has a prominent role in the delayed increase in Ca^{2+} and neuronal death occurring during excitotoxicity.[49] In the model characterized here, we observe calpain activation starting very early during the occlusion of the MCA and a very good correlation with processing of PSD-95 and NR2 subunits. These results strongly suggest that this protease is responsible for the coordinated cleavage of subunits NR2A and NR2B and their associated proteins, spectrin and PSD-95, in the infarcted region during cerebral ischemia. The neuroprotective role of calpain inhibition found in a model of transient focal cerebral ischemia[36] can be explained in the light of the important functions in neuronal survival of substrates of this protease.

This novel mechanism of NMDAR autoregulation induced by excitotoxicity might have an important role in the neuronal death observed not only in stroke

but also other pathologies. The decrease in synaptic NMDAR function might be a general response of neurons to damage, directed to dispose of the severely injured cells. In relation to this, a long-lasting loss of NMDAR function has been described in a mouse model of traumatic brain injury following a short-lived hyperactivation (<1 h).[50] This decrease in NMDAR function might contribute to the cognitive and neurological impairment produced in patients with traumatic or ischemic brain injury and might also explain the failure of clinical trials with competitive antagonists of the NMDAR. These drugs compete with glutamate and glycine at the agonist binding sites, blocking both normal and excessive activation of the NMDAR. Therefore, intolerable side-effects such as drowsiness, hallucinations and coma were observed in clinical trials. In the case of stroke and traumatic brain injury, clinical trials were started despite the fact that NMDA antagonists did not show a significant post-insult neuroprotective time-window in animal models.[17] The results presented here strongly suggest a reason for the failure of those therapies. Neurons surviving an ischemic insult likely have a downregulated synaptic NMDAR function and, thus, further suppression of those receptors by competitive antagonists may facilitate their death instead of protecting them.

Anyway, the NMDAR is still a promising therapeutic target for intervention in neurological diseases. Memantine is a low-affinity uncompetitive NMDAR open-channel blocker that was recently approved by the European Union and the United States Food and Drug Administration (FDA) for treatment of dementia. Phase 3 clinical trials have shown that this drug is effective in treating moderate-to-severe Alzheimer's disease while being clinically tolerated.[16] Currently, memantine is in trials for other neurological disorders, including other types of dementia, and promising results have been also obtained in rat models of stroke. In the future, the development of these drugs or the design of new compounds for the treatment of acute or chronic neurological disorders should take into account the process of NMDAR autoregulation induced by excitotoxicity described in this investigation.

Acknowledgments

This work was supported by grant SAF2003-02327 (MD-G) from 'Dirección General de Investigación Científica y Técnica' (Ministerio de Ciencia y Tecnología). MD-G is a researcher of the Ramón y Cajal Program. SG is a recipient of a predoctoral fellowship from Comunidad de Madrid. We thank Dr T Yamamoto for the generous gift of pME18S-HA-NR2A and pME18S-HA-NR2B, and Dr J Luo for plasmid YFP-NR2B. Plasmid Syn-GFP was generated in collaboration with Dr P Scheiffele and Dr FG Scholl (Columbia University, NY, USA). We are grateful to E Abantos for technical support. We also thank Dr J Renart and T Iglesias for helpful discussions.

References

1 Mori H, Mishina M. Structure and function of the NMDA receptor channel. *Neuropharmacology* 1995; **34**: 1219–1237.
2 Lipton SA, Rosenberg PA. Excitatory amino acids as a final common pathway for neurologic disorders. *N Engl J Med* 1994; **330**: 613–622.
3 Choi DW. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1988; **1**: 623–634.
4 Moghaddam B. Bringing order to the glutamate chaos in schizophrenia. *Neuron* 2003; **40**: 881–884.
5 Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 1994; **12**: 529–540.
6 Westbrook GL. Glutamate receptor update. *Curr Opin Neurobiol* 1994; **4**: 337–346.
7 Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S. Molecular cloning and characterization of the rat NMDA receptor. *Nature* 1991; **354**: 31–37.
8 Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M *et al.* Molecular characterization of the family of the *N*-methyl-D-aspartate receptor subunits. *J Biol Chem* 1993; **268**: 2836–2843.
9 Thomas CG, Miller AJ, Westbrook GL. Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. *J Neurophysiol* 2006; **95**: 1727–1734.
10 Tovar KR, Westbrook GL. The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses *in vitro*. *J Neurosci* 1999; **19**: 4180–4188.
11 Li JH, Wang YH, Wolfe BB, Krueger KE, Corsi L, Stocca G *et al.* Developmental changes in localization of NMDA receptor subunits in primary cultures of cortical neurons. *Eur J Neurosci* 1998; **10**: 1704–1715.
12 Rumbaugh G, Vicini S. Distinct synaptic and extrasynaptic NMDA receptors in developing cerebellar granule neurons. *J Neurosci* 1999; **19**: 10603–10610.
13 Husi H, Ward MA, Choudhary JS, Blackstock WP, Grant SG. Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. *Nat Neurosci* 2000; **3**: 661–669.
14 Hardingham GE, Fukunaga Y, Bading H. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci* 2002; **5**: 405–414.
15 Papadia S, Stevenson P, Hardingham NR, Bading H, Hardingham GE. Nuclear Ca^{2+} and the cAMP response element-binding protein family mediate a late phase of activity-dependent neuroprotection. *J Neurosci* 2005; **25**: 4279–4287.
16 Chen HS, Lipton SA. The chemical biology of clinically tolerated NMDA receptor antagonists. *J Neurochem* 2006; **97**: 1611–1626.
17 Ikonomidou C, Turski L. Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? *Lancet Neurol* 2002; **1**: 383–386.
18 Gascón S, Deogracias R, Sobrado M, Roda JM, Renart J, Rodriguez-Peña A *et al.* Transcription of the NR1 subunit of the N-methyl-D-aspartate receptor is down-regulated by excitotoxic stimulation and cerebral ischemia. *J Biol Chem* 2005; **280**: 35018–35027.
19 Simpkins KL, Guttmann RP, Dong Y, Chen Z, Sokol S, Neumar RW *et al.* Selective activation induced cleavage of the NR2B subunit by calpain. *J Neurosci* 2003; **23**: 11322–11331.
20 Neumar RW, Xu YA, Gada H, Guttmann RP, Siman R. Cross-talk between calpain and caspase proteolytic systems during neuronal apoptosis. *J Biol Chem* 2003; **278**: 14162–14167.
21 Dong YN, Wu HY, Hsu FC, Coulter DA, Lynch DR. Developmental and cell-selective variations in N-methyl-D-aspartate receptor degradation by calpain. *J Neurochem* 2006; **88**: 206–217.
22 Dong YN, Waxman EA, Lynch DR. Interactions of postsynaptic density-95 and the NMDA receptor 2 subunit control calpain-mediated cleavage of the NMDA receptor. *J Neurosci* 2004; **24**: 11035–11045.
23 Wu HY, Yuen EY, Lu YF, Matsushita M, Matsui H, Yan Z *et al.* Regulation of N-Methyl-D-aspartate receptors by calpain in cortical neurons. *J Biol Chem* 2005; **280**: 21588–21593.
24 Qiu S, Hua YL, Yang F, Chen YZ, Luo JH. Subunit assembly of N-methyl-D-aspartate receptors analyzed by fluorescence resonance energy transfer. *J Biol Chem* 2005; **280**: 24923–24930.

25 Hironaka K, Umemori H, Tezuka T, Mishina M, Yamamoto T. The protein-tyrosine phosphatase PTPMEG interacts with glutamate receptor delta 2 and epsilon subunits. *J Biol Chem* 2000; **275**: 16167–16173.
26 Nakazawa T, Komai S, Tezuka T, Hisatsune C, Umemori H, Semba K *et al*. Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR epsilon 2 (NR2B) subunit of the N-methyl-D-aspartate receptor. *J Biol Chem* 2001; **276**: 693–699.
27 Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 2002; **295**: 868–872.
28 Rose K, Goldberg MP, Choi DW. Citotoxicity in murine neocortical cell culture. *Methods Toxicol* 1993; **1A**: 46–60.
29 Chen ST, Hsu CY, Hogan EL, Maricq H, Balentine JD. A model of focal ischemic stroke in the rat: reproducible extensive cortical infarction. *Stroke* 1986; **17**: 738–743.
30 Liu TH, Beckman JS, Freeman BA, Hogan EL, Hsu CY. Polyethylene glycol-conjugated superoxide dismutase and catalase reduce ischemic brain injury. *Am J Physiol* 1989; **256**: H589–H593.
31 Kornau HC, Schenker LT, Kennedy MB, Seeburg PH. Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* 1995; **269**: 1737–1740.
32 Matesic DF, Lin RC. Microtubule-associated protein 2 as an early indicator of ischemia-induced neurodegeneration in the gerbil forebrain. *J Neurochem* 1994; **63**: 1012–1020.
33 Koh JY, Choi DW. Vulnerability of cultured cortical neurons to damage by excitotoxins: differential susceptibility of neurons containing NADPH-diaphorase. *J Neurosci* 1988; **8**: 2153–2163.
34 Williams K. Ifenprodil discriminates subtypes of the N-methyl-D-aspartate receptor: selectivity and mechanisms at recombinant heteromeric receptors. *Mol Pharmacol* 1993; **44**: 851–859.
35 Siman R, Noszek JC. Excitatory amino acids activate calpain I and induce structural protein breakdown *in vivo*. *Neuron* 1988; **1**: 279–287.
36 Hong SC, Goto Y, Lanzino G, Soleau S, Kassell NF, Lee KS. Neuroprotection with a calpain inhibitor in a model of focal cerebral ischemia. *Stroke* 1994; **25**: 663–669.
37 Hong SC, Lanzino G, Goto Y, Kang SK, Schottler F, Kassell NF *et al*. Calcium-activated proteolysis in rat neocortex induced by transient focal ischemia. *Brain Res* 1994; **661**: 43–50.
38 Mizuta I, Katayama M, Watanabe M, Mishina M, Ishii K. Developmental expression of NMDA receptor subunits and the emergence of glutamate neurotoxicity in primary cultures of murine cerebral cortical neurons. *Cell Mol Life Sci* 1998; **54**: 721–725.
39 Sans N, Petralia RS, Wang YX, Blahos II J, Hell JW, Wenthold RJ. A developmental change in NMDA receptor-associated proteins at hippocampal synapses. *J Neurosci* 2000; **20**: 1260–1271.
40 Wechsler A, Teichberg VI. Brain spectrin binding to the NMDA receptor is regulated by phosphorylation, calcium and calmodulin. *EMBO J* 1998; **17**: 3931–3939.
41 Fukaya M, Kato A, Lovett C, Tonegawa S, Watanabe M. Retention of NMDA receptor NR2 subunits in the lumen of endoplasmic reticulum in targeted NR1 knockout mice. *Proc Natl Acad Sci USA* 2003; **100**: 4855–4860.
42 Mori H, Manabe T, Watanabe M, Satoh Y, Suzuki N, Toki S *et al*. Role of the carboxy-terminal region of the GluR epsilon2 subunit in synaptic localization of the NMDA receptor channel. *Neuron* 1998; **21**: 571–580.
43 Sprengel R, Suchanek B, Amico C, Brusa R, Burnashev N, Rozov A *et al*. Importance of the intracellular domain of NR2 subunits for NMDA receptor function *in vivo*. *Cell* 1998; **92**: 279–289.
44 Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K *et al*. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science* 1999; **283**: 70–74.
45 Ikonomidou C, Stefovska V, Turski L. Neuronal death enhanced by N-methyl-D-aspartate antagonists. *Proc Natl Acad Sci USA* 2000; **97**: 12885–12890.
46 Olney JW, Wozniak DF, Jevtovic-Todorovic V, Farber NB, Bittigau P, Ikonomidou C. Drug-induced apoptotic neurodegeneration in the developing brain. *Brain Pathol* 2002; **12**: 488–498.
47 Young D, Lawlor PA, Leone P, Dragunow M, During MJ. Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. *Nat Med* 1999; **5**: 448–453.
48 Vanderklish PW, Bahr BA. The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. *Int J Exp Pathol* 2000; **81**: 323–339.
49 Bano D, Young KW, Guerin CJ, Lefeuvre R, Rothwell NJ, Naldini L *et al*. Cleavage of the plasma membrane Na+/Ca2+ exchanger in excitotoxicity. *Cell* 2005; **120**: 275–285.
50 Biegon A, Fry PA, Paden CM, Alexandrovich A, Tsenter J, Shohami E. Dynamic changes in N-methyl-D-aspartate receptors after closed head injury in mice: implications for treatment of neurological and cognitive deficits. *Proc Natl Acad Sci USA* 2004; **101**: 5117–5122.

Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)