Curcumin Protects against NMDA-Induced Toxicity: A Possible Role for NR2A Subunit

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Purpose. Curcumin, a phenolic compound extracted from the rhizome of *Curcuma longa*, was found to attenuate NMDA-induced excitotoxicity in primary retinal cultures. This study was conducted to further characterize curcumin neuroprotective ability and analyze its effects on NMDA receptor (NMDAr).

Methods. NMDAr modifications were analyzed in primary retinal cell cultures using immunocytochemistry, whole-cell patch-clamp recording and western blot analysis. Cell death was evaluated with the TUNEL assay in primary retinal and hippocampal cultures. Optical fluorometric recordings with Fura 2-AM were used to monitor $[Ca^{2+}]_i$.

Results. Curcumin dose- and time-dependently protected both retinal and hippocampal neurons against NMDA-induced cell death, confirming its anti-excitotoxic property. In primary retinal cultures, in line with the observed reduction of NMDA-induced $[Ca^{2+}]_i$ rise, whole-cell patch-clamp experiments showed that a higher percentage of retinal neurons responded to NMDA with low amplitude current after curcumin treatment. In parallel, curcumin induced an increase in NMDAr subunit type 2A (NR2A) level, with kinetics closely correlated to time-course of neuroprotection and decrease in $[Ca^{2+}]_i$. The relation between neuroprotection and NR2A level increase was also in line with the observation that curcumin neuroprotection required protein synthesis. Electrophysiology confirmed an increased activity of NR2A-containing NMDAr at the plasma membrane level.

Conclusions. These results confirm the neuroprotective activity of curcumin against NMDA toxicity, possibly related to an increased level of NR2A, and encourage further studies for a possible therapeutic use of curcumin based on neuromodulation of NMDArs. (*Invest Ophthalmol Vis Sci.* 2011;52:1070–1077) DOI:10.1167/iovs.10-5966

Curcumin, a phenolic compound extracted from the rhizome of *Curcuma longa*, is used worldwide as spice, flavoring agent, food preservative, coloring agent and, in Asian countries, as herbal medicine. Curcumin is non-toxic even at high dosages and has been classified by the United States Food and Drug Administration as "Generally Recognized As Safe."[1] A large body of evidence suggests that curcumin has a wide range of biological activities and potential therapeutic effects on numerous pathologic disorders, including diabetes,[2,3] rheumatoid arthritis,[4] multiple sclerosis,[5] and cancer.[6] In the CNS, curcumin administration has been reported to attenuate cognitive deficits, neuroinflammation, and plaque pathology in Alzheimer's disease models.[7–9] Moreover, it shows neuroprotective potential in cerebral ischemia[10,11] and against excitotoxicity in cerebral cortical neurons and retinal cultures.[12,13] However, its efficacy has been object of controversy and its mechanism of action remains elusive.

Excitotoxicity, mainly due to an overactivation of NMDA receptors (NMDArs), has been proposed to be relevant in the pathophysiology of CNS diseases, including ophthalmologic disorders, such as retinal ischemia,[14] diabetic retinopathy,[15] and glaucoma.[16,17] We previously reported the protective effect of curcumin against NMDA-induced excitotoxicity in rat retinal cultures, suggesting a mechanism involving modification of NMDAr activity. To gain more insight into curcumin neuroprotective effects, we examined the functional activity of NMDAr combining biochemical and electrophysiological methods. We observed an increase in NMDAr subunit type 2A (NR2A) level, which resulted in a negative modulation of NMDAr activity. Furthermore, the temporal profile of NR2A level increase correlated with the neuroprotection time-course. Our results confirm that NMDAr could be a target in curcumin neuroprotection mechanisms.

Materials and Methods

Primary Cell Cultures, Preparation and Drug Treatments

All animal handling was performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Primary retinal and pure hippocampal cultures were obtained from Wistar rat embryos at gestational day 18 as already described[18,19] and were used at 12–14 day in vitro (DIV). Each cell culture, obtained from the embryos of one pregnant rat, was considered as a single experiment. Retinal cultures were obtained from retinas dissociated in trypsin; cells were seeded onto poly-L-lysine-coated cell culture plates or glass coverslips and grown in synthetic cell culture medium (MEM; Sigma-

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Supported in part by by Alfa Intes S.r.l., Casoria, Naples, Italy (AM).

Submitted for publication May 27, 2010; revised July 30 and August 17, 2010; accepted August 24, 2010.

Disclosure: **A. Matteucci**, None; **R. Cammarota**, None; **S. Paradisi**, None; **M. Varano**, None; **M. Balduzzi**, None; **L. Leo**, None; **G.C. Bellenchi**, None; **C. De Nuccio**, None; **G. Carnovale-Scalzo**, None; **G. Scorcia**, None; **C. Frank**, None; **C. Mallozzi**, None; **A.M. Di Stasi**, None; **S. Visentin**, None; **F. Malchiodi-Albedi**, None

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Aldrich, St. Louis, MO) with 10% fetal calf serum (FCS, Life Technologies, Milan, Italy), giving rise to a mixed glial–neuronal cell population. Hippocampal cultures were obtained from dissected hippocampi. After dissociation in trypsin, cells were seeded on poly-L-lysine-coated 24-well plates in MEM/FCS. To obtain pure neuronal cultures, the medium was replaced after 2 h with neurobasal medium supplemented with B27 (NBM/B27, Life Technologies); at 1 DIV, 5 μM arabinosylcytosine (Sigma-Aldrich) was added to prevent glial proliferation. In these conditions, astrocytes were $< 1\%$.[18] Curcumin (Sigma-Aldrich) was diluted in ethanol as 10 mM stock solution. Cell cultures were pretreated with 5 or 15 μM curcumin for 20 minutes, 2, 4, 6, 24 h, or treated after the agonists. Excitotoxic neuronal damage was examined in "delayed" toxicity protocols, which characteristically triggers an apoptotic pathway of cell death.[20] Cell cultures were treated with NMDA (1 mM) or AMPA (0.5 mM) or Kainate (0.5 mM) for 20 minutes. Medium was then replaced with fresh NBM/B27 and the cells fixed after 24 h. To evaluate protective effects of curcumin against AMPA- and Kainate-induced apoptosis, we treated cells in the presence of the NMDAr inhibitor MK-801 (5 μM). The protein synthesis inhibitor cycloheximide (CHX) was prepared as a stock solution in DMSO (100 mg/mL). {(*R*)-[(*S*)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydro-quinoxalin-5-yl)-methyl}-phosphonic acid (NVP-AAM077, a kind gift of Yves Auberson and Sandra Kirchhoff of the Novartis Institutes for Biomedical Research, Basel, Switzerland), a specific NR2A antagonist, was prepared as a stock solution in 0.1 N NaOH. Ifenprodil (IFN, Sigma-Aldrich), an NMDA receptor subunit type 2B (NR2B) antagonist, was diluted in cell culture medium.

Apoptosis Detection

After fixation in 4% paraformaldehyde in PBS, 0.12 M in sucrose, apoptosis was evaluated in retinal and hippocampal cultures by the terminal transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay (DeadEnd kit, Promega, Madison, WI). Cells undergoing apoptotic cell death were quantified by counting TUNEL-positive nuclei in bright field (Nikon Optiphot microscope; Nikon, Melville, NY). Eight microscopic fields were chosen randomly for a total of at least 300 cells for each coverslip. Two coverslips were scored for each condition. The values obtained for each coverslip were averaged to produce a single mean value for each experiment. Apoptosis was expressed as percentage of apoptotic cells over total cells.

Intracellular Ca^{2+} ($[Ca^{2+}]_i$)

To evaluate $[Ca^{2+}]_i$, optical fluorometric recordings with Fura 2-AM were used. Fura 2-AM stock solutions were obtained by adding 50 mg of Fura 2-AM to 50 mL of 75% DMSO plus 25% pluronic acid. Retinal cultures were bathed for 60 minutes at room temperature with 5 mL of stock solution diluted in 1 mL of extracellular solution (in mM: 125 NaCl, 1 KCl, 5 CaCl2, 1 MgCl2, 8 glucose, and 20 HEPES, pH 7.35) for a final Fura 2-AM concentration of 5 mM. After prolonged wash with fresh buffer, the plates were placed on the microscope plate. Drugs were applied by directly dripping in the bath. To measure fluorescence changes, a computerized analysis system was used (ImagEM; Hamamatsu Photonix, Hamamatsu, Japan). In each experiment, the ratio between the values of light intensity at 340 and 380 nm stimulation (F340/380) was recorded every 6 seconds in at least 12 neurons. The experiments were repeated in at least three cultures.

Electrophysiology

Membrane electrical activity of single retinal cells was studied in the whole-cell configuration of the patch-clamp technique[21]. Glass coverslips bearing retinal cultures were placed in a recording chamber on the stage of a microscope (Axiovert 35 inverted microscope; Zeiss, Göttingen, Germany). Conventional compensation of electrode and whole-cell capacities, as well as of series resistance were used taking advantage of the built in features of an amplifier (Axopatch-2D; Axon Instruments, Foster, CA). An analysis software package (pClamp; Axon Instruments) was used for recording and data analysis and graphing software (Origin Package; OriginLab, Northampton, MA) was used for further analysis and graphic presentation of the data. The bulk solution had the following composition (mM): 140 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, 10 HEPES/NaOH (room temperature, pH 7.4, 290 mOsmol/L). In parallel with a perfusion system allowing the change of the bulk solution of the bath, a local perfusion system was used. It consisted of a set of reservoirs containing different solutions connected to polyethylene tubing with an internal diameter of 300 μm held together in parallel and connected to a rotary motor. The rapid change of the solution bathing the cell in study was made possible by operating on the rotary motor. This allowed the switch between control and test solutions with a minimal contamination of the cells surrounding the area of interest. In the locally applied solution, MgCl2 was replaced by CaCl2 and 10 mM Glycin was added. The electrodes were filled with the following solution (mM): 140 KGluconate, 1 CaCl2, 2 MgCl2, 10 EGTA, 2 MgATP, and 10 HEPES/KOH (pH 7.2, 290 mOsmol/L). When filled with this solution, electrodes had a resistance ranging from 2 to 4 MW. An inward current was induced by applying 1 mM NMDA for 5 to 20 seconds at a holding potential of -70 mV. To evaluate the inhibitory activity of NMDAr antagonists, they were preapplied for 30 seconds before being coapplied together with the agonist.

Immunocytochemistry

Retinal cultures were fixed as described above and incubated with the following primary antibodies: Cy3-conjugated mouse anti-vimentin and rabbit anti-GABA, purchased from Sigma; mouse anti-cellular retinaldehyde binding protein (CRALBP), purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA; rabbit anti-MAP-2, mouse anti-NeuN, mouse anti-synaptophysin, mouse anti-THY1.1, rabbit anti-pNR1 (Ser 897), rabbit anti-NR2A, rabbit anti-NR2B, mouse anti-rhodopsin, all purchased from Millipore, Bedford, MA. After washes in PBS, for immunoperoxidase, anti-pNR1 was revealed by the biotin–streptavidin/ peroxidase sequence (Vector Quick Stain, Vector Laboratories, Burlingame, CA). Samples were observed at a microscope (Nikon Optiphot; Nikon). For immunofluorescence, anti-rabbit 488 or anti-mouse 546 Alexafluor conjugates (Molecular Probes, Eugene, OR) were used as secondary antibodies. Cultures were counterstained with Hoechst 33,258 and observed at a fluorescence microscope (Eclipse 80i Nikon; Nikon), equipped with a Video Confocal system (ViCo).

Electrophoresis and Western Blot Analysis

Retinal cultures were solubilized in RIPA buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 0.1% SDS, 1% sodium-deoxycholate) in the presence of proteases inhibitors mixture (Complete without EDTA, Roche Molecular Biochemicals, Indianapolis, IL) and phosphatases inhibitors 50 mM NaF, 1 mM Na_3VO_4, for 1 hour in ice. The retinal cellular extracts were solubilized in 4 X Laemmli sample buffer and boiled for 5 minutes. Proteins (30–50 μg) were separated on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes at 35 V overnight. The membranes were washed with TTBS buffer (50 mM Tris-Hcl pH 7.4, 150 mM NaCl, 0.05% Tween 20), blocked with 3% BSA (wt/vol)-TTBS for 1 h at room temperature and incubated with the appropriate primary antibodies overnight at 4°C. The immunoreactive bands were detected by chemiluminescence coupled to peroxidase activity (ECL). Protein concentration was determined using a kit (Micro BCATM Protein Assay Kit; Pierce, Rockford, IN). Antibodies were obtained from the following sources: polyclonal anti-NR2A, anti-pNR1 (Ser 897) and anti-NR2B from Millipore; monoclonal anti β-actin from Calbiochem-Oncogene Research Products (MA); peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies from Bio-Rad, Hercules, CA.

Statistical Analysis

Data were expressed as mean $\pm$ SEM of at least three independent experiments. They were analyzed either by Student's *t*-test or by non-parametric Wilcoxon matched pairs test, when the sample size did

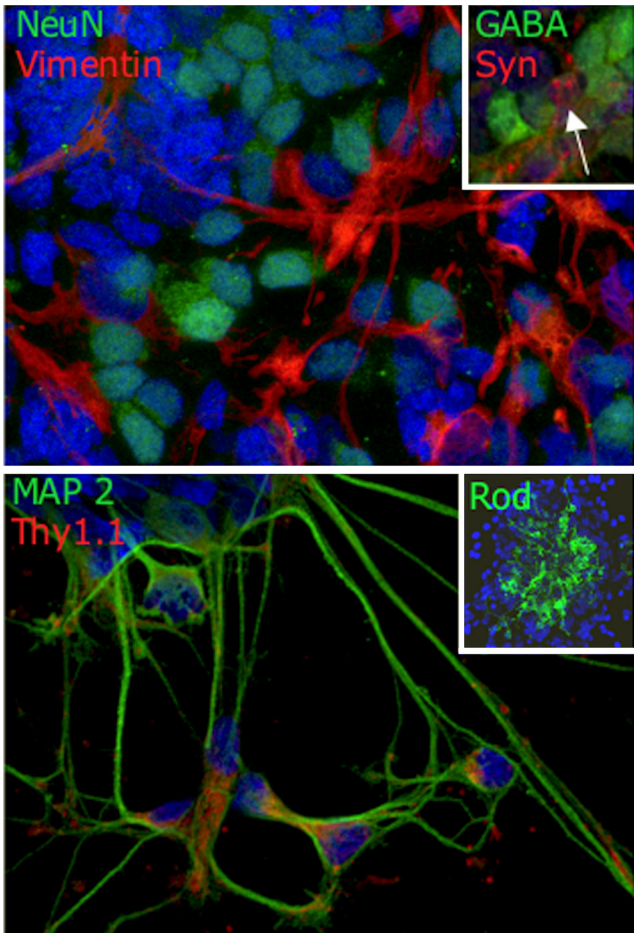

FIGURE 1. Immunocytochemical characterization of retinal cell cultures. *Upper panel*: retinal cultures at 14 DIV were immunolabeled with antibodies against NeuN (*green*), a specific neuronal marker, and vimentin (*red*), which labels Müller glia, showing a mixed neuronal–glial populations. The NeuN-negative nuclei on the *right* belong to Müller glia. Those around the *upper left corner* are probably nuclei of photoreceptors, which are negative NeuN (data not shown). *Insert*: amacrine and horizontal cells are immunolabeled in *green* by antibodies against GABA. Synapses are evident as synaptophysin-positive *red dots* (*arrow*). *Lower panel*: retinal cultures were immunolabeled for MAP-2 (*green*), a marker of neuronal neuritic tree, and THY1.1 (*red*), which identifies ganglion cells. Nuclei are counterstained with Hoechst 33,258. *Insert*: a rhodopsin-positive photoreceptor rosette is shown.

not allow assuming that the sampling distribution was normal. They were considered significantly different at $P < 0.05$.

RESULTS

Characterization of the Cell Population of Primary Retinal Culture

Retinal cultures are composed of mixed neuronal and glial cells. Neuronal cells, immunolabeled by NeuN, elongating a branching MAP-2-positive neuritic tree, were interspersed among vimentin-positive Müller glia. Some of the neuronal cells were positive for GABA (amacrine cells or horizontal cells); ganglion cells, immunolabeled by THY 1.1, were rarely detected. Photoreceptor rosettes, positive for rhodopsin, were present. At 14 DIV, neuronal cells were well differentiated, as shown by the formation of synaptophysin-positive synapses (Fig. 1).

Curcumin Effects on NMDA Currents in Retinal Cultures

In a previous study,[12] we suggested that curcumin neuroprotection against excitotoxicity was due to a modulation of NMDAr activity. Here we used the patch-clamp technique to measure NMDAr currents in primary retinal neurons (Fig. 2). The retinal neurons examined were characterized by the current profile shown in Figures 2A and 2B. This current profile, lacking inward rectifying currents at hyperpolarized potentials, rules out the possibility that Müller cells, which show large inward currents, were recorded. NMDA at the concentration of 1 mM was applied to the cell in study for 20 seconds and the resulting current was recorded (Fig. 2C). As expected, this caused the induction of a fast inwardly directed current that, after reaching a peak, declined to a new steady state. Comparing the average current peak recorded in control and curcumin-treated cultures, the observed decrease in the latter

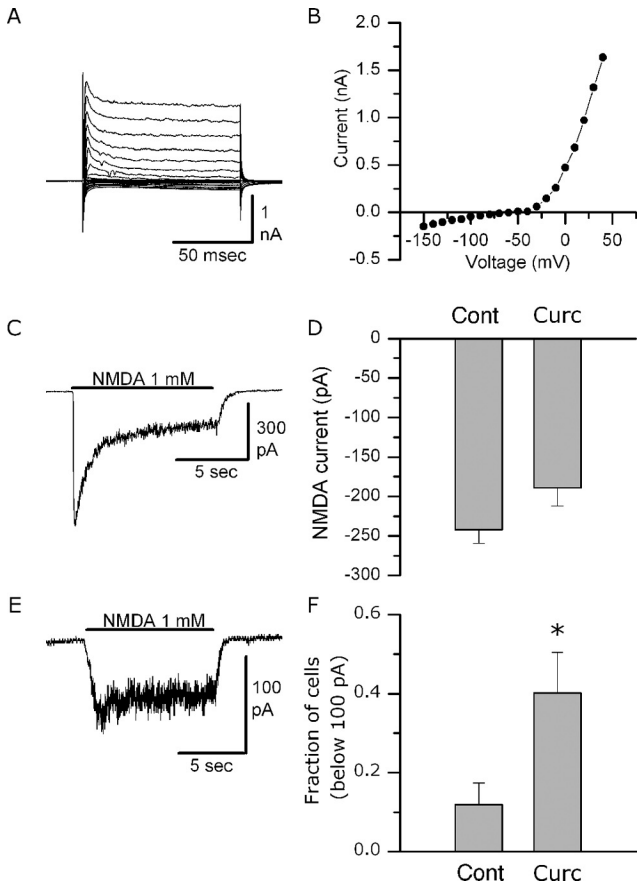

FIGURE 2. Electrophysiological evaluation of curcumin effect on NMDA-induced inward currents. (**A**) An exemplifying current profile of the retinal neurons used is shown. From a holding potential of −70 mV, a series of voltage steps from −150 to +40 mV were applied to the single cell and membrane currents were recorded. The amplitudes of the currents at the end of the voltage pulses (from 80 to 90 ms) were calculated and the IV curve is shown in (**B**). Retinal neurons were treated with 15 μM curcumin for 24 h, voltage-clamped at holding potential of −70 mV and challenged with 1 mM NMDA. An exemplifying recording is shown in (**C**). From the inward currents induced by NMDA, the average of peak amplitude was calculated in control and curcumin-treated cultures (**D**). The percentage of cells showing current amplitudes smaller than 100 pA (an exemplifying current recording is shown in (**E**)) was also calculated and shown in (**F**), demonstrating that the fraction of cells showing NMDA-induced currents with small amplitude (≤100 pA) was higher in curcumin-treated cells than in control cultures. * $P < 0.05$, Student's *t*-test.

condition was not statistically significant (number of cells analyzed: Cont = 74, Curc = 60) (Fig. 2D). However, analyzing the distribution of peak amplitudes, we found that the fraction of cells, recorded in single experiments, showing only NMDA currents with small amplitudes, arbitrarily chosen below 100 pA (Fig. 2E), was significantly higher in curcumin-treated (40.2 ± 10%) than in control (11.9 ± 5%) cultures, identifying a subset of cells where curcumin caused an almost complete inhibition of NMDA-induced currents (Fig. 2F).

Curcumin-Induced Neuroprotection against NMDA, but Not AMPA and Kainate

We have shown that curcumin reduces NMDA-induced apoptosis in primary retinal neurons.[12] Here we studied if different times of curcumin pretreatment modified the effects on NMDA-induced apoptosis. As shown in Fig. 3A, apoptosis was particularly evident around photoreceptor rosettes, where GABAergic neurons are particularly abundant, as already described.[12] Neuroprotection against apoptosis started with 6 h of curcumin pretreatment and was maximal at 24 h (Fig. 3B). Moreover, we tested the effects of curcumin in AMPA- and Kainate-induced apoptosis in the presence of the NMDAr blocker MK-801. We treated primary retinal cultures with 15 μM curcumin for 20 minutes, 6 and 24 hours before administration of agonists or immediately after and apoptotic rate was analyzed. Curcumin was unable to protect against AMPA or Kainate toxicity either administered before or after agonist treatments (Fig. 3C).

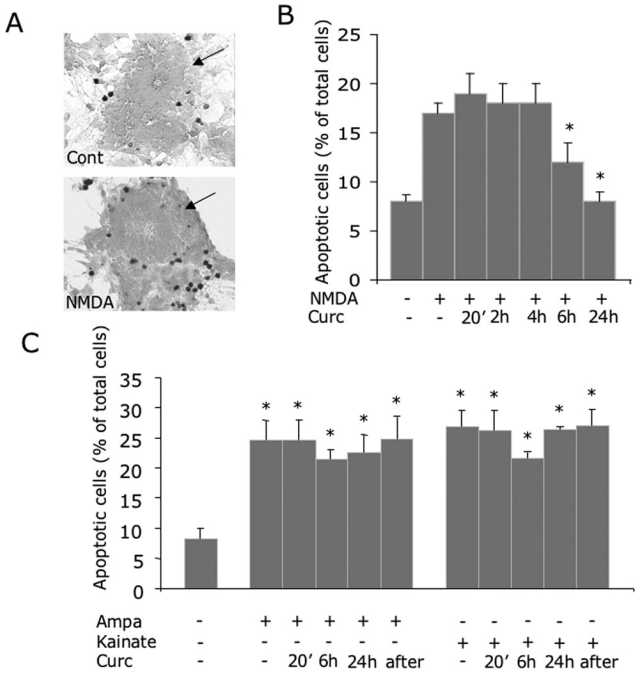

FIGURE 3. Curcumin time-dependently reduces NMDA-, but not AMPA- or Kainate-induced apoptosis. (**A**) A representative image of TUNEL-positive nuclei in mixed retinal cultures treated with 1 mM NMDA for 24 h (NMDA), compared to control cultures (cont). Rosette-forming photoreceptors (*arrows*) are characteristically spared from apoptosis. (**B**) Retinal cultures were pretreated with 15 μM curcumin for 20 minutes, 4, 6, or 24 h. Apoptosis was induced by treating cells with 1 mM NMDA for 20 minutes. The medium was then replaced with fresh NBM/B27 and the cells were fixed after 24 h. Apoptosis was evaluated by the TUNEL assay. Neuroprotection started after 6 h of pretreatment and reached a maximum after 24 h. Bars represent the mean ± SEM of at least five independent cell cultures. $^*P < 0.05$ versus NMDA, Wilcoxon matched pairs test. (**C**) Before the administration of AMPA (0.5 mM) and Kainate (0.5 mM) for 25 minutes, retinal cultures were treated with 15 μM curcumin for 20 minutes, 6 and 24 h before the excitotoxic insult or after the substitution of the medium (fresh NBM/B27). To inhibit NMDA receptor, before AMPA or Kainate treatment, 5 μM MK-801 was used. Apoptosis was evaluated by the TUNEL assay. In these conditions curcumin was not able to reduce apoptosis. Bars represent the mean ± SEM of at least four independent cell cultures.

Curcumin Reduces NMDA-Mediated $[Ca^{2+}]_i$ Rise and NR1 Dephosphorylation

Curcumin treatment induced a decrease of NMDA-mediated $[Ca^{2+}]_i$ rise, measured using Fura 2-AM. The reduction of $[Ca^{2+}]_i$ rise timely correlated with neuroprotection, starting after a pretreatment of 6 h and resulting more evident after a pretreatment of 24 h (Fig. 4A). In retinal primary cultures we had found that curcumin decreased NR1 phosphorylation.[12] To investigate if the decrease in NR1 phosphorylation was related to the decreased susceptibility to NMDA toxicity, we analyzed curcumin-induced NR1 dephosphorylation after 6 and 24 h of pretreatment. By immunocytochemical analysis (Fig. 4B), we found that immunoreactivity for pNR1 (ser 897) decreased after curcumin treatment, reaching a minimum with a 6-h pretreatment. Data were confirmed by Western blot analysis (Fig. 4C). On the basis of these experiments, it seemed unlikely that changes in NR1 phosphorylation could be at the basis of curcumin-induced neuroprotection.

Curcumin Protection Requires De Novo Protein Synthesis

The time required to obtain maximal neuroprotection suggested a relation between curcumin neuroprotection and protein synthesis. To verify this hypothesis, we performed experiments using CHX, a protein synthesis inhibitor. When CHX was delivered after curcumin treatment, together with NMDA, it did not affect curcumin neuroprotection. However, if curcumin and CHX were administered together, no protection was observed and NMDA toxicity increased (Fig. 5). These findings suggest that the neuroprotection exerted by curcumin required protein synthesis.

Curcumin Modifies NR2A Levels

By immunocytochemical analysis, we found that NR2A immunoreactivity increased in retinal neurons after 24 h treatment with curcumin, while NR2B levels did not show significant changes (Fig. 6A). The increase in NR2A reactivity was particularly evident in the neuronal cells surrounding photoreceptor rosettes. NR2A was mainly localized in neurons, as shown by lack of colocalization of NR2A and CRALBP, a specific marker of Müller cells (Fig. 6A, inset). Western blot analysis showed that NR2A levels started to increase at 6 h and reached a maximum level at 24 h, mimicking the temporal profile of neuroprotection, while the level of NR2B was faintly increased (Fig. 6B). The increased NR2A/NR2B ratio further supports the rise of NR2A subunit after 24 h treatment with curcumin (Fig. 6C). To demonstrate that the increased NR2A level was accompanied by enhanced activity of NR2A-containing NMDAr, we used the whole-cell configuration of the patch-clamp technique and assessed the effects of the inhibitors on NMDA-induced inward currents (Fig. 6D). The responses recorded in control and curcumin treated cells did not differ when we used IFN, a specific NR2B inhibitor (Fig. 6D). On the contrary, when we used NVP-AAM077, a selective NR2A blocker, we could discriminate between the two experimental conditions, since NMDA-induced current in curcumin-treated cells resulted significantly more sensitive to receptor inhibition (Fig. 6D). These results sustain that the increased NR2A expression is correlated

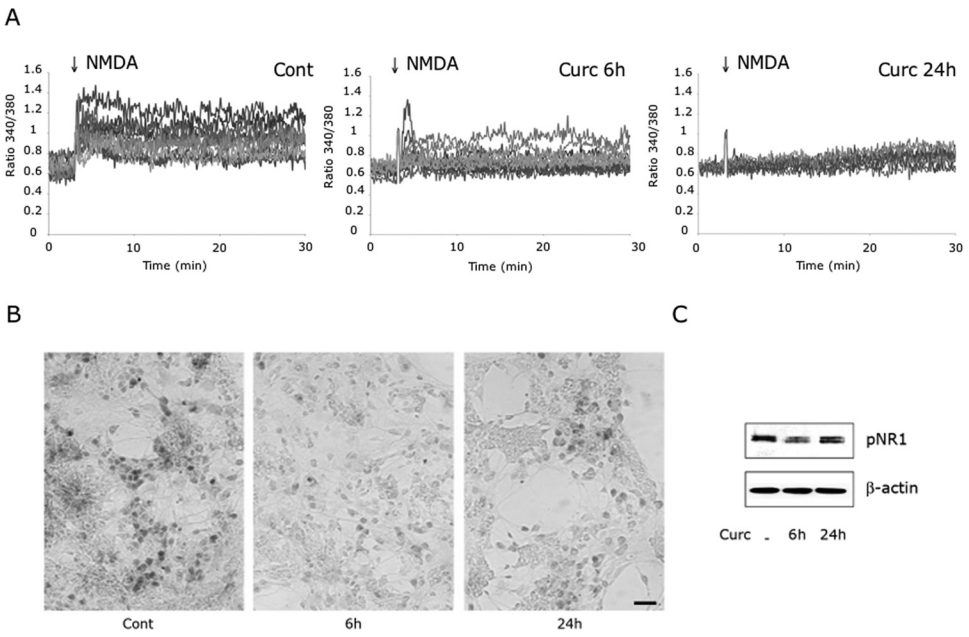

Figure 4. Curcumin modifies NMDA-induced $[Ca^{2+}]_i$ increase and NR1 phosphorylation. (**A**) NMDA-induced $[Ca^{2+}]_i$ increase was analyzed by optical fluorometric recordings with Fura 2-AM. During NMDA application, at the third minute of the experiment, the microscope shutter was closed, inducing an artifact consisting in a spike up to ratio value of 1. Each line represents the ratio value of one neuron. Application of 1 mM NMDA induces a sustained $[Ca^{2+}]_i$ increase. A 6-h pretreatment with curcumin reduces NMDA-induced $[Ca^{2+}]_i$ increase, with a further decrease after a 24-h pretreatment. The experiment was repeated in three different cultures. The time-course of a single representative experiment is shown. (**B**) Retinal cultures were treated with 15 μM curcumin for 6 and 24 h and immunostained for pNR1 (Ser 897). The experiment was repeated in three different cultures and a representative experiment is shown. Respect to control cultures, pNR1 immunoreactivity was lowest after 6-h pretreatment, but increased again after 24-h pretreatment. Bar, 10 μm. (**C**) Whole-cell lysates were prepared from retinal cultures and total proteins resolved on 10% SDS-PAGE and transferred to nitrocellulose. Membranes were probed with anti-pNR1 (Ser 897) and normalized versus β-actin levels. The immunoreactive bands were detected by ECL. The experiment was repeated in three different cultures and a representative experiment is shown. Western blot analysis confirms the immunocytochemical data.

to an increased presence and activity of this subunit in NMDArs at the membrane level.

IFN Reduces NMDA-Induced Apoptosis

It has been suggested that NMDA-induced apoptosis is mainly due to an activation of NR2B-containing NMDAr.[22] Consistently, we found that IFN was able to significantly reduce NMDA-induced apoptosis (Fig. 7A), confirming that these receptors are strictly involved in excitotoxicity in retinal cultures. NVP-AAM077 only slightly modified NMDA-induced toxicity (Fig. 7B).

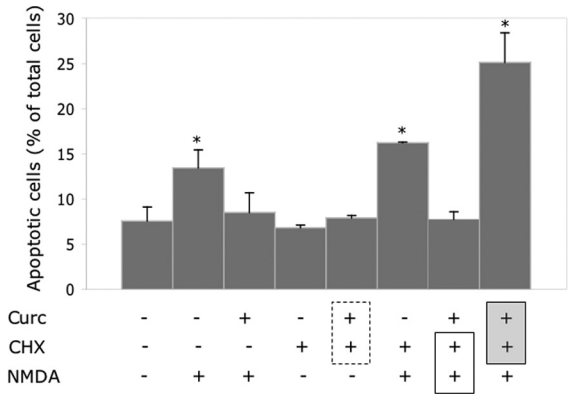

Figure 5. CHX abolishes curcumin neuroprotection. Retinal cultures were treated as follows: 1 mM NMDA for 20 minutes; 15 μM curcumin for 24 h followed by NMDA; 1 mg/mL CHX for 24 h; curcumin and CHX for 24 h (*dashed box*), CHX for 24 h before NMDA for 20 minutes; curcumin for 24 h before NMDA and CHX for 20 minutes (*empty box*), curcumin and CHX (*filled box*) for 24 h before NMDA for 20 minutes. Different boxes are used to indicate simultaneous treatments with two different agents. The medium was then replaced with fresh NBM/B27 and the cells were fixed after 24 h. Apoptosis was evaluated by the TUNEL assay. When CHX was delivered after curcumin treatment, together with NMDA, it did not affect curcumin neuroprotection (*empty box*). On the contrary, when curcumin and CHX were administered simultaneously, no protection was observed (*filled box*). No toxicity was observed for curcumin/CHX treatment (*dashed box*). Bars represent the mean ± SEM of at least five independent cell cultures. $^*P < 0.05$ versus control, Wilcoxon Matched Pairs test.

Curcumin Protects Hippocampal Neurons against Excitotoxicity

To validate curcumin as a general neuroprotectant against excitotoxicity, we analyzed its activity in a different cell culture model. Hippocampal neurons, pretreated or not with 5 and 15 μM curcumin for 24 h, were exposed for 20 minutes to 50 or 75 μM NMDA, respectively. Curcumin preincubation resulted in a consistent dose-dependent reduction of apoptotic cells (Fig. 8A). The NMDA-induced apoptosis was completely abolished in neurons preincubated with 15 μM curcumin and treated with 50 μM NMDA. Moreover analyzing the decrease of NMDA-induced apoptosis after 6 and 24 h of curcumin pretreatment, we confirmed that neuroprotection started with 6 h of curcumin pretreatment and was maximal at 24 h (Fig. 8B).

Discussion

Curcumin possesses many biological activities, some of which validate the research interest on this molecule as possible tool against neurodegenerative diseases. Here we confirmed and further characterized its anti-excitotoxic effect in retinal cell cultures. In addition, we described its neuroprotective ability in a different neuronal cell model, such as primary hippocampal cell cultures, a well-described in vitro model for excitotoxic cell damage.[20]

We[12] and others[23] have put in relation curcumin neuroprotection against NMDA excitotoxicity with a reduced NMDAr activity. By measuring NMDAr currents in primary retinal neurons, we could confirm that curcumin affected NMDAr activity, since after curcumin treatment a larger proportion of cells responded to NMDA with currents of small amplitude. These results point to NMDAr as a target for curcumin neuroprotection. This was further sustained by the observation that cur-

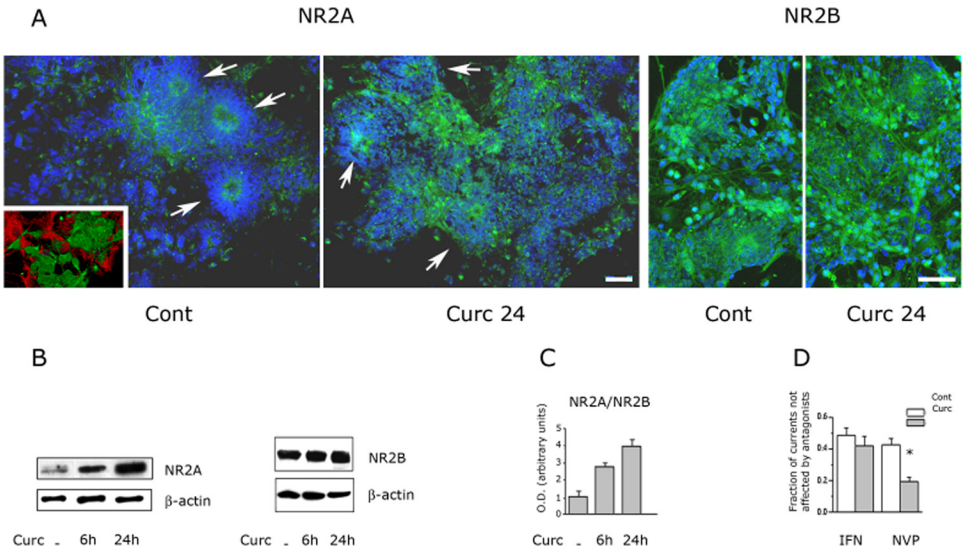

FIGURE 6. Curcumin induces an increase in NR2A level. (**A**) After treatment with 15 μM curcumin for 24 h, retinal cultures were immunolabeled for NR2A or NR2B and counterstained with Hoechst 33258. In curcumin-treated samples, enhanced reactivity for NR2A is evident, especially in cells surrounding photoreceptors rosettes (*arrows*). NR2A was primarily expressed in neurons, as shown by lack of co-immunostaining of NR2A and CRALBP, a specific marker of Müller cells (*inset*). NR2B is similarly immunolabeled in curcumin-treated and control cultures. Bars, 30 μm. (**B**) Whole-cell lysates were prepared from retinal cultures, untreated or treated with curcumin for 6 or 24 h. Equal amounts of total protein from each lysate were resolved on 10% SDS-PAGE and transferred to nitrocellulose. Membranes were probed with the indicated antibodies. The immunoreactive bands were detected by ECL. The experiment was repeated in three different cultures and a representative experiment is shown. An increase in NR2A labeling after curcumin treatment is evident at 6 and 24 h, while NR2B is slightly modified. (**C**) Densitometric analysis of NR2A/NR2B ratio, normalized against the respective β-actin levels. Bars are mean ± SEM of three different experiments. The result further confirms the increase in NR2A. (**D**) NVP-AAM077 significantly reduces NMDA-induced inward currents in curcumin-pretreated retinal neurons. The inhibitory effect of the NVP-AAM077 (0.1 μM) and IFN (1 μM), NR2A and NR2B antagonists respectively, was evaluated in control and curcumin-treated retinal neurons by means of the whole-cell patch-clamp approach. The antagonists were pre-applied 30 seconds before and then co-applied with NMDA. The fraction of NMDA-induced inward currents not affected by the antagonists is shown. IFN showed similar current sensitivity in neurons pretreated or not with curcumin, while NVP-AAM077 significantly reduced the fraction of currents in curcumin-pretreated cells, with respect to control cultures, depicting a more elevated presence of NR2A subunits in NMDAr. At least 30 cells were recorded for each condition. $^*P < 0.001$, Student's *t*-test.

cumin was unable to protect against AMPA or Kainate-induced apotosis.

Curcumin neuroprotection required a long preincubation, starting for 6 h and reaching a peak for 24 h curcumin pretreatment. An analogous time-course was observed for the reduction of NMDA-induced $[Ca^{2+}]_i$ rise. The long preincubation with curcumin required to obtain neuroprotection suggested that protein synthesis might be involved. This was confirmed by lack of curcumin neuroprotection when protein synthesis was inhibited in retinal cultures with CHX.

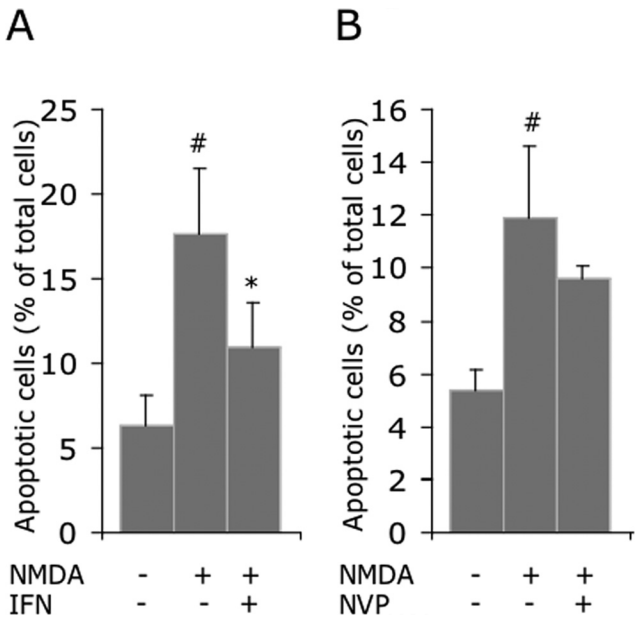

FIGURE 7. IFN protects against NMDA-induced apoptosis in primary retinal cultures. Before administration of 1 mM NMDA for 20 minutes, the cultures were treated with 1 μM IFN (**A**) or 0.1 μM NVP-AAM077 (**B**). Apoptosis was evaluated by the TUNEL assay. In these conditions only IFN was able to reduce NMDA-induced apoptosis. Bars represent the mean ± SEM of at least five independent cell cultures. # $P < 0.05$ versus control; $^*P < 0.05$ versus NMDA, Wilcoxon matched pairs test.

Our previous results showed that curcumin treatment caused a decreased phosphorylation of the NR1 subunit,[12] which could account for a reduced receptor activity. However, here we found that while maximal effect for the reduction of NMDA-induced of $[Ca^{2+}]_i$ increase and neuroprotection was obtained for a pretreatment of 24 h, NR1 dephosphorylation peaked at 6 h, while at 24 h it was higher than its minimum value. The kinetics of the curcumin-induced NR1 dephosphorylation, thus, differs from the time-courses of neuroprotection or from the reduction of $[Ca^{2+}]_i$ rise, suggesting that different mechanisms could be involved.

Analyzing the protein levels of NMDAr subunits, we observed that while the levels of NR1[12]and NR2B were unaltered, NR2A immunoreactivity increased. Interestingly, the kinetics of NR2A level increase nicely fitted with the neuroprotection time-course, both starting at 6 h of pretreatment and reaching its maximum level at 24 h. The modification in the NR2A levels in curcumin-treated retinal neurons was accompanied by an altered NMDAr subunit composition, as shown using selective NR2A and NR2B antagonists in whole cell patch-clamp experiments. The NR2B blocker IFN similarly inhibited NMDA-induced currents in untreated or curcumin-treated neurons, while curcumin treated cells were significantly more sensitive to the NR2A antagonist NVP-AAM077 than control cultures. This finding suggests that the total amount of functional NMDAr in retinal cultures treated with curcumin contained a higher proportion of NR2A subunits. Different NR2 subunits are expressed in vertebrate retina,[24–26] and both NR2A and NR2B are present in the inner plexiform layer.[27] Immunocyto-

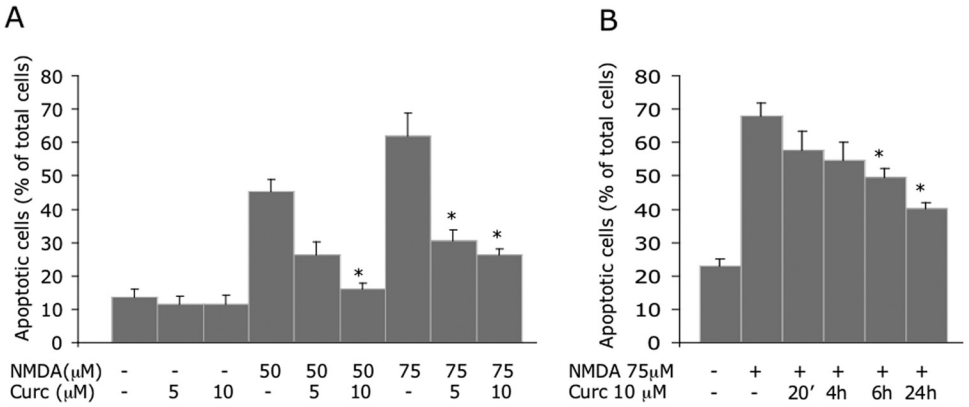

Figure 8. Curcumin protects hippocampal neurons against NMDA-induced apoptosis. (**A**) Hippocampal cultures were pretreated with 5 and 10 μM curcumin for 24 h. Apoptosis was induced by treating cells with 50 and 75 μM NMDA for 20 minutes. The medium was then replaced with fresh NBM/B27 and the cells were fixed after 24 h. Apoptosis was evaluated by the TUNEL assay. Curcumin dose-dependently protected against NMDA-induced apoptosis. (**B**) Hippocampal cultures were pretreated with 10 μM curcumin for 20 minutes, 4, 6, or 24 h. Apoptosis was induced by treating cells with 75 μM NMDA for 20 minutes. The medium was then replaced with NBM/B27 and the cells were fixed after 24 h. Apoptosis was evaluated by the TUNEL assay. Bars represent the mean ± SEM of at least five independent cell cultures. $^*P < 0.05$ versus NMDA, Wilcoxon matched pairs test.

chemistry showed that in retinal cultures the NR2A increase was particularly evident in GABAergic cells surrounding the photoreceptor rosettes. This is in line with data of the literature, which report that NR2A is detected in GABAergic neurons[28] and with our previous results, which showed that in our model GABAergic neurons undergo apoptosis after NMDA treatment and are the cells protected by curcumin.[12]

Different NR2 subunit composition confers distinct electrophysiological and pharmacological properties to the receptors, differently contributing to excitotoxic neuronal cell damage. In various studies, activation of either synaptic or extrasynaptic NR2A-containing NMDArs has been found to promote protection against NMDA-induced neuronal damage, or at least to be less involved in NMDA induced toxicity,[29] while activation of NR2B-containing NMDArs results in increased neuronal apoptosis.[22] Developmental changes in susceptibility to NMDA neurotoxicity is linked to developmental changes in subunit composition, supporting the idea that activation of NR2B-containing NMDArs is more critical for NMDA-mediated neurotoxicity.[29] Our results obtained in retinal cultures are in line with these observations, since we found that NVP-AAM077 did not block NMDA-induced apoptosis, while IFN significantly counteracted it, suggesting that NMDA toxicity is mainly mediated by the NR2B containing NMDAr. In our hypothesis, curcumin treatment reduces NMDA-induced excitotoxicity by determining an increase in functionally active NR2A-containing NMDAr. This is in line with the observation that curcumin protection requires protein synthesis. Moreover, since the activation of NR1/NR2B results in a greater transfer of charge and Ca^{2+} influx,[30,31] an increased level of NR2A could also explain the decrease in NMDA currents, as shown by electrophysiology, and the reduction of $[Ca^{2+}]_i$ rise, as shown by the experiments with Fura 2-AM.

Our results suggest a possible therapeutic use of curcumin based on neuromodulation of NMDArs for the treatment of ophthalmologic diseases associated with excitotoxicity.

Acknowledgments

The authors thank Tamara C. Petrucci for helpful suggestions.

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