Cell Death and Differentiation (2005) 12, 761–772

www.nature.com/cdd

NF-κB factor c-Rel mediates neuroprotection elicited by mGlu5 receptor agonists against amyloid β-peptide toxicity

M Pizzi*[,1], I Sarnico[1], F Boroni[1], M Benarese[1], N Steimberg[2], G Mazzoleni[2], GPH Dietz[3], M Bähr[3], H-C Liou[4] and PF Spano[1]

[1] Division of Pharmacology and Experimental Therapeutics, Department of Biomedical Sciences and Biotechnologies, School of Medicine, University of Brescia, 25123, Italy
[2] Division of General Pathology and Immunology, Department of Biomedical Sciences and Biotechnologies, School of Medicine, University of Brescia, 25123, Italy
[3] Neurologische Universitatsklinik, Gottingen, Germany
[4] Department of Medicine, Division of Immunology, Weill Cornell University, New York, USA
* Corresponding author: M Pizzi, Division of Pharmacology, Department of Biomedical Sciences and Biotechnologies, Viale Europa, 11, 25123 Brescia, Italy. Tel: +39-030-3717501; Fax: +39-030-3701157; E-mail: pizzi@med.unibs.it

Received 20.9.04; revised 28.12.04; accepted 19.1.05; published online 01.4.05
Edited by P Nicotera

Abstract

Opposite effects of nuclear factor-κB (NF-κB) on neuron survival rely on activation of diverse NF-κB factors. While p65 is necessary for glutamate-induced cell death, c-Rel mediates prosurvival effects of interleukin-1β. However, it is unknown whether activation of c-Rel-dependent pathways reduces neuron vulnerability to amyloid-β (Aβ), a peptide implicated in Alzheimer's disease pathogenesis. We show that neuroprotection elicited by activation of metabotropic glutamate receptors type 5 (mGlu5) against Aβ toxicity depends on c-Rel activation. Aβ peptide induced NF-κB factors p50 and p65. The mGlu5 agonists activated c-Rel, besides p50 and p65, and the expression of manganese superoxide dismutase (MnSOD) and Bcl-X_L. Targeting c-Rel expression by RNA interference suppressed the induction of both antiapoptotic genes. Targeting c-Rel or Bcl-X_L prevented the prosurvival effect of mGlu5 agonists. Conversely, c-Rel overexpression or TAT-Bcl-X_L addition rescued neurons from Aβ toxicity. These data demonstrate that mGlu5 receptor activation promotes a c-Rel-dependent antiapoptotic pathway responsible for neuroprotection against Aβ peptide.

Cell Death and Differentiation (2005) **12,** 761–772.
doi:10.1038/sj.cdd.4401598
Published online 1 April 2005

Keywords: NF-κB; amyloid-β; mGlu5 receptor; CHPG; MnSOD; Bcl-X_L

Abbreviations: Aβ, amyloid-β peptide; CHPG, (R,S)-2-chloro-5-hydroxyphenylglycine; 3HPG, (*S*)-3-hydroxy-phenyl-glycine; IL-1β, interleukin-1β; mGlu, metabotropic glutamate; MnSOD, manganese superoxide dismutase; MPEP, 2-methyl-6-phenyl-ethynyl-pyridine; NF-κB, nuclear factor-κB; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling; GFP, green fluorescent protein

Introduction

Nuclear factors-κB (NF-κB) are widely expressed in developing and mature central nervous system,[1] where they regulate the adaptive responses to environmental factors stimulus-dependently.[2,3] The NF-κB family of dimeric transcription factors include p50, p52, p65 or RelA, RelB and c-Rel proteins, which use the Rel homology domain for dimerization and DNA binding.[4] In resting cells, NF-κB factors interact with inhibitory IκB proteins. Cellular stimulation results in phosphorylation, ubiquitination and degradation of IκB, allowing NF-κB dimers to translocate to the nucleus where they regulate transcriptional function. Besides regulating long-term changes to neuronal function in response to synaptic transmission,[5,6] NF-κB factors participate in pathological events associated with neurodegeneration.[2,7–9] Increased NF-κB activation has been observed in neurons exposed to trauma and ischemia,[9–12] as well as in the brains of patients with Parkinson's or Alzheimer's disease.[13–15] However, accumulating evidence has pointed to NF-κB as a survival determinant for neurons.[16–18] It has been shown that NF-κB mediates neuroprotection elicited by tumor necrosis factor in hippocampal cells[19,20] and promotes neuron resistance to excitotoxicity[21,22] and amyloid β (Aβ)-induced apoptosis.[23]

We hypothesized that the opposite regulation of neuron survival by NF-κB in response to proapoptotic or antiapoptotic stimuli might depend on the activation of distinct combinations of subunits resulting in transcriptional regulation of diverse subsets of genes dictating the cell response.[24–26] By studying glutamate and interleukin-1β (IL-1β), we showed that the neurotoxic effect produced by *N*-methyl-D-aspartate receptor activation in cerebellar granule cells and hippocampal slices was associated with activation of NF-κB dimers composed of p50 and p65 proteins, whereas neuroprotection elicited by IL-1β correlated with activation of c-Rel-containing dimers. Targeting p65 expression with antisense oligodeoxynucleotides prevented glutamate-mediated cell death, while targeting c-Rel abolished IL-1β neuroprotection.[27] Our present study sheds light on the prosurvival role of c-Rel factor in neuronal cells and shows that activation of the c-Rel-dependent antiapoptotic pathway reduces neuronal vulnerability to Aβ 1–40 peptide. We demonstrate that neuroprotective activity of agonists at metabotropic glutamate receptor type 5 (mGlu5) against Aβ-mediated apoptosis relies on c-Rel activation. The post-transcriptional silencing of c-Rel gene by RNA interference technique, as its deletion in c-$Rel^{-/-}$ neurons, abolished mGlu5-mediated neuroprotection as well

as manganese superoxide dismutase (MnSOD) and Bcl-X_L expression. Conversely, c-Rel overexpression, or the application of TAT-Bcl-X_L protein, reduced Aβ-mediated apoptosis.

Results

Activation of mGlu5 receptors prevented Aβ toxicity and induced NF-κB activation in mouse cortical neurons and in human SK-N-SH cells

The treatment of primary cultures of mouse cortical neurons with different concentrations of 3HPG, a group I mGlu receptor agonist, prevented neuronal cell death induced by 48 h exposure to 5 μM Aβ. By using MTT reduction as an index of neuron viability we found about 55% of cell survival in Aβ-treated cells and more than 90% in cultures co-exposed to 50 μM 3HPG (Figure 1b). The data obtained by MTT reduction were very similar to those found by counting TUNEL positive cells (Figure 1a). The TUNEL staining of neuronal cells showed a large number of nuclei displaying chromatin fragmentation in Aβ-treated cultures (46 $\pm$ 3% of total cells), but nearly no staining in cultures co-exposed to 3HPG (5 $\pm$ 2% of total cells), as in control culture (4 $\pm$ 3% of total cells). The neuroprotective effect of 3HPG was prevented by the selective mGlu5 receptor antagonist MPEP used at 5 μM concentration, and was reproduced by the selective mGlu5 receptor agonist CHPG at 1 mM concentration (Figure 3b, d). These data indicated that mGlu5 subtype of mGlu receptors expressed in mouse cortical neurons[28] is mainly responsible for the neuroprotection elicited by 3HPG against Aβ toxicity. To verify this phenomenon in another neuronal model, we used SK-N-SK cells differentiated by retinoic acid treatment. We found that neuronal SK-N-SH cells, besides expressing functional *N*-methyl-D-Aspartate receptors,[29] expressed mGlu1 and mGlu5 receptors. The RT-PCR analysis revealed the expression of both mGlu1 and mGlu5 receptor mRNAs in SK-N-SH cells, when compared to human thalamus templates (Figure 1c). The immunoblot analysis of total cell extracts confirmed the presence of the receptor proteins (Figure 1d). The application of 3HPG at 50 μM concentration prevented the release of cytochrome *c* into the cytosol (Figure 1e) as well as the toxic effect produced by 24 h exposure to Aβ peptide (Figure 1f). The neuroprotective response of 3HPG was blocked by MPEP, confirming that mGlu5 receptor signalling had a role in modulating the cell vulnerability to Aβ peptide in different types of neuronal cells. No modification of cell survival was produced by MPEP alone in the two neuronal cultures (Figure 1b, f).

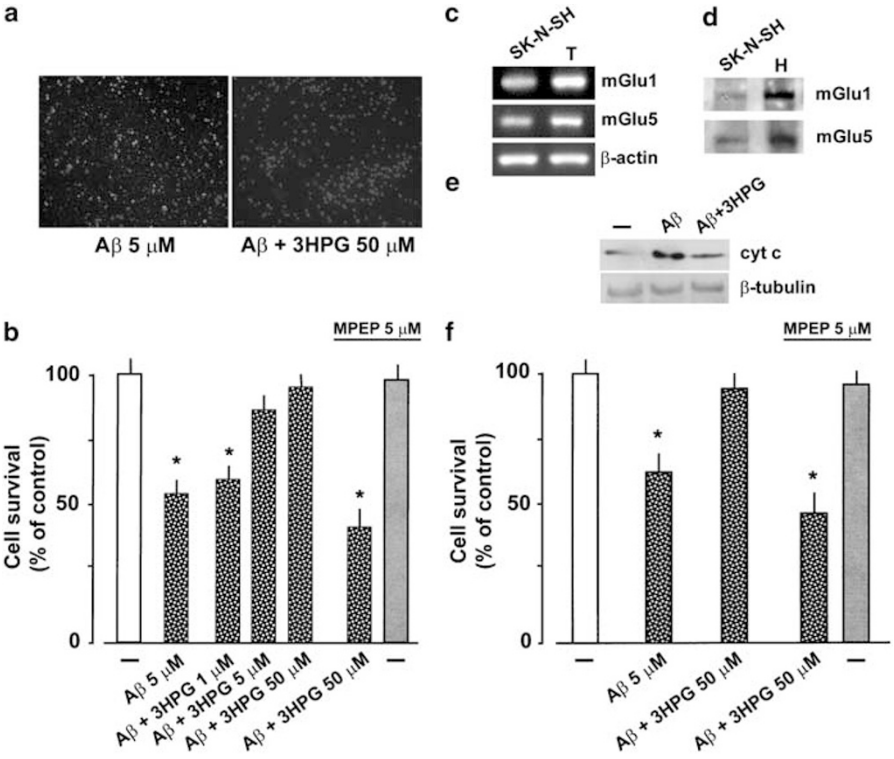

Figure 1 The activation of mGlu5 receptors prevents Aβ toxicity in primary cultures of mouse cortical neurons and in neuronal SK-N-SH cells. (**a**) TUNEL staining in cortical neurons exposed to Aβ with or without 3HPG for 48 h. (**b**) Cell survival was measured as MTT reduction in cortical neurons exposed to Aβ with or without 3HPG (1–50 μM) for 48 h. MPEP completely prevented the neuroprotection by 3HPG, while *per se* it did not modify the cell viability. (**c**) RT-PCR analysis of the expression of mGlu1 (292 bp band) and mGlu5 receptors (513 bp band) or β-actin (270 bp band) in templates from SK-N-SH neuronal cells and human thalamic nucleus (T). (**d**) Cell extracts from SK-N-SH cells and rat hippocampus (H) were immunoblotted against mGlu1 and mGlu5 receptor antibodies. Blots reprobed with an anti β-tubulin antibody indicated equal amounts of proteins in the different lanes (data not shown). (**e**) 3HPG attenuated the release of cytochrome *c* induced by exposure of SK-N-SH neuronal cells to Aβ for 24 h. (**f**) Cell survival was measured in differentiated SK-N-SH cells exposed to Aβ with or without 3HPG for 24 h. The 3HPG effect was prevented by MPEP. MPEP alone did not modify cell viability. Similar results were obtained in three separate experiments run in quadruplicate. *$P \leqslant 0.05$ *versus* corresponding control value

Then, we investigated the effect produced by mGlu5 receptor stimulation on the activity of NF-κB transcription factor. An ELISA-based analysis of DNA-binding interactions for the different NF-κB factors was performed in nuclear extracts prepared from cortical neurons (Figure 2b). By this procedure we obtained results similar to those found by EMSA and super shift analysis (Figure 2a). A 20-min application of 50 μM 3HPG or 1 mM CHPG to cortical cells activated NF-κB, as shown by the nuclear translocation of NF-κB factors p50, p65 and c-Rel (Figure 2b). Besides, in line with previous evidence,[15,30,31] 20-min application of the Aβ peptide induced NF-κB, but restricted to p50 and p65 subunits. An ELISA-based assay specific for human cells confirmed that both 3HPG and CHPG application to SK-N-SH neurons activated p50, p65 and c-Rel. No activation of RelB or p52 factors was detected (Figure 2c). To elucidate the specific composition of NF-κB complexes, we carried out co-immunoprecipitation studies. Using antibodies against p50, p65 and c-Rel, we identified the p50/p65, p50/c-Rel and p65/c-Rel dimers in nuclear extracts from unstimulated cells (Figure 2d). Control antibodies to IgG alone were unable to immunoprecipitate any NF-κB proteins. The CHPG treatment increased the nuclear amount of the c-Rel-containing complexes p50/c-Rel and p65/c-Rel, while it left unchanged the nuclear content of p50/p65. Although this approach did not allow us to detect the activation of p50/p50 homodimer, it could be excluded by the lack of a lower band totally supershifted by p50 antibody in the EMSA analysis of NF-κB-DNA complexes. These results indicated that while Aβ peptide activates dimers of p50 and p65 subunits, the mGlu5 receptor signalling leads to selective activation the NF-κB dimers p50/c-Rel and p65/c-Rel.

c-Rel silencing or c-Rel knockout suppressed the mGlu5-mediated neuroprotection in cortical cells

Synthetic siRNAs can be readily introduced into primary cortical neurons and effectively inhibit the expression of endogenous genes.[32] A double-stranded siRNA targeting c-Rel expression (c-Rel-siRNA) or a control non-siRNA were designed as described in Material and Methods and added to 8 DIV cortical cells. The treatment with a fluorescein-conjugated siRNA showed a transfection efficiency of about

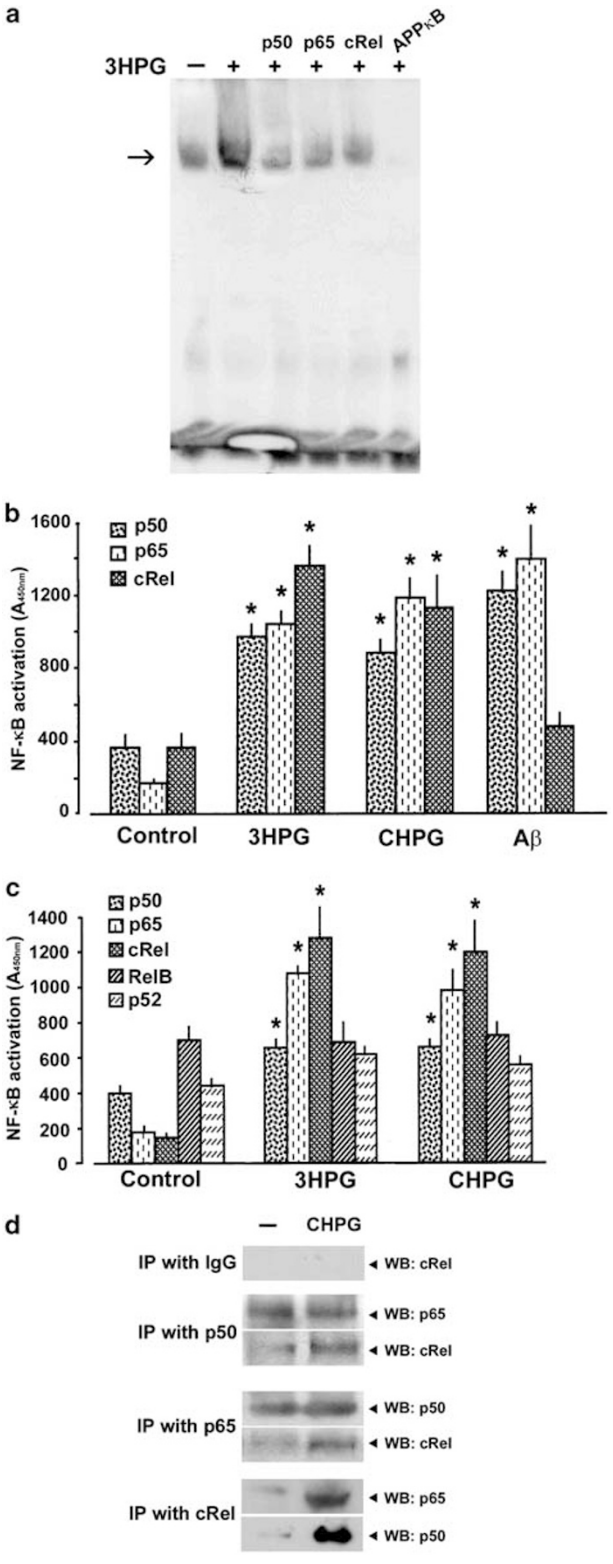

Figure 2 The stimulation of mGlu5 receptors activates NF-κB factors in mouse cortical neurons and neuronal SK-N-SH cells. (**a**) EMSA analysis was performed in nuclear extracts from cortical cells treated with vehicle or 50 μM 3HPG for 20 min, followed by incubation in fresh medium for an additional 30 min. Competition with 100-fold excess of APPκB demonstrated specificity of binding (arrow). The molecular composition of NF-κB complexes was investigated by incubating nuclear extracts in the presence of antibodies raised against p50, p65 and c-Rel. (**b**) EMSA results were confirmed by an ELISA-based analysis of DNA-binding interactions for the different NF-κB factors. Nuclear extracts were from cortical cells exposed to 50 μM 3HPG, 1 mM CHPG and 5 μM Aβ in cortical cells. (**c**) ELISA-based analysis of human NF-κB factors in nuclear extracts from SK-N-SH cells exposed to 50 μM 3HPG and 1 mM CHPG. Absorbance data represent the means of three separate experiments. $^*P \leqslant 0.05$ *versus* corresponding control value. (**d**) Co-immunoprecipitation analysis of NF-κB complexes in nuclear extracts from SK-N-SH cells. The efficiency of immunoprecipitations was confirmed by immunoblot analysis with antibodies raised against the immunoprecipitated proteins (data not shown). The antibody to p50 immunoprecipitated p65 and c-Rel. The p65 antibody immunoprecipitated p50 and c-Rel, while the c-rel antibody immunoprecipitated p65 and p50. The antibody to IgG did not immunoprecipitate c-Rel or other NF-κB factors (data not shown). Treatment with 1 mM CHPG enhanced the nuclear translocation of NF-κB complexes p50/c-Rel and p65/c-Rel, but not p50/p65

80% (data not shown). After 3 h, the medium was changed to Neurobasal and the c-Rel expression was evaluated by immunoblot analysis (Figure 3a) and immunocytochemistry (data not shown). The RNA interference significantly reduced c-Rel expression time-dependently. The decrease of c-Rel was clearly evident at 48 h (data not shown) and 72 h (Figure 3a). The c-Rel siRNA did not decrease the cellular amounts of p50, p65 or β-tubulin, indicating the specificity of c-Rel silencing. Indeed, the amounts of p50 and p65 appeared slightly increased in c-Rel silenced neurons, suggesting that their expression could compensate the lack of c-Rel in siRNA-treated cells. The siRNA treatment did not affect *per se* the neuron viability. At 48 h after application of siRNA, cells were exposed for an additional 48 h to Aβ with or without mGlu5 receptor agonists. As shown in Figure 3b, the c-Rel targeted cells were vulnerable to Aβ toxicity, as were cells treated with non-siRNA used as a negative control. Conversely, the c-Rel targeted cells became totally resistant to mGlu5 receptor-mediated neuroprotection. A higher vulnerability trend was observed in c-Rel silenced neurons co-exposed to Aβ and 3HPG or CHPG. Parallel experiments were carried out in primary cultures of cortical neurons prepared from c-Rel$^{-/-}$ embryos (Figure 3c). As reported in Figure 3d, the Aβ-mediated toxicity was preserved in c-Rel$^{-/-}$ cells, while the neuroprotective activity of mGlu5 receptor agonists was completely lost. These results support the observation that c-Rel factor is dispensable for Aβ toxicity, but also absolutely necessary for mGlu5 receptor-mediated neuroprotection.

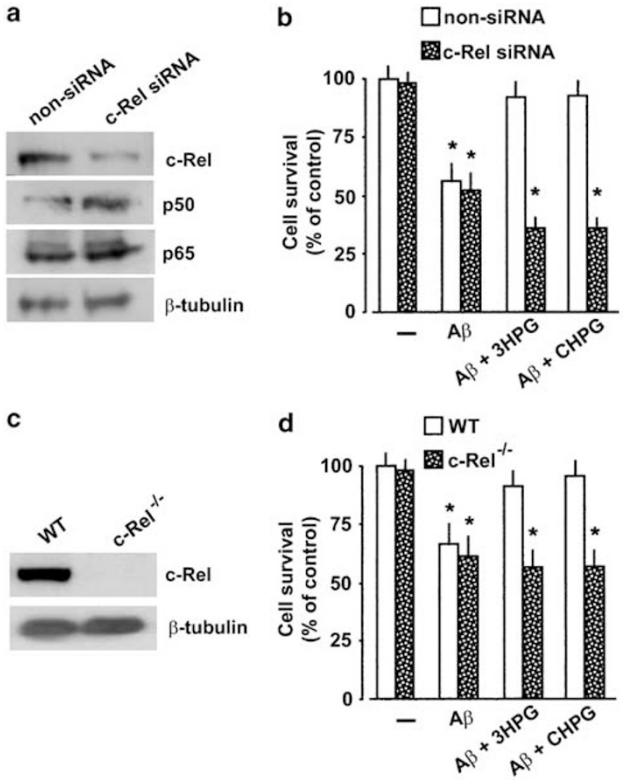

Figure 3 Targeting c-Rel expression abolished mGlu5 receptor-mediated neuroprotection. (**a**) Primary cortical neurons were transfected with siRNA cognate to c-Rel gene (c-Rel siRNA) or with a negative control (non-siRNA) for 3 h, as described in Material and Methods. The cell extracts were prepared 72 h later and immunoblotted with c-Rel antibody. Blots probed with p50, p65 antibodies indicated specificity of c-Rel targeting by siRNA. Blots re-probed with an anti-β-tubulin antibody indicated equal amounts of proteins in the different lanes. (**b**) At 2 days after siRNA transfection, primary cortical neurons were exposed to 5 μM Aβ with or without 50 μM 3HPG or 1 mM CHPG for 48 h. The protective effect of 3HPG and CHPG, observed in non-siRNA treated cells, was suppressed in cells exposed to c-Rel siRNA. (**c**) Immunoblot analysis of c-Rel expression in extracts from primary cortical neurons prepared from WT or c-Rel$^{-/-}$ mice. (**d**) Cortical neurons from WT or c-Rel$^{-/-}$mice were exposed to Aβ with or without mGlu5 receptor agonists for 48 h as in (**b**). Data represent the means $\pm$ S.E.M. of three separate experiments run in triplicate. *$P \leqslant 0.05$ *versus* corresponding vehicle-treated samples

Overexpression of c-Rel reduced SK-N-SH cell vulnerability to Aβ exposure

As knocking down c-Rel expression abolished neuroprotective response mediated by mGlu5 receptor stimulation, we investigated the effect of direct c-Rel activation on neuron vulnerability. SK-N-SH neuronal cells were transiently transfected with expression plasmid coding for human c-Rel protein, pSG-cRel, or with control pSG5 expression vector. At 24 h after transfection, the overexpression of c-Rel was monitored by immunoblot analysis in total cell extracts (Figure 4a). The cells were exposed to Aβ for an additional 24 h. As reported in Figure 4b, control cultures and pSG5 transfected cells showed a similar decrease of cell survival after Aβ exposure. Conversely, c-Rel overexpressing cells showed a reduced vulnerability to Aβ toxicity (Figure 4b). Counting TUNEL positive cells in pSG5 and pSG-cRel-transfected cultures confirmed the protective effect of c-Rel against Aβ toxicity (Figure 4c).

CHPG induced Bcl-X_L and MnSOD expression in SK-N-SH cells by induction of c-Rel

Bcl-X_L and MnSOD are two prosurvival genes under the transcriptional control of NF-κB c-Rel factor in non-neuronal cells.[33–35] To demonstrate the participation of c-Rel in neuroprotection elicited by mGlu5 receptor activation, we investigated the effect of CHPG on MnSOD and Bcl-X_L expression in neuronal cells pretreated with control non-siRNA or c-Rel-siRNA. As shown in Figure 5a, the RT-PCR analysis revealed increased amounts of both MnSOD and Bcl-X_L mRNAs in SK-N-SH cells exposed for 1 or 4 h to 1 mM CHPG (Figure 5a). Accordingly, the relative protein contents appeared increased 4 h after CHPG application (Figure 5c). Treatment with c-Rel-siRNA efficiently downregulated c-Rel expression (Figure 5c) and prevented the increase of both MnSOD and Bcl-X_L by CHPG. A relative estimate of PCR products was carried out by evaluating the ratio between MnSOD or Bcl-X_L and corresponding β-actin bands for each template (Figure 5b). Relative variations in protein amounts were measured as ratio between MnSOD or Bcl-X_L bands and corresponding β-tubulin (Figure 5d).

To verify the direct prosurvival role for at least one of these proteins on Aβ-mediated cell death, we investigated the effect of TAT-Bcl-X_L obtained by the fusion of the TAT protein transduction domain with the antiapoptotic Bcl-X_L protein.[36] As shown in Figure 6a, the treatment with TAT-Bcl-X_L at

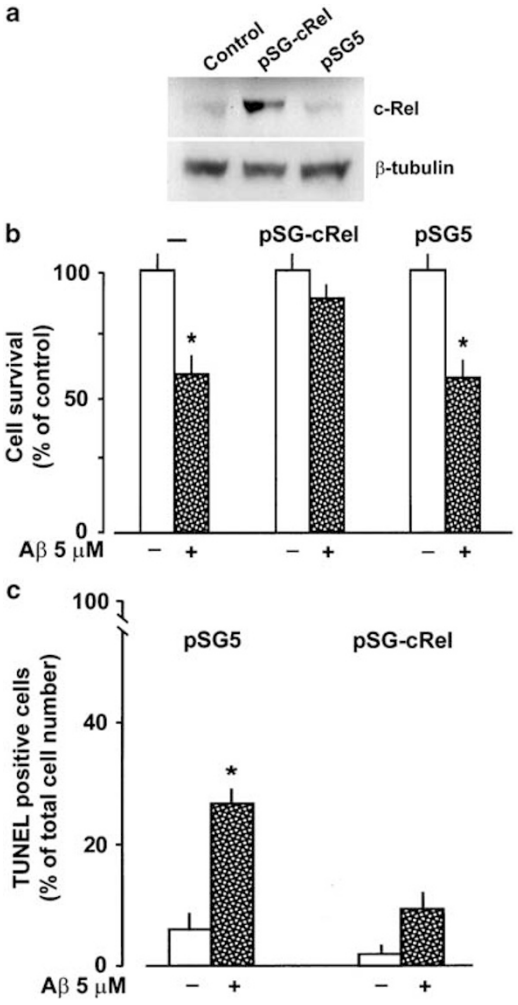

Figure 4 c-Rel overexpression counteracted Aβ-mediated toxicity in SH-N-SH neuronal cells. (**a**), SK-N-SH cells were transfected with pSG-cRel or pSG5 vector for 24 h. Cell extracts, which were immunoblotted with c-Rel antibody, revealed higher c-Rel expression in pSG-cRel-transfected cells. Blots re-probed with an anti β-tubulin antibody indicated equal amounts of proteins in the different lanes. (**b**) At the end of the transfection period, control cells and transfected cultures were exposed to Aβ peptide dissolved in fresh serum-free medium for additional 24 h. Cell viability was measured by MTT reduction assay. (**c**) TUNEL positive cells were measured in sister cultures transfected with pSG5 or pSG-cRel and exposed for 24 h to Aβ peptide. The Hoechst staining revealed similar number of cells in the wells exposed to the different treatments. Data represent the means $\pm$ S.E.M. of three separate experiments run in triplicate. *$P \leqslant 0.05$ *versus* corresponding vehicle-treated samples

concentrations ranging from 100 to 300 nM completely rescued SK-N-SH cells from Aβ neurotoxicity. Conversely, no protection against the Aβ peptide was found in cells pretreated with a control TAT-GFP protein. Finally, to confirm the participation of Bcl-X_L in the CHPG-mediated neuroprotection, we silenced the Bcl-X_L expression in SK-N-SH neuronal cells by using a specific Bcl-X_L siRNA.[37] The siRNA application reduced the protein content of Bcl-X_L after 24 and 48 h (Figure 6b), without significantly modify the basal cell survival (Figure 6d). The exposure to Aβ peptide induced the apoptosis of neuronal cultures pretreated with non-siRNA or Bcl-X_L siRNA, as shown by the analysis of cytochrome *c* release (Figure 6c) and MTT reduction (Figure 6d). However,

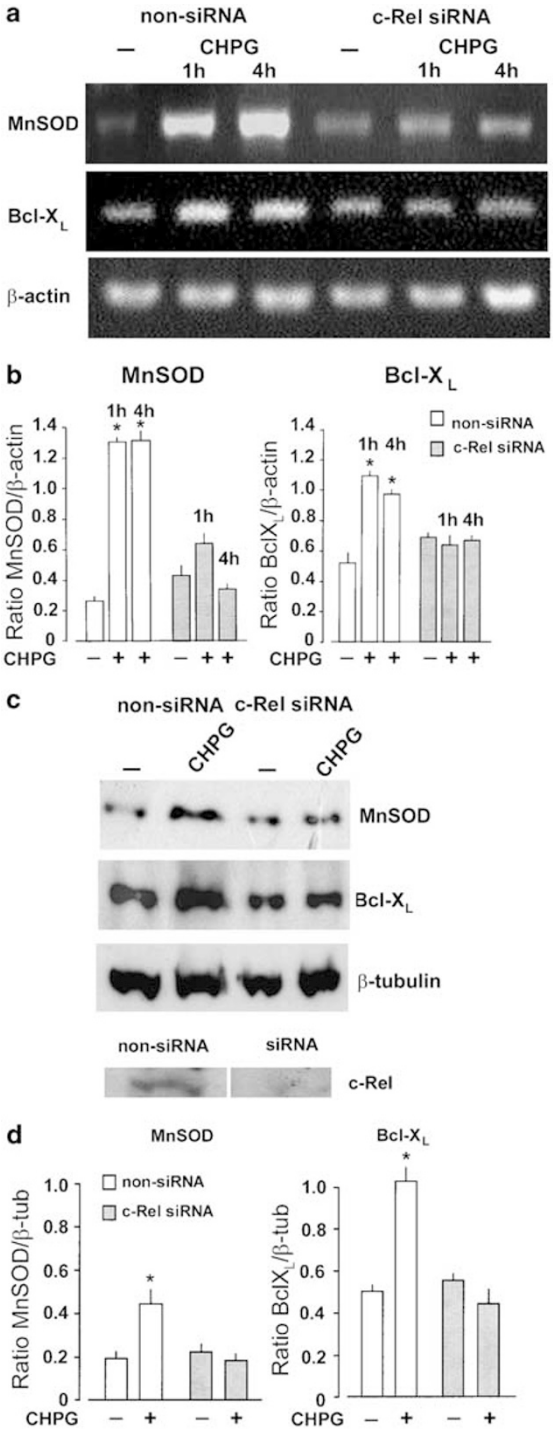

Figure 5 Targeting c-Rel expression abolished the CHPG-mediated increase of MnSOD and Bcl-X_L. SK-N-SH cells were transfected with c-Rel siRNA or with non-siRNA for 3 h. The cells were returned to a fresh medium and 72 h later they were exposed to 1 mM CHPG for 1 or 4 h. (**a**) RT-PCR analysis of the expression of MnSOD (446 bp band), Bcl-X_L (129 bp band) or β-actin (270 bp band) at 1 or 4 h of exposure to CHPG. (**b**) Data from the densitometry analysis of PCR products are expressed as ratio of MnSOD or Bcl-X_L to β-actin. (**c**) Protein cell extracts were immunoblotted with MnSOD, Bcl-X_L, c-Rel or β-tubulin antibodies. (**d**) Data from densitometry analysis of MnSOD or Bcl-X_L immunoblots are expressed as ratio of MnSOD or Bcl-X_L to β-tubulin. Columns represent the means $\pm$ S.E.M. of three experiments *$P \leqslant 0.05$ *versus* corresponding control value

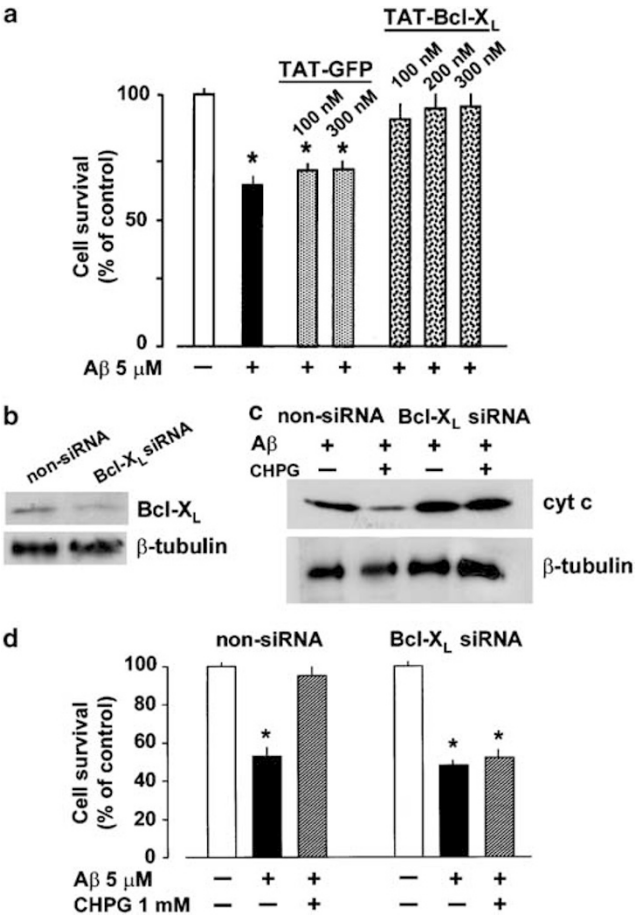

Figure 6 Induction of Bcl-X_L preserved cell viability from Aβ toxicity. (**a**) SK-N-SH cells were exposed to Aβ with or without different concentrations of TAT-Bcl-X_L for 24 h. To exclude the possibility that the TAT domain itself could cause inhibition of Aβ toxicity, we assessed the effect of a TAT-GFP protein. (**b**) SK-N-SH cells were transfected with non-siRNA or with Bcl-X_L siRNA. The level of Bcl-X_L protein was strongly reduced 48 h after transfection. (**c**) At 24 h after Bcl-X_L siRNA application, the SK-N-SH cells were exposed to 5 μM Aβ with or without 1 mM CHPG. The release of cytochrome *c* was evaluated 24 h later. (**d**) Cell survival was measured as MTT reduction in SK-N-SH cells silenced for Bcl-X_L and exposed for 48 h to Aβ with or without CHPG. Data represent the means $\pm$ S.E.M. of three separate experiments run in triplicate. $^*P \leqslant 0.05$ *versus* corresponding vehicle-treated samples

the protective effect of CHPG elicited in control cells was abolished in Bcl-X_L silenced cultures. These results lead to the conclusion that the cell resistance to Aβ toxicity induced by stimulation of mGlu5 receptors relies on c-Rel-mediated activation of Bcl-X_L.

Discussion

Our data show that stimulation of mGlu5 receptors rescues neuronal cells from Aβ-induced apoptosis. This effect requires activation of NF-κB transcription factor c-Rel and involves Bcl-X_L and MnSOD induction.

Though the mechanisms underlying neurodegeneration in Alzheimer's disease remain unclear, a growing body of evidence implicates Aβ in a pivotal role.[38,39] A matter of controversy is whether the pathological cascade is initiated by accumulation of Aβ in the extracellular space or by its intraneuronal generation. However, demonstration that wild-type neuronal tissue, when grafted into brain of APP-transgenic mice, also develops amyloid deposits and signs of neurodegeneration,[40] implicates the pathological contribution of extracellular Aβ. A lot of evidence has proved the proapoptotic effect of the two main constituents of amyloid plaques, Aβ 1-40 and Aβ 1-42, in cultured neurons,[41–45] and their capability to induce accumulation of intracellular Aβ.[46,47] Although Aβ 1–42 is considered the form initially deposited in diffuse plaques, the subsequent deposition of Aβ 1–40 characterizes the formation of fibril-rich neuritic plaques with surrounding cytopathology.[38]

For our study we used two different cell-based models of Aβ-mediated toxicity, the primary cultures of mouse cortical cells and differentiated human SK-N-SH cells. In both neuronal cell types, activation of mGlu5 receptors prevented the cell loss produced by exposure to the Aβ peptide. The precise role of group I mGlu receptors, including mGlu1 and mGlu5 subtypes, in regulating neuronal cell death is still debated. Most studies, addressed to evaluating poorly selective group I receptor ligands in models of excitotoxic or ischemia-induced injury, produced conflicting results.[48] The recent availability of more selective compounds has generated evidence suggesting that activation of mGlu1, but not mGlu5, is likely to play a major role in neurodegeneration secondary to brain ischemia.[49] The role of mGlu5 receptors is less clear, possibly because we lack mGlu5 agonists suitable for *in vivo* studies and most mGlu5 antagonists were found to block NMDA receptors, at high concentrations.[50–53] By means of either pharmacological blockers or strategies targeting the receptor expression, it was shown that endogenous activation of mGlu5 receptors elicits antiapoptotic effect and sustains neuronal survival during early brain development.[54,55] However, endogenous stimulation of mGlu5 receptors also contributes to neurotoxicity induced by the Aβ 25–35 soluble fragment in cultured neurons[56] and amplifies nigro-striatal damage induced by methamphetamine or MPTP.[57,58] More homogeneous but opposite evidence rise from studies investigating the effects of exogenous stimulation of mGlu5 receptors by selective agonists. It was shown that CHPG is neuroprotective in a rat model of cerebral ischemia when administered intraventricularly.[52] Activation of mGlu5 receptors protects neurons from oxidative stress,[59] as well as from neurotoxicity mediated by glutamate and by a variety of proapoptotic agents[60–62] including the Aβ peptide.[63] It can be argued that, while endogenous stimulation of mGlu5 receptors, possibly associated with the activation of other glutamate receptor subtypes, is detrimental, selective exogenous stimulation of mGlu5 by pharmacological agonists leads to neuroprotection. This is in line with studies indicating that under sustained activation, the function of group I metabotropic receptors in the control of glutamate transmission and cell death can switch from a facilitating to an inhibiting mode.[64–67] Transient stimulation of receptors can exacerbate neuron vulnerability. Tonic stimulation of receptors induces the desensitization of the facilitatory activity, switching the receptor function to the inhibitory activity and neuroprotection. Although not completely elucidated, the signalling pathway

responsible for that switch in function involves protein kinase C activation.[65]

We have here demonstrated that neuroprotection elicited by mGlu5 agonists was associated with activation of NF-κB factor c-Rel. In line with our previous evidence that peculiar composition of NF-κB dimers may contribute to establish neuronal cell fate in response to environmental stimuli,[27] and according to Akama *et al*.,[68] we found that the neurotoxic Aβ peptide activated only p50 and p65 subunits. Prevention of p50/p65 translocation, by a decoy oligonucleotide containing the κB regulatory sequence, prevented the Aβ toxicity (data not shown). Conversely, by co-immunoprecipitation studies we demonstrated that the mGlu5 agonists activated p50/c-Rel and p65/c-Rel dimers. The activation of c-Rel-containing dimers was an absolute requirement for prosurvival effect elicited by mGlu5 receptor agonists. This was demonstrated by targeting c-Rel expression through either RNA interference or gene knockout techniques. Both conditions completely suppressed the prosurvival effect of mGlu5 agonists, while they did not affect the Aβ-induced cell death. The involvement of c-Rel activation in NF-κB-preserved cell survival was further confirmed by overexpressing c-Rel in cultured neurons. The overexpression of c-Rel reproduced the neuroprotective effect of mGlu5 agonists in SK-N-SH cells, as previously found for the antiapoptotic response of nerve growth factor in sympathetic neurons[17,69] or insulin-like growth factor-1 in cerebellar granule cells.[70]

Consistent with our evidence, Movsesyan *et al*.,[63] recently showed that mGlu5 receptor activation prevents the Aβ-mediated apoptosis in rat cortical cells and reduces cytochrome *c* translocation, caspase-3 activation and apoptosis-inducing factor release. We have here demonstrated that neuroprotection by mGlu5 agonists was associated with increased expression of the antioxidant enzyme MnSOD and the antiapoptotic protein Bcl-X_L. MnSOD is a mitochondrial protein that catalyzes the dismutation of the anion superoxide radical to oxygen and hydrogen peroxide, a mechanism that may be responsible for cell resistance to apoptogenic burst of reactive oxygen species produced under Aβ exposure.[30,71] Neuronal cells overexpressing MnSOD exhibit nearly complete resistance to apoptosis induced by Aβ.[71,72] Bcl-X_L is a bcl-x gene product generated, together with Bcl-X_S, by alternative splicing of bcl-x transcript.[73] It is generally accepted that Bcl-X_L preserves cell survival by controlling mitochondrial membrane ion permeability and cytochrome *c* release.[74] Additionally, it acts downstream of cytochrome c release through binding to the apoptosis protease-activating factor-1 that prevents procaspase-9 and subsequent caspase-3 activation.[75] Both MnSOD and Bcl-X_L are expressed in neuronal tissues where their relative expression level can determine cell commitment to apoptosis.[19,76,77] We found that the intracellular increase of Bcl-X_L, through exogenous application of a fusion protein of Bcl-X_L with TAT protein transduction domain,[36] completely prevented the Aβ-mediated cell death, as already reported for staurosporine-, glutamate- or ischemia-induced apoptosis.[78,79] Both MnSOD and Bcl-X_L genes were shown to be transcriptional targets of c-Rel[33–35] in non-neuronal cells. Though the transcriptional regulation of Bcl-X_L and MnSOD by c-Rel has never been investigated in neurons, the p50/c-Rel dimers were found to bind to bcl-x gene promoter during brain ischemia. The p50/cRel activation appeared to be area-specific and correlated with increased Bcl-X_L expression and higher neuronal resistance to hypoxic insult.[80] Indeed, we found that the silencing of c-Rel abolished the increases of both MnSOD and Bcl-X_L induced by CHPG. Besides, the silencing of Bcl-X_L suppressed the neuroprotective activity of CHPG. These results give compelling evidence about the primary role of c-Rel in the antiapoptotic program activated by CHPG, which includes the expression of Bcl-X_L and MnSOD.

The evidence that c-Rel-containing dimers are required for neuroprotection and induction of antiapoptotic genes is in line with the view that specific combination of NF-κB subunits within the NF-κB complex is responsible for activating specific subsets of genes.[24,26] Though, an additional level of regulation of NF-κB-target genes, which may involve post-translational modification of nuclear NF-κB subunits and protein–protein interaction with other promoter-bound factors, has recently emerged.[26] As recently reviewed,[81] both phosphorylation and acetylation of NF-κB factors are required to generate a fully active NF-κB complex. Phosphorylation of p65 has been found to promote the recruitment of the transcriptional coactivators, p300/CPB (CREB binding protein) and p300/CPB-associated factor, which, in turn, acetylate both p65 and histones surrounding the promoter of NF-κB-target genes. It results in chromatin remodelling and increased transcriptional response. Likewise, phosphorylation of c-Rel is necessary for NF-κB-mediated transactivation and modulation of cell apoptosis in response to tumor necrosis factor-α.[82] It can be inferred that post-translational modifications, together with cooperation with other transcription factors, may integrate the signalling cascades activated by extracellular stimuli, to provide specificity and versatility in the regulation of genes and cellular responses by distinct NF-κB factors.

The specific role of c-Rel in neuronal pathophysiology emerges from our data and from a recent study dealing with molecular mechanisms of memory formation. Through a bioinformatics analysis and the use of c-Rel$^{-/-}$ mice, it was shown that c-Rel-mediated gene transcription is specifically required for the consolidation of long-term memory in the hippocampus.[6] Thus, clarifying the signalling cascade responsible for c-Rel activation, or for post-translational modifications implicated in the interaction of c-Rel with the transcriptional machinery in the brain, will unravel new potential targets for pharmacological treatment of neurodegenerative diseases and memory dysfunction.

Materials and Methods

Cell culture

Primary cultures of mouse cortical neurons

Fifteen-day embryonic mice were taken with cesarean section from anesthetized pregnant dams. C57/BL6 mice were purchased from Charles River, Italia, and c-Rel$^{-/-}$ mice (background strain C57/BL6) were provided by H-C Liou.[83] Cerebral cortices were isolated and dissociated by manual dispersion with a fire-polished Pasteur pipette. Cells were plated at a density of 1.5×10^5 cells/cm^2, in 8 cm^2 culture dishes (NUNC) for Western blot, EMSA and ELISA analyses, and in 2 cm^2 tissue culture

dishes for viability studies. Culture dishes were coated with 10 μg/ml poly-L-lysine. The cells were plated in Neurobasal medium (Invitrogen Corporation) supplemented with 2% B27 (Invitrogen Corporation), 0.5 mM L-glutamine and 50 U/ml penicillin/streptomicin (Invitrogen Corporation). At 3 days after plating, 50% of the medium was changed with fresh medium and subsequently 50% of the medium was changed twice a week, until 11 days *in vitro.*

SK-N-SH cell culture

The human SK-N-SH neuroblastoma cell line was purchased from American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM as previously described.[29] Cells were plated at a density of $25 \times 10^3/cm^2$ in 8 cm^2 dishes (NUNC) and neuronally differentiated by addition of 50 μM RA for 12–15 days.

Beta-amyloid toxicity

Soluble Aβ 1-40 (Bachem, Switzerland) was dissolved in water to a final concentration of 2.5 mM, divided into aliquots and frozen. Before being used it was left to aggregate for a week, at 37°C. For our experiments, 11 DIV mouse cortical neurons or differentiated SK-N-SH cells were preincubated at 37°C for 5 min with the following ligands for mGlu receptors (Tocris, UK), (*S*)-3-hydroxy-phenyl-glycine (3HPG) or (R,S)-2-chloro-5-hydroxyphenylglycine (CHPG). When present, 2-methyl-6-phenylethynyl-pyridine (MPEP) was co-administered. TAT-Bcl-X_L and TAT-GFP (green fluorescent protein),[36] kindly provided by Dr. S Cheng (Weill Medical College of Cornell University, New York, USA), were dissolved in serum-free medium and added to SK-N-SH cells during Aβ exposure. The Aβ 1–40 was then added at a concentration of 5 μM and incubation was carried out for 48 h in a Neurobasal/B27 medium for cortical neurons, or for 24 h in a serum-free medium for SK-N-SH cells. At the end of these periods, cell viability was determined by various techniques. The conversion of tetrazolium bromide (MTT) (Sigma) to purple formazan was measured. The reaction product obtained after 1 h of cell incubation with 0.5 mg/ml MTT was analyzed spectrophotometrically at 540 nm with an automated microplate reader. MTT is a water-soluble tetrazolium salt that is converted to insoluble purple formazan by dehydrogenase enzymes. As active mitochondrial dehydrogenases of living cells, but not of dead cells, cause MTT conversion, this method is used to measure cell viability according to Hansen *et al.*[84] Terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) was performed using the kit purchased by Roche Molecular Biochemicals according to the manufacturer's instructions. The TUNEL positive cells were counted in eight different fields for each well taken from experiments that were run in triplicate. The ratio between TUNEL positive cells and Hoechst labelled neurons was calculated. The release of cytochrome *c* was measured according to Movsesyan *et al.*,[63] by immunoblot analysis of cytosolic extracts.

Transfection with expression plasmids

Transfection of differentiated SK-N-SH cells was carried out according to the manufacturer's instructions with LipofectAMINE 2000 Reagent (LF 2000, Invitrogen Corporation). The day before transfection cultures were incubated with normal growth medium containing serum and without antibiotics. Cells were transfected with expression plasmids encoding c-Rel (pSG-cRel), or with the expression vector pSG5[85] as a negative control. For each cell well, 1 μg of DNA was diluted into 50 μl of Opti-MEM (Invitrogen Corporation) and 3 μl of LF 2000 Reagent into 50 μl of Opti-MEM. The two solutions were mixed and incubated for 20 min at room temperature to form the transfection complex. After washing the cells with serum-free medium, the transfection complex was added to the cells at a final volume of 1 ml in DMEM without serum and antibiotics. Cells were incubated at 37°C under an atmosphere of 5% CO_2, 95% air for 24 h, before undergoing the experiment with the Aβ peptide.

Transfection with siRNA

Double-stranded siRNA corresponding to homologous sequences of human and mouse c-Rel gene was designed as recommended,[86] with 5′ phosphate, 3′ hydroxyl, and two base overhangs on each strand. It was synthesized by Qiagen (Qiagen-Xeragon, Germantown, MD, USA). The following gene-specific sequences were used successfully: c-Rel siRNA sense 5′-CCGUGCUCCAAAUACUGCA-3′ and antisense 3′-UGCA GUAUUUGGAGCACGG-5′. Bcl-X_L siRNA sense 5′-GAGAAUCACUAAC CAGAGATT-3′ and antisense 3′-UCUCUGGUUAGUGAUUCUCTT-5′.[37] As a negative control (non-siRNA) the following sequences were used: sense 5′-UUCUCCGAACGUGUCACGU-3′ and antisense 3′-ACGUGA-CACGUUCGGAGAA-5′. The siRNAs were dissolved in buffer (100 mM potassium acetate, 30 mM HEPES-potassium hydroxide, 2 mM magnesium acetate, pH 7.4) to a final concentration of 20 μM, heated at 90°C for 60 s and incubated at 37°C for 60 min before use, to disrupt any higher aggregates forming during the synthesis. Cell transfection was carried out as follows: for every 2 cm^2 dishes, c-Rel siRNA (0.8 μg) or Bcl-X_L siRNA (1.2 μg) were condensed with 6 μl RNAiFect Transfection Reagent (Qiagen), for 15 min at room temperature, according to the manufacturer's instructions. To transfect cortical cultures, the transfection complex was diluted in 300 μl of Neurobasal/B27 and added directly to the cells. It was replaced with fresh Neurobasal/B27 3 h later. To transfect SK-N-SH cells, the transfection complex was added in DMEM without serum and antibiotics. It was replaced with complete DMEM 3 h later.

NF-κB activation

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Neuronal cultures were exposed to the test drugs for 20 min and to the fresh medium for additional 30 min. Nuclear extracts were prepared as previously described.[27] The protein concentration was assessed by the Bio-Rad Bradford assay according to the manufacturer's instructions. In total, 5 μg of nuclear extracts were combined with 15 fmol of biotin-labelled κB oligonucleotides (APP1, 5′-TAGAGACGGGGTTTCACCGTGTTA-3′) in binding buffer using the LightShift Chemilumineshent EMSA kit (Pierce Biotechnology, Rockford, IL, USA) containing poly(dI · dC) 25 ng/μl and 1 μg bovine serum albumin in a total volume of 20 μl. In competition experiments, 100-fold molar excess of unlabelled competitor oligonucleotides were added together with biotinilated probes. Reactions were carried out for 20 min at room temperature, and protein–DNA complexes were resolved on nondenaturing 4% polyacrylamide gels in 0.5 × TBE buffer. Gels were then transferred electrophoretically onto a nylon Hybond-N + membrane (Amersham Biociences, UK). The crosslink of transferred DNA was performed by exposing the membrane to 1.2 J/cm^2 UV light. The biotin-labelled κB DNA was detected according to the manufacturer's instructions. In supershift experiments, nuclear proteins were incubated with antibodies (5 μg) against various NF-κB subunits for 45 min at room temperature. Then, other components of the reaction mixture were added and incubated for an additional 20 min at room temperature. Rabbit polyclonal antibodies against p50, p65 and c-Rel were from Santa Cruz Biotechnology (Germany).

ELISA

Binding of human NF-κB subunits to the NF-κB binding consensus sequence was measured with the ELISA-based Trans-Am NF-κB kit (Active Motif, Carlsbad, CA, USA). The mouse NF-κB subunits were analyzed using the Mercury TransFactor kit (BD Biosciences, Clontech, Palo Alto, CA, USA). The Preparation of cell extracts and analysis procedures were performed as recommended by the manufacturer. Aliquots of nuclear extracts (5 μg) were transferred to 96-well plates containing high-density immobilized κB oligonucleotides. The active forms of either the NF-κB subunits in whole-cell extracts were detected using antibodies specific for the subunit bound to the target DNA. The addition of primary antibody was followed by the addition of an HRP-conjugated secondary antibody. Developing solutions were added and the samples were read by spectrophotometry. The specificity of protein–DNA interaction was checked by measuring the ability of soluble wild-type or mutated oligonucleotides to inhibit the binding. Data are expressed as the difference of absorbance observed in the presence of nuclear extracts and that observed in the absence of nuclear extracts. The ELISA-based analysis of DNA-binding activity of diverse NF-κB factors correlated very well with the EMSA analysis.

Western blot assay

SK-N-SH cells and mouse cortical neuron cells were harvested in 100 μl of lysis buffer (pH 6.9) containing 1 mM methyl sulfonyl fluoride, 1 μg/ml leupeptin, 5 μg/ml aprotinin and 1 μg/ml pepstatin. The suspension was sonicated for 30 s at full power and centrifuged at 21 000 $\times$ *g* for 30 min at 4°C. Total proteins present in the supernatant (25 μg proteins/sample) were suspended in 62.5 mM Tris-HCl, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.02% bromophenol blue (pH 7.5), and resolved by 10% SDS/polyacrylamide gel. The proteins were transferred electrophoretically onto nitrocellulose membrane. Immunodetection was performed by incubating the membrane O/N at 4°C, with the following primary antibodies: polyclonal anti-c-Rel antibody (1 μg/ml, Santa Cruz Biotechnology), polyclonal anti-MnSOD antibody (0.2 μg/ml StressGen Biotechnology Corporation), polyclonal anti-Bcl-X_L antibody (4 μg/ml, Santa Cruz Biotechnology), polyclonal anti-mGluR1α antibody (1 μg/ml, Chemicon International, Inc.), polyclonal anti-mGluR5 antiserum specifically recognizing the carboxy-terminal portion mGluR5 (1 : 500),[87] kindly provided by Dr. Rainer Kuhn and anti-β-tubulin antibody (1 : 1500, NeoMarkers). The immunoreaction was revealed by 1 h incubation at 4°C with secondary antibodies coupled to horseradish peroxidase (1 : 1500; Santa Cruz Biotechnology) and chemoluminescence detection using ECL Western blotting reagents (Amersham, Italy). Immunoblot of cytochrome *c* in cytosolic cell extracts was performed with the anticytochrome *c* monoclonal antibody (sc-13156, 1 : 500, Santa Cruz Biotechnology).

Immunoprecipitation

Co-immunoprecipitation studies were carried out in nuclear extracts from SK-N-SH neuronal cells, either untreated or treated with a CHPG, in RIPA buffer composed of 10 mM tris-HCl pH 8, 140 mM NaCl, 0.5% (v/v) Nonidet P-40, 1 mM sodium orthovanodato, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1% protease inhibitor cocktail. The antibodies used for immunoprecipitation and immunoblot analysis were: rabbit anti-p50 (Abcam, Cambridge, Science park Cambridge, UK), goat or rabbit anti-c-Rel (c-Rel-C, Santa Cruz Biotechnology), goat or rabbit anti-p65 (C-20-G, Santa Cruz Biotechnology) and anti-rabbit IgG (Chemicon), as control antiserum. Nuclear extracts (50–100 μg) were incubated at 4°C overnight with 2 μg/0.5 ml of corresponding antibodies. After incubation with the antibody, 25 μl of protein A/G (Santa Cruz Biotechnology) was added to the reaction mixture and rotated for 2 h at 4°C. Immunoprecipitates were collected by centrifuging at 1000 $\times$ *g* for 5 min followed by washing four times with RIPA buffers. Following the final wash, all the liquids that adhered to the protein A/G beads were removed. Samples were then resuspended in the sample loading buffer, subjected to SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane, and the immunoprecipitated proteins were then detected by Western blotting.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from RA-differentiated SK-N-SH cells, using QuickPrep Total RNA extraction kit (Amersham) according to the procedures suggested by the manufacturer. Human thalamic RNA was purchased by BD Clontech (Germany) and used as internal control. Reverse transcriptase reaction was performed using ImProm II™ Reverse transcriptase kit (Promega Corp., USA), according to the manufacturer's instructions using 2 μg of RNA as template and a mixture of antisense oligonucleotides (7.8 μM each) for mGlu1 (5′-AGGCCGTCT CATTGGTCTTCA-3′), mGlu5 receptor (5′-GGACCATACTTCATCAT CATC-3′)[88] and human β-actin (5′-TGATCTTCATTCTGCTGGGTG-3′). For MnSOD and Bcl-X_L mRNA analysis, cDNA was synthesized from 2 μg total RNA per sample, using oligo dT primers. PCR was performed in a Perkin-Elmer Model 480 Thermocycler (Emeryville, CA, USA) by incubating 0.2–0.4 μg of the templates with 6.2 μM of specific primers for mGlu1 and mGlu5 receptors,[88] for β-actin (sense 5′-GAAGAGCTAC GAGCTGCCTGA-3′; antisense 5′-TGATCTTCATTCTGCTGGGTG-3′), 6.6 μM of specific primers for MnSOD (sense 5′-GGCGCCCTGGAACCT CACAT-3′; antisense 5′-ACACATCAATCCCCAGCAGT-3′) according to Bernard *et al.*,[33] and 7.3 μM of specific primers for Bcl-X_L (sense 5′-GGATGGCCACTTACCTGA-3′; antisense 5′-CGGTTGAAGCGTTCCTG-3′) according to Aerbajinai *et al.*[89] The templates were heated at 94°C for 5 min. In total, 37 (mGlu1, mGlu5) or 35 (MnSOD, Bcl-X_L, β-actin) temperature cycles were conducted as follows: denaturation at 94°C for 1 min, annealing at 56°C (mGlu1, mGlu5, MnSOD, β-actin) or 61°C (Bcl-X_L) for 1 min and extension at 72°C for 1 min. Additional extension was carried out at 72°C for 5 min. PCR products were resolved on 1.2% ethidium bromide-stained agarose gel.

Statistics

Columns in the figures represent means $\pm$ S.E.M. The cell viability values are means of at least three separate experiments run in triplicate. Statistical significance of the differences was analyzed by Kruskal–Wallis nonparametric ANOVA with adjustment for multiple comparisons.

Acknowledgements

This work was supported by grants from the National Research Council (CNR) CT 9704566; Italian Ministry of Education, University and Scientific Research- COFIN 2002, and 2004 and FIRB 2001; Center of Study and Research on Ageing, Brescia; MURST Center of Excellence for Innovative Diagnostics and Therapeutics (IDET) of Brescia University. We would like to thank Dott Robert Coates of the Centro Linguistico of Brescia University for his linguistic revision.

References

1. Kaltschmidt B, Baeuerle PA and Kaltschmidt C (1993) Potential involvement of the transcription factor NF-kappa B in neurological disorders. Mol. Aspects Med. 14: 171–190
2. O' Neill LAJ and Kaltschmidt C (1997) NF-κB: a crucial transcription factor for glial and neuronal cell function. TINS 20: 252–258
3. West AE, Griffith EC and Greenberg ME (2002) Regulation of transcription factors by neuronal activity. Nat. Rev. Neurosci. 3: 921–931
4. Gosh S, May MJ and Kopp EB (1998) NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. Ann. Rev. Immunol. 16: 225–260
5. Meffert MK, Chang JM, Wiltgen BJ, Fanselow MS and Baltimore D (2003) NF-kappa B functions in synaptic signaling and behavior. Nat. Neurosci. 6: 1072–1078
6. Levenson JM, Choi S, Lee SY, Cao YA, Ahn HJ, Worley KC, Pizzi M, Liou HC and Sweatt JD (2004) A bioinformatics analysis of memory consolidation reveals involvement of the transcription factor c-rel. J. Neurosci. 24: 3933–3943
7. Grilli M, Pizzi M, Memo M and Spano PF (1996) Neuroprotection by salicylate through blockade of NF-κB activation. Science 274: 1383–1385
8. Qin ZH, Chen RW, Wang Y, Nakai M, Chuang DM and Chase TN (1999) Nuclear factor kappaB nuclear translocation upregulates c-Myc and p53 expression during NMDA receptor-mediated apoptosis in rat striatum. J. Neurosci. 19: 4023–4033
9. Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T and Schwaninger M (1999) NF-kappaB is activated and promotes cell death in focal cerebral ischemia. Nat. Med. 5: 554–559
10. Clemens JA, Stephenson DT, Smalstig EB, Dixon EP and Little SP (1997) Global ischemia activates nuclear factor-kappa B in forebrain neurons of rats. Stroke 28: 1073–1080
11. Bethea JR, Castro M, Keane RW, Lee TT, Dietrich WD and Yezierski RP (1998) Traumatic spinal cord injury induces nuclear factor-kappaB activation. J. Neurosci. 18: 3251–3260
12. Nurmi A, Lindsberg PJ, Koistinaho M, Zhang W, Juettler E, Karjalainen-Lindsberg ML, Weih F, Frank N, Schwaninger M and Koistinaho J (2004) Nuclear factor-kappaB contributes to infarction after permanent focal ischemia. Stroke 35: 987–991
13. Terai K, Matsuo A, McGeer EG and McGeer PL (1996) Enhancement of immunoreactivity for NF-kappa B in human cerebral infarctions. Brain Res. 739: 343–349
14. Hunot S, Brugg B, Ricard D, Michel PP, Muriel MP, Ruberg M, Faucheux BA, Agid Y and Hirsch EC (1997) Nuclear traslocation of NF-κB is increased in dopaminergic neurons of patients with Parkinson's disease. Proc. Natl. Acad. Sci. USA 94: 7531–7536
15. Kaltschmidt B, Uherek M, Volk B, Baeuerle PA and Kaltschmidt C (1997) Transcription factor NF-κB is activated in primary neurons by amyloid β peptides and in neurons surrounding early plaques from patients with Alzheimer's disease. Proc. Natl. Acad. Sci. USA 94: 2642–2647
16. Lezoualc'h F, Sagara Y, Holsboer F and Behl C (1998) High costitutive NF-κB activity mediates resistance to oxidative stress in neuronal cells. J. Neurosci. 18: 3224–3232
17. Maggirwar SB, Sarmiere PD, Dewhurst S and Freeman RS (1998) Nerve growth factor-dependent activation of NF-kappaB contributes to survival of sympathetic neurons. J. Neurosci. 18: 10356–10365
18. Koulich E, Nguyen T, Johnson K, Giardina CA and D'Mello SR (2001) NF-kappaB is involved in the survival of cerebellar granule neurons: association of IkappaBbeta [correction of Ikappabeta] phosphorylation with cell survival. J. Neurochem. 76: 1188–1198
19. Mattson MP, Goodman Y, Luo H, Fu W and Furukawa K (1997) Activation of NF-kappaB protects hippocampal neurons against oxidative stress-induced apoptosis: evidence for induction of manganese superoxide dismutase and suppression of peroxynitrite production and protein tyrosine nitration. J. Neurosci. Res. 49: 681–697
20. Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, Miyake S, Mizuno T and Tohyama M (1999) Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NF-kappaB activation in primary hippocampal neurons. J. Biol. Chem. 274: 8531–8538
21. Yu ZF, Zhou D, Bruce-Keller AJ, Kindy MS and Mattson MP (1999) Lack of the p50 subunit of nuclear factor-κB increases the vulnerability of hippocampal neurons to excitotoxic injury. J. Neurosci. 19: 8856–8865
22. Fridmacher V, Kaltschmidt B, Goudeau B, Ndiaye D, Rossi FM, Pfeiffer J, Kaltschmidt C, Israel A and Memet S (2003) Forebrain-specific neuronal inhibition of nuclear factor-kappaB activity leads to loss of neuroprotection. J. Neurosci. 23: 9403–9408
23. Kaltschmidt B, Uherek M, Wellmann H, Volk B and Kaltschmidt C (1999) Inhibition of NF-κB potentiates amyloid β-mediated neuronal apoptosis. Proc. Natl. Acad. Sci. USA 96: 9409–9414
24. Perkins ND (1997) Achieving transcriptional specificity with NF-kappa B. Int. J. Biochem. Cell Biol. 29: 1433–1448
25. Lin SC, Wortis HH and Stavnezer J (1998) The ability of CD40L, but not lipopolysaccharide, to initiate immunoglobulin switching to immunoglobulin G1 is explained by differential induction of NF-kappaB/Rel proteins. Mol. Cell. Biol. 18: 5523–5532
26. Hoffmann A, Leung TH and Baltimore D (2003) Genetic analysis of NF-kappaB/Rel transcription factors defines functional specificities. EMBO J. 22: 5530–5539
27. Pizzi M, Goffi F, Boroni F, Benarese M, Perkins SE, Liou HC and Spano P (2002) Opposing roles for NF-kappa B/Rel factors p65 and c-Rel in the modulation of neuron survival elicited by glutamate and interleukin-1beta. J. Biol. Chem. 277: 20717–20723
28. Strasser U, Lobner D, Behrens MM, Canzoniero LM and Choi DW (1998) Antagonists for group I mGluRs attenuate excitotoxic neuronal death in cortical cultures. Eur. J. Neurosci. 10: 2848–2855
29. Pizzi M, Boroni F, Bianchetti A, Moraitis C, Sarnico I, Benarese M, Goffi F, Valerio A and Spano P (2002) Expression of functional NR1/NR2B-type NMDA receptors in neuronally differentiated SK-N-SH human cell line. Eur. J. Neurosci. 16: 2342–2350
30. Behl C, Davis JB, Lesley R and Schubert D (1994) Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 77: 817–827
31. Du Yan S, Zhu H, Fu J, Yan SF, Roher A, Tourtellotte WW, Rajavashisth T, Chen X, Godman GC, Stern D and Schmidt AM (1997) Amyloid-beta peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: a proinflammatory pathway in Alzheimer disease. Proc. Natl. Acad. Sci. USA 94: 5296–5301
32. Krichevsky AM and Kosik KS (2002) RNAi functions in cultured mammalian neurons. Proc. Natl. Acad. Sci. USA 99: 11926–11929
33. Bernard D, Quatannens B, Begue A, Vandenbunder B and Abbadie C (2001) Antiproliferative and antiapoptotic effects of crel may occur within the same cells via the up-regulation of manganese superoxide dismutase. Cancer Res. 61: 2656–2664
34. Chen C, Edelstein LC and Gelinas C (2000) The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). Mol. Cell. Biol. 20: 2687–2695
35. Kirito K, Watanabe T, Sawada K, Endo H, Ozawa K and Komatsu N (2002) Thrombopoietin regulates Bcl-xL gene expression through Stat5 and phosphatidylinositol 3-kinase activation pathways. J. Biol. Chem. 277: 8329–8337
36. Dietz GP, Kilic E and Bahr M (2002) Inhibition of neuronal apoptosis *in vitro* and *in vivo* using TAT-mediated protein transduction. Mol. Cell. Neurosci. 21: 29–37
37. Zhang L, Zhao H, Sun A, Lu S, Liu B, Tang F, Feng Y, Zhao L, Yang R and Han ZC (2004) Early down-regulation of Bcl-xL expression during megakaryocytic differentiation of thrombopoietin-induced CD34+ bone marrow cells in essential thrombocythemia. Haematologica 89: 1199–1206
38. Selkoe DJ (2001) Alzheimer's disease: genes, proteins, and therapy. Physiol. Rev. 81: 741–766
39. Hardy J and Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297: 353–356
40. Meyer-Luehmann M, Stalder M, Herzig MC, Kaeser SA, Kohler E, Pfeifer M, Boncristiano S, Mathews PM, Mercken M, Abramowski D, Staufenbiel M and Jucker M (2003) Extracellular amyloid formation and associated pathology in neural grafts. Nat. Neurosci. 6: 370–377
41. Loo DT, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ and Cotman CW (1993) Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. Proc. Natl. Acad. Sci. USA 90: 7951–7955
42. Estus S, Tucker HM, van Rooyen C, Wright S, Brigham EF, Wogulis M and Rydel RE (1997) Aggregated amyloid-beta protein induces cortical neuronal apoptosis and concomitant 'apoptotic' pattern of gene induction. J. Neurosci. 17: 7736–7745

43. Cotman CW (1998) Apoptosis decision cascades and neuronal degeneration in Alzheimer's disease. Neurobiol. Aging 19: S29–S32
44. Copani A, Condorelli F, Caruso A, Vancheri C, Sala A, Giuffrida Stella AM, Canonico PL, Nicoletti F and Sortino MA (1999) Mitotic signaling by beta-amyloid causes neuronal death. FASEB J. 13: 2225–2234
45. Lu DC, Soriano S, Bredesen DE and Koo EH (2003) Caspase cleavage of the amyloid precursor protein modulates amyloid beta-protein toxicity. J. Neurochem. 87: 733–741
46. Yang AJ, Chandswangbhuvana D, Shu T, Henschen A and Glabe CG (1999) Intracellular accumulation of insoluble, newly synthesized Aβn-42 in amyloid precursor protein-transfected cells that have been treated with Aβ1–42. J. Biol. Chem. 274: 20650–20656
47. Hayes GM, Howlett DR and Griffin GE (2002) Production of beta-amyloid by primary human foetal mixed brain cell cultures and its modulation by exogenous soluble beta-amyloid. Neuroscience 113: 641–646
48. Nicoletti F, Bruno V, Catania MV, Battaglia G, Copani A, Barbagallo G, Cena V, Sanchez-Prieto J, Spano PF and Pizzi M (1999) Group-I metabotropic glutamate receptors: hypotheses to explain their dual role in neurotoxicity and neuroprotection. Neuropharmacology 38: 1477–1484
49. Pellegrini-Giampietro DE (2003) The distinct role of mGlu1 receptors in post-ischemic neuronal death. Trends Pharmacol. Sci. 24: 461–470
50. O'Leary DM, Movsesyan V, Vicini S and Faden AI (2000) Selective mGluR5 antagonists MPEP and SIB-1893 decrease NMDA or glutamate-mediated neuronal toxicity through actions that reflect NMDA receptor antagonism. Br. J. Pharmacol. 131: 1429–1437
51. Movsesyan VA, O'Leary DM, Fan L, Bao W, Mullins PG, Knoblach SM and Faden AI (2001) mGluR5 antagonists 2-methyl-6-(phenylethynyl)-pyridine and (E)-2-methyl-6-(2-phenylethenyl)-pyridine reduce traumatic neuronal injury *in vitro* and *in vivo* by antagonizing *N*-methyl-D-aspartate receptors. J. Pharmacol. Exp. Ther. 296: 41–47
52. Bao WL, Williams AJ, Faden AI and Tortella FC (2001) Selective mGluR5 receptor antagonist or agonist provides neuroprotection in a rat model of focal cerebral ischemia. Brain Res. 922: 173–179
53. Gasparini F, Kuhn R and Pin JP (2002) Allosteric modulators of group I metabotropic glutamate receptors: novel subtype-selective ligands and therapeutic perspectives. Curr. Opin. Pharmacol. 2: 43–49
54. Copani A, Casabona G, Bruno V, Caruso A, Condorelli DF, Messina A, Di Giorgi Gerevini V, Pin JP, Kuhn R, Knopfel T and Nicoletti F (1998) The metabotropic glutamate receptor mGlu5 controls the onset of developmental apoptosis in cultured cerebellar neurons. Eur. J. Neurosci. 10: 2173–2184
55. Catania MV, Bellomo M, Di Giorgi-Gerevini V, Seminara G, Giuffrida R, Romeo R, De Blasi A and Nicoletti F (2001) Endogenous activation of group-I metabotropic glutamate receptors is required for differentiation and survival of cerebellar Purkinje cells. J. Neurosci. 21: 7664–7673
56. Bruno V, Ksiazek I, Battaglia G, Lukic S, Leonhardt T, Sauer D, Gasparini F, Kuhn R, Nicoletti F and Flor PJ (2000) Selective blockade of metabotropic glutamate receptor subtype 5 is neuroprotective. Neuropharmacology 39: 2223–2230
57. Battaglia G, Fornai F, Busceti CL, Aloisi G, Cerrito F, De Blasi A, Melchiorri D and Nicoletti F (2002) Selective blockade of mGlu5 metabotropic glutamate receptors is protective against methamphetamine neurotoxicity. J. Neurosci. 22: 2135–2141
58. Battaglia G, Busceti CL, Molinaro G, Biagioni F, Storto M, Fornai F, Nicoletti F and Bruno V (2004) Endogenous activation of mGlu5 metabotropic glutamate receptors contributes to the development of nigro-striatal damage induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice. J. Neurosci. 24: 828–835
59. Sagara Y and Schubert D (1998) The activation of metabotropic glutamate receptors protects nerve cells from oxidative stress. J. Neurosci. 18: 6662–6671
60. Montoliu C, Llansola M, Cucarella C, Grisolia S and Felipo V (1997) Activation of the metabotropic glutamate receptor mGluR5 prevents glutamate toxicity in primary cultures of cerebellar neurons. J. Pharmacol. Exp. Ther. 281: 643–647
61. Allen JW, Knoblach SM and Faden AI (2000) Activation of group I metabotropic glutamate receptors reduces neuronal apoptosis but increases necrotic cell death *in vitro*. Cell Death Differ. 7: 470–476
62. Lea IV PM and Faden AI (2003) Modulation of metabotropic glutamate receptors as potential treatment for acute and chronic neurodegenerative disorders. Drug News Perspect. 16: 513–522
63. Movsesyan VA, Stoica BA and Faden AI (2004) MGLuR5 activation reduces beta-amyloid-induced cell death in primary neuronal cultures and attenuates translocation of cytochrome *c* and apoptosis-inducing factor. J. Neurochem. 89: 1528–1536
64. Herrero I, Miras-Portugal MT and Sanchez-Prieto J (1998) Functional switch from facilitation to inhibition in the control of glutamate release by metabotropic glutamate receptors. J. Biol. Chem. 273: 1951–1958
65. Rodriguez-Moreno A, Sistiaga A, Lerma J and Sanchez-Prieto J (1998) Switch from facilitation to inhibition of excitatory synaptic transmission by group I mGluR desensitization. Neuron 21: 1477–1486
66. Sistiaga A, Herrero I, Conquet F and Sanchez-Prieto J (1998) The metabotropic glutamate receptor 1 is not involved in the facilitation of glutamate release in cerebrocortical nerve terminals. Neuropharmacology 37: 1485–1492
67. Bruno V, Battaglia G, Copani A, Cespedes VM, Galindo MF, Cena V, Sanchez-Prieto J, Gasparini F, Kuhn R, Flor PJ and Nicoletti F (2001) An activity-dependent switch from facilitation to inhibition in the control of excitotoxicity by group I metabotropic glutamate receptors. Eur. J. Neurosci. 13: 1469–1478
68. Akama KT, Albanese C, Pestell RG and Van Eldik LJ (1998) Amyloid beta-peptide stimulates nitric oxide production in astrocytes through an NFkappaB-dependent mechanism. Proc. Natl. Acad. Sci. USA 95: 5795–5800
69. Sarmiere PD and Freeman RS (2001) Analysis of the NF-kappa B and PI 3-kinase/Akt survival pathways in nerve growth factor-dependent neurons. Mol. Cell. Neurosci. 18: 320–331
70. Heck S, Lezoualc'h F, Engert S and Behl C (1999) Insulin-like growth factor-1-mediated neuroprotection against oxidative stress is associated with activation of nuclear factor kappaB. J. Biol. Chem. 274: 9828–9835
71. Keller JN, Kindy MS, Holtsberg FW, St Clair DK, Yen HC, Germeyer A, Steiner SM, Bruce-Keller AJ, Hutchins JB and Mattson MP (1998) Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. J. Neurosci. 18: 687–697
72. Guo Q, Fu W, Holtsberg FW, Steiner SM and Mattson MP (1999) Superoxide mediates the cell-death-enhancing action of presenilin-1 mutations. J. Neurosci. Res. 56: 457–470
73. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nunez G and Thompson CB (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74: 597–608
74. Shimizu S, Narita M and Tsujimoto Y (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. Nature 399: 483–487
75. Hu Y, Benedict MA, Wu D, Inohara N and Nunez G (1998) Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. Proc Natl. Acad. Sci. USA 95: 4386–4391
76. Bredesen DE (1995) Neural apoptosis. Ann. Neurol. 38: 839–851
77. Glasgow JN, Qiu J, Rassin D, Grafe M, Wood T and Perez-Pol JR (2001) Transcriptional regulation of the BCL-X gene by NF-kappaB is an element of hypoxic responses in the rat brain. Neurochem. Res. 26: 647–659
78. Asoh S, Ohsawa I, Mori T, Katsura K, Hiraide T, Katayama Y, Kimura M, Ozaki D, Yamagata K and Ohta S (2002) Protection against ischemic brain injury by protein therapeutics. Proc. Natl. Acad. Sci. USA 99: 17107–17112
79. Cao G, Pei W, Ge H, Liang Q, Luo Y, Sharp FR, Lu A, Ran R, Graham SH and Chen J (2002) *In vivo* delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis. J. Neurosci. 22: 5423–5431
80. Qiu J, Grafe MR, Schmura SM, Glasgow JN, Kent TA, Rassin DK and Perez-Polo JR (2001) Differential NF-kappa B regulation of bcl-x gene expression in hippocampus and basal forebrain in response to hypoxia. J. Neurosci. Res. 64: 223–634
81. Chen LF and Greene WC (2004) Shaping the nuclear action of NF-kappaB. Nat. Rev. Mol. Cell 5: 392–401
82. Martin AG and Fresno M (2000) Tumor necrosis factor-alpha activation of NF-kappa B requires the phosphorylation of Ser-471 in the transactivation domain of c-Rel. J. Biol. Chem. 275: 24383–24391
83. Liou HC, Jin Z, Tumang J, Andjelic S, Smith KA and Liou ML (1999) c-Rel is crucial for lymphocyte proliferation but dispensable for T cell effector function. Int. Immunol. 11: 361–371

84. Hansen MB, Nielsen SE and Berg K (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods 119: 203–210
85. Crenon I, Beraud C, Simard P, Montagne J, Veschambre P and Jalinot P (1993) The transcriptionally active factors mediating the effect of the HTLV-I Tax transactivator on the IL-2R alpha kappa B enhancer include the product of the c-rel proto-oncogene. Oncogene 8: 867–875
86. Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W and Tuschl T (2001) Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J. 20: 6877–6888
87. Valerio A, Rizzonelli P, Paterlini M, Moretto G, Knopfel T, Kuhn R, Memo M and Spano P (1997) mGluR5 metabotropic glutamate receptor distribution in rat and human spinal cord: a developmental study. Neurosci. Res. 28: 49–57
88. Aronica E, Gorter JA, Ijlst-Keizers H, Rozemuller AJ, Yankaya B, Leenstra S and Troost D (2003) Expression and functional role of mGluR3 and mGluR5 in human astrocytes and glioma cells: opposite regulation of glutamate transporter proteins. Eur. J. Neurosci. 17: 2106–2118
89. Aerbajinai W, Giattina M, Lee YT, Raffeld M and Miller JL (2003) The proapoptotic factor Nix is coexpressed with Bcl-xL during terminal erythroid differentiation. Blood 102: 712–717