Opposing Roles for NF-κB/Rel Factors p65 and c-Rel in the Modulation of Neuron Survival Elicited by Glutamate and Interleukin-1β*

Marina Pizzi‡§, Francesca Goffi‡, Flora Boroni‡, Marina Benarese‡, Scott E. Perkins¶‖, Hsiou-Chi Liou, and PierFranco Spano‡**

*From the ‡Division of Pharmacology and Experimental Therapeutics, Department of Biomedical Sciences and Biotechnologies, School of Medicine, University of Brescia, 25123 Brescia, Italy and the **Department of Microbiology and Immunology and the ¶Laboratory and Animal Facility, Weill Medical College of Cornell University, New York, New York 10021*

The nuclear transcription factors NF-κB/Rel have been shown to function as key regulators of either cell death or survival in neuronal cells. Here, we investigated whether selective activation of diverse NF-κB/Rel family members might lead to distinct effects on neuron viability. In both cultured rat cerebellar granule cells and mouse hippocampal slices, we examined NF-κB/Rel activation induced by two opposing modulators of cell viability: 1) interleukin-1β (IL-1β), which promotes neuron survival and 2) glutamate, which can elicit toxicity. IL-1β produced a prolonged stimulation of NF-κB/Rel factors by inducing both IκBα and IκBβ degradation. Glutamate produced a delayed and transient activation of NF-κB/Rel, which was associated with a brief loss of IκBα. Moreover, IL-1β activated the p50, p65, and c-Rel subunits of NF-κB/Rel, whereas glutamate activated only the p50 and p65 proteins. The inhibition of NF-κB/Rel protein expression by antisense oligonucleotides in cerebellar granule cells showed that p65 was involved in glutamate-mediated cell death, whereas c-Rel was essential for IL-1β-preserved cell survival. Furthermore, the depletion of c-Rel in cultured neurons as well as in the hippocampus from the c-Rel⁻/⁻ mouse converted the IL-1β effect into toxicity. These findings suggest that, within a single neuron, the balance between cell death and survival in response to external stimuli may rely on the activation of distinct NF-κB/Rel proteins.

The transcription factors NF-κB/Rel play a key role in regulating a diverse array of genes involved in cell growth, differentiation, and adaptive responses to environmental factors that are cell- and stimulus-specific (1). In the central nervous system, NF-κB/Rel proteins are ubiquitously expressed in neurons and glia (2, 3) where, in addition to regulating physiological processes, they participate in pathological events associated with neurodegeneration (3, 4). Increased NF-κB/Rel levels have been observed in the dying neurons of brains exposed to trauma and ischemia (5–8) as well as in brains of patients with Alzheimer's disease and Parkinson's disease (9–11). Whether NF-κB/Rel participates in a neurodegenerative program or otherwise in a neuroprotective process by increasing neuronal resistance to various noxae is still debated. Although many studies support the antiapoptotic effects of NF-κB/Rel in cultured neurons (12–15), conflicting evidence has emerged from experimental models of pathological conditions affecting adult neurons. For example, some studies showed that NF-κB/Rel mediates the neuroprotection elicited by the tumor necrosis factor in hippocampal cells (16, 17) and promotes neuronal resistance to excitotoxicity (18) and β-amyloid-induced apoptosis (19). Other studies demonstrated that the activation of NF-κB/Rel triggers neuronal degeneration after cerebral ischemia (6, 8) and mediates the glutamate-activated cell death program during excitotoxic insults to central neurons (4, 20, 21).

NF-κB/Rel proteins are a family of transcription factors composed of several members, including p50, p52, p65/RelA, RelB, and c-Rel, that form homo- and heterodimers capable of transmitting receptor signals to the nucleus (3, 22). In resting cells, NF-κB/Rel factors are retained in the cytoplasm by association with the inhibitory IκB proteins. In stimulated cells, IκB is phosphorylated and degradated, thus allowing the release and nuclear translocation of NF-κB dimers. Recently, a more complex regulation of NF-κB/Rel activation that involves modulatory phosphorylations has been emerging. The phosphorylation of NF-κB/Rel components may operate to optimize their DNA binding and transcriptional activities, as well as functional interaction with coactivators (23). The diverse phenotypes of different NF-κB/Rel knockout mice suggest that each NF-κB/Rel member serves unique physiological roles *in vivo*, presumably via the regulation of distinct sets of target genes. Thus, the opposite regulation of neuron survival by NF-κB/Rel may very well depend on the activation of a distinct combination of subunits, resulting in the differential regulation of target genes and the induction of diverse genetic programs that dictate the cell fate (24–26).

In this study, we investigated the contribution of different NF-κB/Rel proteins to the cell survival of brain neurons exposed to IL-1β[1] and glutamate, two common activators of NF-

§ To whom correspondence should be addressed: Dept. of Biomedical Sciences and Biotechnologies, Via Valsabbina 19, 25123 Brescia, Italy. Tel.: 39-030-3717501; Fax: 39-030-3701157; E-mail: pizzi@med.unibs.it.

‖ Present address: Division of Laboratory Animal Medicine, Tufts University School of Medicine, Boston, MA 02111

[1] The abbreviations used are: IL-1β, interleukin-1β; APP, amyloid precursor protein; NMDA, *N*-methyl-D-aspartate; EMSA, electrophoretic mobility shift assay; ODN, oligodeoxynucleotide; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; WT, wild-type; IKK, IκB kinase; NeuN, neuron-specific nuclear protein.

Opposing Modulation of Neuron Survival by p65 and c-Rel

κB/Rel (27) that exert opposite effects on neuronal viability. IL-1β, undetectable in healthy brains, increases after a variety of neuronal insults. It was found to primarily elicit neuroprotection against excitotoxicity at concentrations lower than 13,000 units/ml and exhibits toxicity only when applied for a prolonged period at much higher concentrations (28). On the other hand, glutamate is widely recognized for its ability to trigger neuronal cell death and to mediate most neurodegenerative events in brain injuries (29). We demonstrated here that the opposite effects of IL-1β and glutamate on cell survival rely on the activation of different NF-κB/Rel proteins. Glutamate activation of a p50/p65 dimer promoted cell death, whereas IL-1β activation of p50/c-Rel, in addition to p50/p65, preserved cell survival in both primary neurons and mouse hippocampal slices. Treatment of the cells with p65 antisense reduced glutamate-mediated cell death. The deletion of c-Rel, as in c-Rel-null neurons, made IL-1β as toxic as glutamate. We conclude that within a single neuron the balance between p65 and c-Rel activation may account for the differential modulation of cell survival elicited by glutamate and IL-1β.

EXPERIMENTAL PROCEDURES

Primary Neuronal Cultures—Primary cultures of cerebellar granule cells were prepared from 8-day-old rat cerebella (Sprague-Dawley) (30). Experiments were carried out after culturing the neurons for 12 days *in vitro*. In a neurotoxicity assay, cultures were exposed to 100 μM glutamate or to 250 units/ml human recombinant IL-1β (Roche Molecular Biochemicals) for 15 min in Mg^{2+}-free Locke's solution. Cell viability was measured 18–24 h later by intravital staining with a fluorescein diacetate (15 μg/ml) and propidium iodide (80 μg/ml) mixture and was expressed as a percentage of the total cell number (30).

Hippocampal Slices from Adult Mice—Hippocampal slices were prepared from 40-day-old mice. C57BL/6 mice were purchased from Charles River Italia, and c-Rel$^{-/-}$ mice (background strain C57BL/6) were provided by H. -C. Liou (31). Animals were anesthetized briefly with ether and decapitated. Hippocampi were rapidly removed and cut at a thickness of 600 μm, and experiments were performed as described previously (20, 33). Slices were submerged in 2 ml of Krebs solution containing 11 mM glucose, equilibrated with 95% O_2, 5% CO_2 (pH 7.4), and preincubated at 37 °C for 5 min. Then, 100 μM N-methyl-D-aspartate (NMDA) was added, and incubation was carried out for 15 min. At the end of this period, slices were washed and further incubated in fresh buffer for 35 min. When present, IL-1β was left for the entire period (50 min). At the end of the experiment, slices were fixed and embedded in paraffin. Sections were cut at a thickness of 5 μm, stained with methylene blue and azure II, and examined by optical microscopy. To quantify cell loss, adjacent cells were counted in cell layer fields taken from the CA1 region of at least seven different slices for each treatment. Normal pyramidal neurons were recognized by their characteristic size and morphology. These neurons appeared to be homogeneous and compact with a blue cytoplasm and a brighter nucleus. Lesioned neurons appeared dark and pycnotic and were intermixed with edematous and vacuolated cells. These cell layer fields measured 1.5×10^4 μm^2. The percentage of cell survival was calculated as the ratio of living cells to the total number of cells (33).

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared as previously described (34). Five μg of nuclear extracts were combined with 20,000 cpm (0.1 ng) of γ ^{32}P-labeled κB oligonucleotides (APP1, 5′-TAGAGACGGGGTT-TCACCGTGTTA-3′) in lipage buffer (17.5 mM HEPES, pH 7.5, 5 mM KCl, 103 mM NaCl, 1 mM dithiothreitol, 0.35 mM EDTA, 10% glycerol) containing 0.5 μg of poly(dI·dC) in a total volume of 10 μl. In supershift experiments, nuclear proteins were incubated with antibodies (4 μg) against different NF-κB subunits for 1 h at room temperature before the addition of the other components of the reaction mixture. Incubation proceeded for an additional 20 min. Rabbit polyclonal anti-p50 antibody was kindly provided by M. Grilli. Polyclonal anti-p52, anti-p65, anti-RelB, and anti-c-Rel antibodies were from Santa Cruz Biotechnology.

Synthetic Oligonucleotides—Antisense phosphorothioate oligodeoxynucleotides (ODNs) were synthesized by Sigma Genosys (Cambridge, UK). The antisense sequences were 5′-ggggaacagttcgtccatgcc-3′ for p65, 5′-tacgcaccggaggccatggct-3′ for c-Rel, and 5′-ttaccgcgccgta-gacgggca-3′ for the scrambled oligonucleotide (26). The ODN sequences exhibited no similarity to any other known mammalian genes (BLAST

search). Antisense ODNs (0.3 μM) were added as a complex with lipofectin (5 μg/ml, Invitrogen) 18 h before the experiments at 37 °C in a 95% O_2, 5% CO_2 atmosphere.

Western Blot Analysis—Nuclear proteins (35) from the CA1 region of the hippocampal slices (50 μg/lane) and cell extracts from cerebellar granule cells were resolved on SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with rabbit polyclonal anti-p65 (dilution 1:200) or anti-c-Rel (dilution 1:100) antibodies for 1 h. A secondary horseradish peroxidase-conjugated antibody (1:2000, Santa Cruz Biotechnology) and ECL Western blotting reagents (Pierce) were used for band detection. Immunoblot analysis of IκB proteins was performed on cytoplasmic proteins (35); 1 μM leupeptin, 10 μM aprotinin, and 1 μg/ml pepstatin were present during the extraction procedure. The primary antibodies were anti-IκBα and -IκBβ (C-21 and C-20, respectively; 1:500 dilution; Santa Cruz Biotechnology). A secondary horseradish peroxidase-linked antibody (1:1000) was used and visualized by ECL.

Immunostaining—Primary cultures of cerebellar granule cells were fixed in 4% paraformaldehyde in phosphate-buffered saline and incubated with Triton X-100 (0.2% in phosphate-buffered saline) for 20 min. Hippocampal sections were deparaffinized, rehydrated in a series of ethanol concentrations, and incubated in methanol containing 10% hydrogen peroxide for l0 min and with Triton X-100 (0.2% in phosphate-buffered saline) for an additional 20 min. Overnight incubation with polyclonal antibodies against p65 (1:100) or c-Rel (1:100) was performed at 4 °C. Biotinylated anti-rabbit immunoglobulins (1:300, DAKO) and an ABC kit (DAKO) were used for detection following the manufacturer's instructions. For double immunofluorescence analysis, a monoclonal antibody against NeuN (1:1000, Chemicon International) and polyclonal antibodies against p65 or c-Rel were used and detected, respectively, with Cy-2- and Cy-3-marked secondary antibodies. Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) was performed using the kit purchased from Roche Molecular Biochemicals according to the manufacturer's instructions.

Statistics—Data in all figures are expressed as means ± S.E. The values for cell viability are the means of at least three separate experiments run in quadruplicate (primary cultures of cerebellar granule cells) or in septuplicate (hippocampal slices). Data were analyzed by Wilcoxon's rank sum test. $p \leq 0.05$ was considered statistically significant.

RESULTS

Opposite Regulation of Neuron Survival by Glutamate and IL-1β in Cerebellar Granule Cells Is Associated with a Distinct NF-κB/Rel Activation Pattern—In primary cultures of rat cerebellar granule cells, a 15-min application of 100 μM glutamate in the absence of extracellular magnesium has been well documented as reducing cell survival by ~60% through activation of the NMDA type of glutamate receptor (30). In this system, an application of 250 units/ml IL-1β for 15 min (Fig. 3*B*, control), as well as for 24 h, did not affect cell viability but prevented glutamate toxicity (data not shown) in line with previous evidence (28).

Despite their different effects on cell survival, both glutamate and IL-1β activated NF-κB/Rel in cerebellar granule cells. Nuclear extracts were prepared from replicate cultures 30 min, 1 h, and 3 h after the treatments (Fig. 1*A*) and then assayed by EMSA for their DNA binding activity to the APPκB site from the *APP* gene (27). NF-κB/Rel induction elicited by these two agonists displayed different kinetics, varying in the rate of onset and persistence of NF-κB/Rel binding activity. As previously described in cerebellar granule cells (27), within 30 min IL-1β highly activated APPκB binding activity that remained constant at higher than the control level for 3 h. Glutamate activated NF-κB/Rel in a slow and transient fashion; the activation was minimal at 30 min, maximal at 1 h, and declined completely to the basal level within 3 h.

The difference in NF-κB/Rel activation kinetics in response to glutamate and IL-1β was associated with differential degradation of IκBα and IκBβ. Cytoplasmic extracts were immunoblotted with specific IκB antiserum, and the blots were subjected to densitometry analysis (Fig. 1*B*). The level of IκBα appeared to dramatically decrease 30 min after glutamate ex-
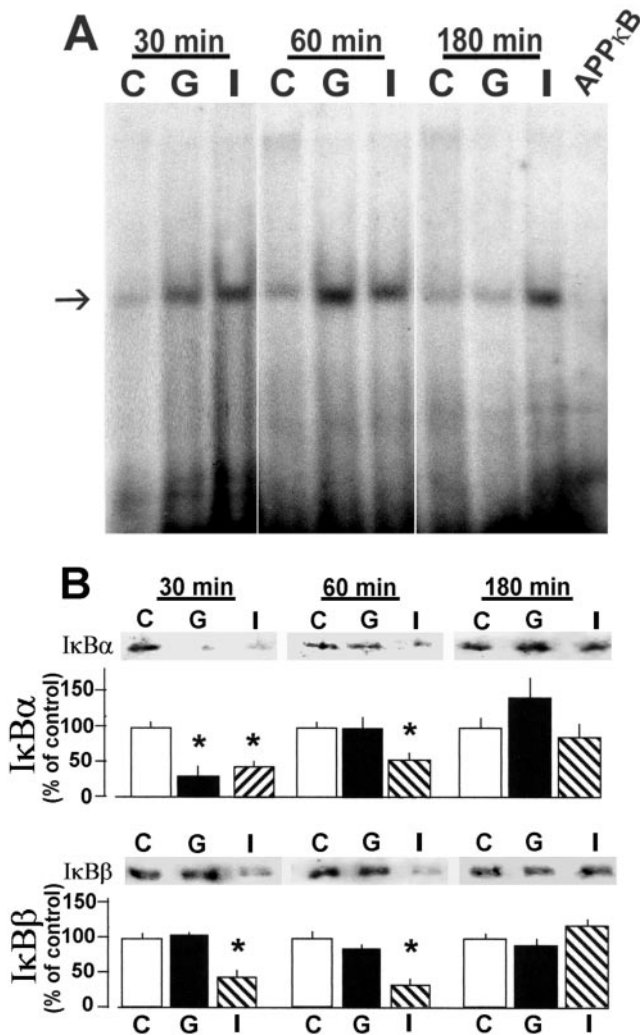
FIG. 1. **NF-κB/Rel activation in cerebellar granule cells.** *A*, nuclear extracts from primary neurons were analyzed by EMSA. This *picture* was taken from a representative experiment showing the time course of NF-κB/Rel activation in vehicle- (*C*), glutamate- (*G*), and IL-1β-treated (*I*) cells. Competition with a 100-fold excess of APPκB demonstrates the specificity of binding (*arrow*). *B*, the effect of glutamate and IL-1β on IκBα and IκBβ degradation in cerebellar granule cells. Cytoplasmic extracts were prepared after the indicated times and immunoblotted with specific antibodies directed against IκBα and IκBβ polyclonal antibodies. Immunoblots are from representative experiments. Data from a densitometry analysis of immunoblots are expressed as a percentage of control value and are expressed as means ± S.E. of three separate experiments. *, $p \leq 0.05$.

posure and returned to the control value within 1 h. No modification of the IκBβ amount was produced by glutamate. In contrast, both IκBα and IκBβ levels rapidly reduced after IL-1β exposure and returned to control levels within 3 h.

Distinct NF-κB/Rel Proteins Are Involved in the Opposing Modulation of Cell Survival Elicited by Glutamate and IL-1β in Cerebellar Granule Cells—To investigate whether distinct NF-κB/Rel proteins are important components of the signaling machinery responsible for cell death or survival, we first established the composition of the APPκB complex activated by glutamate and IL-1β in primary neurons. We selected antibodies specific for different members of the NF-κB/Rel family for their ability to interfere with DNA binding activity (Fig. 2*A*). The p50 antiserum totally inhibited the formation of the APPκB binding complex in both resting and stimulated cultures and caused a slight supershifted band. The anti-c-Rel antibody eliminated the majority of binding activity in nuclear

extracts from cells exposed to IL-1β but did not modify the binding activity of nuclear extracts from cells treated with glutamate, suggesting that only the IL-1β-induced complexes contain c-Rel. The anti-p65 antibody significantly reduced the APPκB binding complexes activated by both glutamate and IL-1β. Finally, anti-p52 and anti-RelB antisera did not affect APPκB binding activity at all. The densitometry analysis of EMSA results revealed the relative contribution of individual NF-κB/Rel proteins to the APPκB binding complexes (Fig. 2*B*). Glutamate preferentially induced NF-κB/Rel dimers composed of p50 and p65 proteins, whereas IL-1β activated NF-κB/Rel dimers that contained p50, p65, and c-Rel subunits.

To determine the role of p65 and c-Rel in glutamate and IL-1β modulation of neuron survival, cerebellar granule cells were pretreated for 18 h with selective antisense ODNs directed to c-Rel or p65 subunits or with a scrambled ODN. Antisense ODNs to p65 and c-Rel efficiently reduced the relative protein amounts as detected by either immunocytochemical (Fig. 3*A*) or immunoblot (Fig. 3*B*) analysis of treated cells. In particular, p65 antisense ODN specifically reduced p65 expression without modifying the c-Rel content (Fig. 3*B*). Likewise, c-Rel antisense ODN suppressed c-Rel but not p65 expression.

In both control or scrambled ODN-pretreated cultures, glutamate reduced cell survival to ∼40%, whereas IL-1β produced no toxicity (Fig. 3*C*). In cultures exposed to c-Rel antisense ODN, neither the viability of resting cells nor glutamate toxicity appeared to be modified. Rather, cells pretreated with c-Rel antisense displayed a high vulnerability to IL-1β application. Conversely, pretreatment with p65 antisense ODN completely prevented glutamate-mediated toxicity without affecting the viability of both resting and IL-1β-treated cells (∼90% of cell viability). These experiments suggest that p65 is involved in glutamate-mediated toxicity, whereas c-Rel is crucial for maintaining neuron survival upon IL-1β stimulation.

IL-1β Preserves Neuron Survival in the Hippocampus of Wild Type Mice but Is Toxic to the Hippocampus of c-Rel Knockout Mice—We next investigated the modulation of neuron survival elicited by glutamate and IL-1β in hippocampal slices from the adult mouse brain, a system that more closely represents *in vivo* conditions. The exposure of hippocampal slices to the glutamate receptor agonist, NMDA (100 μM), for 15 min followed by a recovery period of 35 min damaged about 70% of CA1 pyramidal neurons (Fig. 6*C*). Lesioned neurons showed internucleosomal DNA fragmentation as revealed by a TUNEL assay (Fig. 4*A*). This effect was not observed by a 50-min exposure to either 500 (data not shown) or 1000 units/ml IL-1β (Figs. 4*A* and 6*E*).

NMDA and IL-1β rapidly induced the nuclear accumulation of NF-κB/Rel proteins. The immunoblotting of nuclear extracts prepared at the end point of the experiment revealed increased levels of the p65 subunit by both treatments, but increased levels of c-Rel occurred only with IL-1β treatment (Fig. 4*B*). To further investigate the distinct activation of p65 and c-Rel in neurons, we applied the immunocytochemical technique. This type of single cell analysis is superior when studying signal transduction in brain slices because it helps to identify the cell types involved. Double labeling with antibodies against the neuron-specific NeuN protein and p65 or c-Rel indicated colocalization in neuronal cells (Fig. 4*C*). The hippocampal neurons of unstimulated sections displayed staining of the two NF-κB/Rel subunits in the cytoplasm but weak or no staining in the nuclei (Fig. 4*C*). Exposure to NMDA induced a marked increase of immunoreactivity for p65 but not for c-Rel in the nuclei. In comparison, exposure to IL-1β increased the nuclear staining for both p65 and c-Rel.
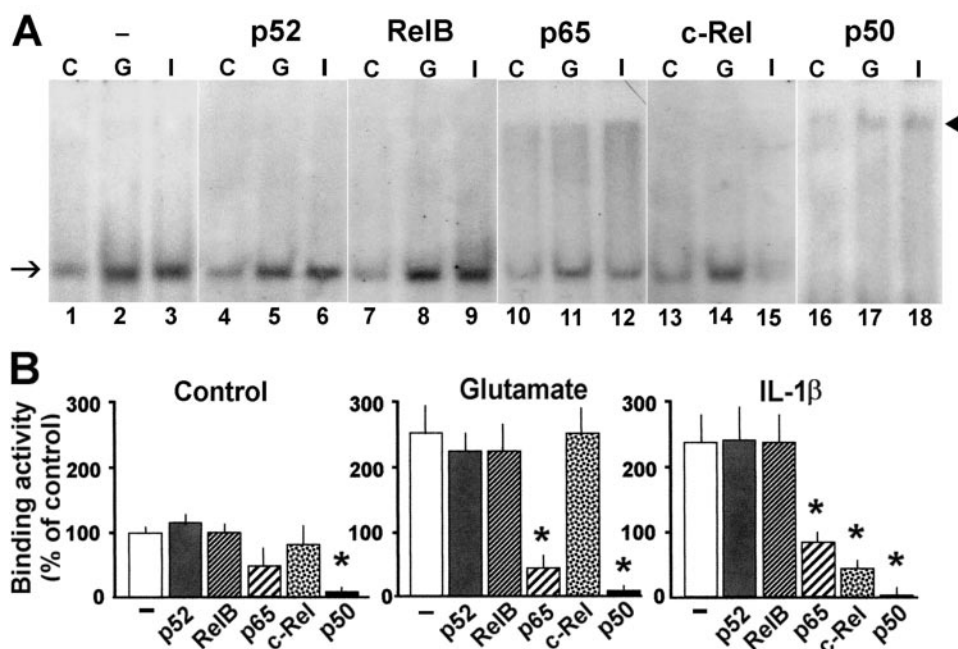
Opposing Modulation of Neuron Survival by p65 and c-Rel

FIG. 2. **Characterization of NF-κB/Rel activation elicited by glutamate and IL-1β in cerebellar granule cells.** *A*, EMSA and supershift analyses were performed in vehicle-treated cells (*C*) and cells treated with glutamate (*G*) or IL-1β (*I*). The molecular composition of the NF-κB/Rel complexes was investigated by incubating nuclear extracts in the presence of antibodies raised against p52, RelB, p65, c-Rel, and p50 subunits. The *arrow* and the *arrowhead* indicate specific binding and supershift bands, respectively. *B*, densitometry analyses of the NF-κB/Rel complexes from EMSA results. The values of binding activity were expressed as a percent of the control value (vehicle-treated cells, *lane 1*). Data are means ± S.E. of at least three experiments carried out in different cell preparations. *, $p \leq 0.05$ *versus* corresponding binding values obtained in the absence of an antibody (*lane 1* for control, *lane 2* for glutamate, *lane 3* for IL-1β).

To confirm the functional relevance of c-Rel in the prosurvival effects of IL-1β, we used mice deficient in the c-Rel protein (c-Rel$^{-/-}$). Mice with a targeted disruption of this subunit have no developmental abnormalities and only demonstrate specific defects in immune responses (31). The immunoblotting of c-Rel$^{-/-}$ brain extracts confirmed the lack of the c-Rel protein (Fig. 5A). Hippocampal slices from wild type (WT) and c-Rel$^{-/-}$ mice were exposed to NMDA, IL-1β, or both and were examined for p65 activation and cell viability. Similar to the occurrences in WT slices, either NMDA or IL-1β activated the p65 subunit (Fig. 5B) in c-Rel$^{-/-}$ hippocampal neurons, suggesting that the deletion of c-Rel did not compromise the NF-κB machinery that leads to the nuclear translocation of p65. By evaluating the cell viability, we found that the property of NMDA to induce neuronal cell death was comparable in both WT (Fig. 6C) and c-Rel$^{-/-}$ (Fig. 6D) slices. Conversely, significant differences in the two groups were disclosed by IL-1β exposure. As expected, IL-1β elicited no toxicity (Fig. 6E) but rather prevented NMDA-mediated neuronal damage in WT slices (Fig. 6G). In contrast, in c-Rel$^{-/-}$ hippocampal slices, IL-1β did not display any protection when added in combination with NMDA (Fig. 6H). Moreover, it caused toxicity even when added alone (Fig. 6F). The quantitation of pyramidal cell loss in the CA1 region of WT and c-Rel$^{-/-}$ hippocampal slices after the different treatments is shown in Fig. 6I. These results further support the crucial role of c-Rel in the prosurvival effect elicited by IL-1β in neuronal cells.

DISCUSSION

The common participation of NF-κB/Rel in either cell survival (16, 17, 32) or cell death programs (6, 8, 20, 21) strongly supports the relevance of this signaling pathway in regulating neuronal vulnerability. The data presented here demonstrate that the activation of distinct NF-κB/Rel proteins can yield an opposite modulation of neuron survival. The nuclear translocation of dimers containing p50 and p65 subunits, as triggered

by glutamate, is associated with cell death. Additional activation of the c-Rel subunit, as induced by IL-1β, switches the cell response from death to survival.

The study was conducted both on primary cultures of pure cerebellar granule cells and in hippocampal slices from adult mice. In both models, NMDA receptor activation by excitatory amino acids could cause neuronal degeneration (30), whereas the IL-1β cytokine preserved cell survival and protected against excitotoxic injury (28). The characterization of NF-κB/Rel activation elicited by the two stimuli revealed significant kinetic differences. Glutamate induced a delayed and transient stimulation of NF-κB/Rel, whereas IL-1β produced a more persistent effect. The transient activation of NF-κB mediated by glutamate correlated with a brief loss of IκBα as described previously in striatal neurons (36). The sustained translocation of NF-κB/Rel promoted by IL-1β, however, was associated with both IκBα and IκBβ degradation. The difference in NF-κB/Rel activation kinetics by the two stimuli may be partially explained by the diverse features of IκBα and IκBβ. Indeed, because *IκBα* is a κB-responsive gene, upon NF-κB/Rel stimulation the protein can rapidly recover and accumulate in the cytoplasm via an autoregulatory feedback loop (22). In addition, the newly synthesized IκBα can shuttle between the nucleus and the cytoplasm, thus operating an active export of NF-κB/Rel proteins from the nucleus through interaction with the nuclear export receptor CRM1 (37). These properties are not shared by IκBβ (37), which is less efficient in exporting nuclear NF-κB/Rel proteins and can allow a persistent activation of the transcription factor (22).

The different kinetics of NF-κB/Rel in response to glutamate and IL-1β were associated with the activation of distinct NF-κB/Rel proteins to form active κB binding dimers. Excitotoxic stimuli mainly activated p50 and p65 subunits, as previously found in the hippocampus from kainate-injected mice (18) or in mouse brains exposed to focal ischemia (8). In contrast, IL-1β
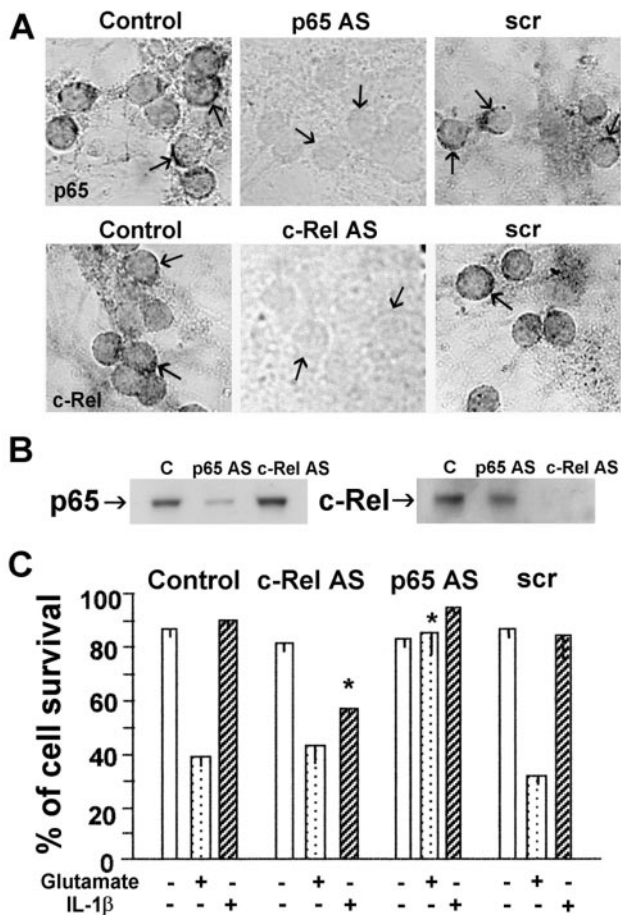
FIG. 3. **Effect of the antisense ODN to p65 and c-Rel on glutamate and IL-1β modulation of cell survival.** Cerebellar granule cells were treated for 18 h with specific antisense ODNs (*AS*) or with scrambled ODNs (*scr*). *A*, immunoreactivity to p65 and c-Rel was particularly evident in the cytoplasm of resting neurons (*arrows*), although it was also present in the nucleus. Treatments with p65 AS and c-Rel AS abolished the immunoreactivity to respective target proteins. No modification was produced by scrambled ODN. *B*, cell extracts from control and AS-treated cells were immunoblotted against p65 and c-Rel antibodies. Both p65 AS and c-Rel AS produced a specific decrease of the target protein. Blots reprobed with an anti-α-tubulin antibody indicated equal amounts of proteins in the different lanes (data not shown). *C*, after ODN treatment, cells received a 15-min pulse of glutamate or IL-1β, and 18 h later they were evaluated for cell viability by propidium iodide and fluorescein diacetate intravital staining. Percentages of cell survival after exposure to glutamate or IL-1β in control, c-Rel AS-, p65 AS-, or scrambled ODN-treated cultures were quantified. Similar results were obtained in three separate experiments run in quadruplicate. *, $p < 0.05$ *versus* the corresponding control value.

FIG. 4. **Effect of NMDA and IL-1β on neuron survival and NF-κB/Rel activation in the CA1 region of hippocampal slices from C57BL/6 mice.** After a 15-min exposure to NMDA (100 μM), hippocampal slices were left to recover for 35 min in fresh buffer. Exposure to IL-1β (1000 units/ml) was maintained for the entire period (50 min). *A*, immunohistochemistry and TUNEL labeling in the CA1 region of mouse hippocampal slices. Cells of the pyramidal layer appear immunopositive to the specific neuronal marker NeuN. Cell nuclei were particularly stained. The TUNEL labeling of CA1 neurons indicates the presence of DNA fragmentation in the nuclei of slices exposed to NMDA (*arrows*) but not in control or IL-1β−treated slices (*arrows*). *B*, Western blot analysis of the p65 and c-Rel proteins in nuclear extracts of hippocampal slices. At the end of the experiments, the CA1 regions of hippocampi were dissected from the slices and used for the preparation of nuclear extracts. The NMDA treatment increased the nuclear amount of the p65 subunit, whereas IL-1β stimulation increased the nuclear levels of both p65 and c-Rel. Blots reprobed with an anti-α-tubulin antibody indicated equal amounts of proteins in the different lanes (data not shown). *C*, double immunofluorescence analysis of NF-κB activation in the CA1 region of mouse hippocampal slices. Antibodies to NeuN (Cy-2 *green*) and c-Rel or p65 (both Cy-3 *red*) demonstrate colocalization. The nuclear localization (*arrows*) of NF-κB proteins in hippocampal neurons indicates that NMDA stimulation activates only the p65 subunit, whereas IL-1β exposure promotes the nuclear translocation of both p65 and c-Rel proteins.

activated dimers mainly composed of p50, p65, and c-Rel proteins. The complete disappearance of the NF-κB/Rel binding complex in nuclear extracts supershifted with the p50 antibody suggests a common participation of p50 to form both p50/p65 and p50/cRel dimers.

To delineate the specific involvement of different NF-κB subunits in mediating cell death or cell survival, we treated primary neuronal cultures with antisense oligonucleotides directed to p65 or c-Rel proteins. The antisense technique was successfully applied to demonstrate specific NF-κB/Rel regulation of proinflammatory mediators in peripheral cells (26). We found a differential role of p65 and c-Rel in modulating neuronal survival. Antisense to p65 completely reversed glutamate-mediated cell death. Antisense to c-Rel showed no effect on glutamate injury but did convert IL-1β neuroprotection into toxicity. The functional balance between c-Rel and p65 in IL-1β-promoting cell survival was further supported by experi-
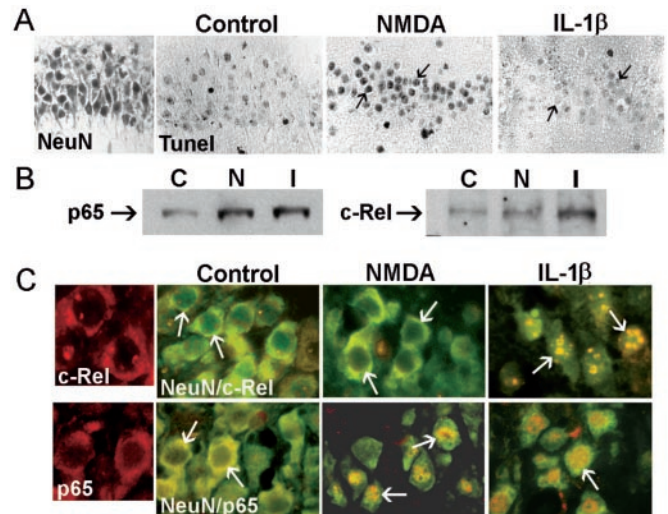
ments on hippocampal slices from c-Rel$^{-/-}$ mice. The lack of the c-Rel subunit did not compromise either p65 activation by both stimuli or NMDA-mediated toxicity. Conversely, the knockout of the c-Rel protein made hippocampal neurons become vulnerable to IL-1β. Thus, by specifically targeting the p65 or the c-Rel subunit, the opposite regulation elicited by NF-κB/Rel on cell survival of mature neurons was unmasked. Dimers mainly composed of p50 and p65 can mediate neuronal death. The inclusion of c-Rel in a dimer combination switches the NF-κB/Rel effect into neuroprotection. Consistent with this proposal, c-Rel overexpression mediates cell viability and reproduces the anti-apoptotic response of the nerve growth factor in sympathetic neurons (14) or of insulin-like growth factor-1 in immortalized hippocampal cells and in cerebellar granule cells (38). Finally, the common participation of p50 in both cell death and cell survival dimers might account for the opposing results reported on neuron vulnerability in p50 knockout mice (8, 18).

To our knowledge, these data provide the first direct evidence for a dual role for NF-κB/Rel proteins as either cell death- or cell survival-promoting factors within a single neuron. Moreover, they demonstrate that the activation of distinct NF-κB/Rel proteins can be a functional "end point," dictating neuronal cell death or survival in response to external stimuli.

These findings raise and leave unanswered many general questions. The mechanisms by which glutamate and IL-1β regulate specific NF-κB/Rel dimers remain elusive, as well as

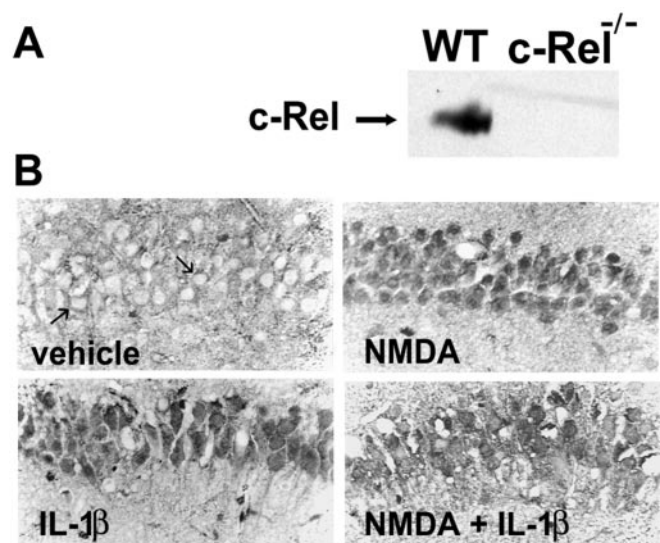FIG. 5. **NF-κB in hippocampal slices from c-Rel knockout mice.** *A*, Western blot analysis of the c-Rel protein in total brain extracts from WT and c-Rel$^{-/-}$ mice. *B*, p65 immunoreactivity was absent in cell nuclei (*arrows*) of vehicle-treated hippocampal slices from c-Rel$^{-/-}$ mice but increased in slices exposed to either NMDA (100 μM) or IL-1β (1000 units/ml) or both drugs.

the mechanism by which they differently regulate the breakdown of IκBs. Here, we report that the calcium-permeable NMDA receptor channel can only engage in IκBα degradation. This can presumably occur via activation of the canonical IKKα·IKKβ·IKKγ complex as well as by activation of more cell type- and stimulus-specific IκB kinases (39). A recent study that investigated the NMDA receptor-mediated activation of NF-κB in striatal neurons likewise reported a selective degradation of IκBα (36). Because the participation of proteasomes in NMDA-mediated IκBα degradation appeared negligible, the existence of an alternative signaling pathway was also thought to contribute to NF-κB induction in neurons exposed to excitotoxic stimuli (36). Based on structural information, it has been shown that IκBα and IκBβ differentially associate with homo- and heterodimers containing c-Rel and p65 (22, 40), although to date most of the divergence appears in their capability to respond to incoming signals and to dictate the timing of the onset and duration of the response (22, 37). Thus, it is likely that IL-1β, by activating different signal transduction pathways from NMDA receptors such as the phosphatidylinositol 3-kinase/AKT pathway, may engage the activation of different IKK complexes that trigger both IκBα and IκBβ degradation (22, 39) in addition to direct NF-κB/Rel subunit phosphorylation and transactivation (23, 41). Identifying specific IKK complexes that associate with each type of receptor, together with all IKK molecular targets, will help clarify the issue of NF-κB/Rel subunit specificity.

How specific NF-κB proteins might exert opposing regulation of neuron survival is another topic of primary importance. Indeed, in conjunction with the selective activation of NF-κB/Rel complexes, other regulatory mechanisms may contribute to the specificity of the NF-κB/Rel response. NF-κB/Rel proteins can cooperate with a large number of heterologous transcription factors to either enhance (such as with the CCAAT/enhancer-binding protein β, SP1, and members of Fos/Jun family) or repress (such as with Egr1) NF-κB-mediated transactivation (24, 42). In addition, the NF-κB function may be modulated through interaction with non-DNA binding transcriptional co-activators, such as the p300/CREB-binding protein, or through phosphorylation of NF-κB subunits achieved by signaling pathways that modulate transcription activities (23, 24, 41). All
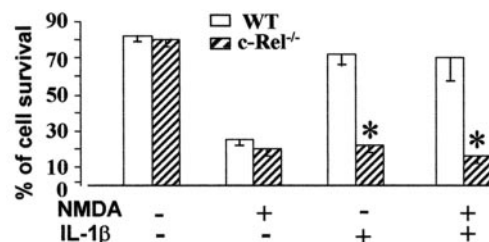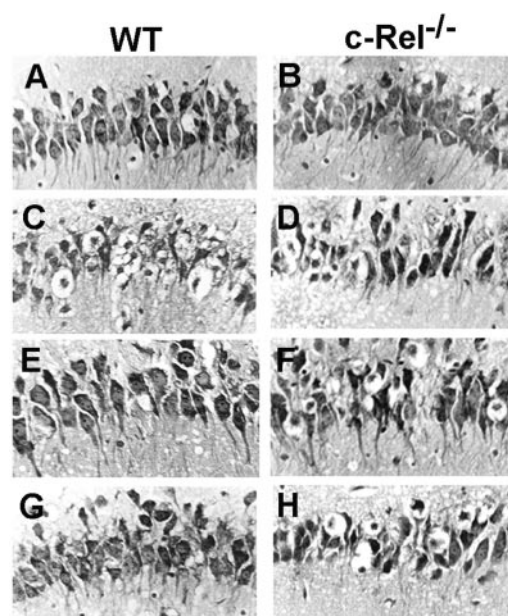

FIG. 6. **IL-1β (1000 units/ml) elicits neuroprotection against NMDA (100 μM)-mediated excitotoxicity in hippocampal slices from WT mice but exerts toxicity in slices from c-Rel$^{-/-}$ mice (c-Rel$^{-/-}$).** Photographs were taken from the CA1 region of hippocampal sections stained with methylene blue and azure II. *A* and *B*, vehicle-treated slices. *C* and *D*, slices exposed to NMDA. *E* and *F*, slices exposed to IL-1β. *G* and *H*, slices exposed to NMDA plus IL-1β. Superimposable results were obtained from three separate experiments. Graph, a quantitative analysis of NMDA and IL-1β-induced cell loss in the CA1 hippocampal region of WT and c-Rel$^{-/-}$ mice. *, $p < 0.05$ versus the corresponding values of WT group.

these regulatory mechanisms may integrate NF-κB/Rel activation with other signaling molecules, thus adding selectivity and specificity to cell response. It is not known whether and which of these mechanisms operate in neurons exposed to glutamate or IL-1β.

Among the κB-responsive genes possibly involved in the control of neuronal cell death, various pro-apoptotic genes such as *p53*, c-*Myc*, or the Fas ligand and its receptor (FAS/CD95) genes are activated upon excitotoxic stimulation (4, 8, 21) and might mediate NF-κB/Rel-induced cell death in neurons. On the other hand, other NF-κB/Rel target genes endowed with anti-apoptotic function might be involved in NF-κB-promoted neuron survival. These include the manganese superoxide dismutase (16), which is responsive to IL-1β in insulin-producing cells (43), the calcium-binding protein calbindin (44), members of the inhibitors of apoptosis (IAP) family (45), and Bcl-x$_L$ and Bfl-1, two members of Bcl2 family acting at the mitochondrial level (46–49). In particular, manganese superoxide dismutase (50) or Bcl-x$_L$ and Bfl-1 (46–48) were found to be direct transcriptional targets of the c-Rel protein. Most of this evidence has been obtained in non-neuronal cells. However, a recent study also suggests that in the hippocampus, p50/c-Rel activation after hypoxia is associated with Bcl-x$_L$ expression. In that situation, hippocampal neurons display higher cell resistance

to hypoxia when compared with basal forebrain neurons, wherein only p50/p50 and p50/p65 dimers are activated (51). Thus, the possibility that these groups of genes may be differentially involved in the opposing modulation of neuron survival by glutamate and IL-1β is intriguing and deserves further investigation. Understanding how distinct NF-κB/Rel dimers can differently switch on arrays of cell death or cell survival genes by acting individually or cooperatively with other transcriptional factors might have a relevant implication for intervention in neurodegenerative diseases.

REFERENCES

1. Liou, H.-C. & Baltimore, D. (1993) *Curr. Opin. Cell Biol.* **5,** 477–487
2. Kaltschmidt, B., Baeuerle, P. A. & Kaltschmidt, C. (1993) *Mol. Aspects Med.* **14,** 171–190
3. O'Neill, L. A. J. & Kaltschmidt, C. (1997) *Trends Neurosci.* **20,** 252–258
4. Grilli, M. & Memo, M. (1999) *Cell Death Differ.* **6,** 22–27
5. Terai, K., Matsuo, A., McGeer, E. G. & McGeer, P. L. (1996) *Brain Res.* **739,** 343–349
6. Clemens, J. A., Stephenson, D. T., Smalstig, E. B., Dixon, E. P. & Little, S. P. (1997) *Stroke* **28,** 1073–1080
7. Bethea, J. R., Castro, M., Keane, R. W., Lee, T. T., Dietrich, W. D. & Yezierski, R. P. (1998) *J. Neurosci.* **18,** 3251–3260
8. Schneider, A., Martin-Villalba, A., Weih, F., Vogel, J., Wirth, T. & Schwaninger, M. (1999) *Nat. Med.* **5,** 554–559
9. Terai, K., Matsuo, A. & McGeer, P. L. (1996) *Brain Res.* **735,** 159–168
10. Hunot, S, Brugg, B., Ricard, D., Michel, P. P., Muriel, M. P., Ruberg, M., Faucheux, B. A., Agid, Y. & Hirsch, E. C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 7531–7536
11. Kaltschmidt, B., Uherek, M., Volk, B., Baeuerle, P. A. & Kaltschmidt C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 2642–2647
12. Lezoualc'h, F., Sagara, Y., Holsboer, F. & Behl, C. (1998) *J. Neurosci.* **18,** 3224–3232
13. Koulich, E., Nguyen, T., Johnson, K., Giardina, C. A. & D'Mello, S. R. (2001) *J. Neurochem.* **76,** 1188–1198
14. Maggirwar, S. B., Sarmiere, P. D., Dewhurst, S. & Freeman, R. S. (1998) *J. Neurosci.* **18,** 10356–10365
15. Middleton, G., Hamanoue, M., Enokido, Y., Wyatt, S., Pennica, D., Jaffray, E., Hay, R. T. & Davies, A. M. (2000) *J. Cell Biol.* **148,** 325–332
16. Mattson, M. P., Goodman, Y., Luo, H., Fu, W. & Furukawa, K. (1997) *J. Neurosci. Res.* **49,** 681–697
17. Tamatani, M., Che, Y. H., Matsuzaki, H., Ogawa, S., Okado, H., Miyake, S., Mizuno, T. & Tohyama, M. (1999) *J. Biol. Chem.* **274,** 8531–8538
18. Yu, Z. F., Zhou, D., Bruce-Keller, A. J., Kindy, M. S. & Mattson, M. P. (1999) *J. Neurosci.* **19,** 8856–8865
19. Kaltschmidt, B., Uherek, M., Wellmann, H., Volk, B. & Kaltschmidt, C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 9409–9414
20. Grilli, M., Pizzi, M., Memo, M. & Spano, P. F. (1996) *Science* **274,** 1383–1385
21. Qin, Z. H., Chen, R.-W., Wang, Y., Nakai, M., Chuang, D.-M. & Chase, T. N. (1999) *J. Neurosci.* **19,** 4023–4033
22. Gosh, S., May, M. J. & Kopp, E. B. (1998) *Annu. Rev. Immunol.* **16,** 225–260
23. Schmitz, M. L., Bacher, S. & Kracht, M. (2001) *Trends Biochem. Sci.* **26,** 186–190
24. Perkins, N. D. (1997) *Int. J. Biochem. Cell Biol.* **29,** 1433–1448
25. Lin, S. C., Wortis, H. H. & Stavnezer, J. (1998) *Mol. Cell. Biol.* **18,** 5523–5532
26. Roshak, A. K., Jackson, J. R., McGough, K., Chabot-Fletcher, M., Mochan, E. & Marshall, L. A. (1996) *J. Biol. Chem.* **271,** 31496–31501
27. Grilli, M., Goffi, F., Memo, M. & Spano P. F. (1996) *J. Biol. Chem.* **271,** 15002–15007
28. Strijbos, P. J. L. M. & Rothwell, N. J. (1995) *J. Neurosci.* **15,** 3468–3474
29. Lipton, S. A. & Rosenberg, P. A. (1994) *N. Engl. J. Med.* **330,** 613–622
30. Pizzi, M., Galli, P., Consolandi, O., Arrighi, V., Memo, M. & Spano, P. F. (1996) *Mol. Pharmacol.* **49,** 586–594
31. Liou, H.-C., Jin, Z., Tumang, J., Andjelic, S., Smith, K. A. & Liou, M. L. (1999) *Int. Immunol.* **11,** 361–371
32. Kaltschmidt, B., Uherek, M., Wellmann, H., Volk, B. & Kaltschmidt, C. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 9409–9414
33. Pizzi, M., Consolandi, O., Memo, M. & Spano, P. F. (1996) *Eur. J. Neurosci.* **8,** 1516–1521
34. Andrews, N. C. & Faller, D. V. (1991) *Nucleic Acids Res.* **19,** 2499–2503
35. Brooks, P. J., Marietta, C. & Goldman, D. (1996) *J. Neurosci.* **16,** 939–945
36. Qin, Z.-H., Wang, Y. & Chasea, T. N. (2000) *Mol. Brain Res.* **80,** 111–122
37. Tam, W. F. & Sen, R. (2001) *J. Biol. Chem.* **276,** 7701–7704
38. Heck, S., Lezoualc'h, F., Engert, S. & Behl, C. (1999) *J. Biol. Chem.* **274,** 9828–9835
39. Israël, A. (2000) *Trends Cell Biol.* **10,** 129–133
40. Phelps, C. B., Sengchanthalangsy, L. L., Huxford, T. & Ghosh, G. (2000) *J. Biol. Chem.* **275,** 29840–29846
41. Sizemore, N., Lerner, N., Dombrowski, N., Sakurai, H. & Stark, G. R. (2002) *J. Biol. Chem.* **277,** 3863–3869
42. Chapman, N. R. & Perkins, N. D. (2000) *J. Biol. Chem.* **275,** 4719–4725
43. Darville, M. I., Ho, Y. S. & Eizirik, D. L. (2000) *Endocrinology* **141,** 153–162
44. Cheng, B., Christakos, S. & Mattson, M. P. (1994) *Neuron* **12,** 139–153
45. Stehlik, C., de Martin, R., Kumabashiri, I., Schmid, J. A., Binder, B. R. & Lipp, J. (1998) *J. Exp. Med.* **188,** 211–216
46. Chen, C., Edelstein L. C. & Gélinas, C. (2000) *Mol. Cell. Biol.* **20,** 2687–2695
47. Grumont, R. J., Rourke, I. J. & Gerondakis, S. (1999) *Genes Dev.* **13,** 400–411
48. Zong, W.-X., Edelstein, L. C., Chen, C., Bash, J. & Gelinas, C. (1999) *Genes Dev.* **13,** 382–387
49. Owyang, A. M., Tumang, J. R., Schram, B. R., Hsia, C. Y., Behrens, T. W., Rothstein, T. L. & Liou, H. C. (2001) *J. Immunol.* **167,** 4948–4956
50. Bernard, D., Quattannens, B., Begue, A., Vanderbunder, B. & Abbadie, C. (2001) *Cancer Res.* **61,** 2656–2664
51. Qiu, J., Grafe, M. R., Schmura, S. M., Glasgow, J. N., Kent, T. A., Rassin, D. K. & Perez- Polo, J. R. (2001) *J. Neurosci. Res.* **64,** 223–234

Opposing Roles for NF-κB/Rel Factors p65 and c-Rel in the Modulation of Neuron Survival Elicited by Glutamate and Interleukin-1 β

Marina Pizzi, Francesca Goffi, Flora Boroni, Marina Benarese, Scott E. Perkins,
Hsiou-Chi Liou and PierFranco Spano

Access the most updated version of this article at doi: 10.1074/jbc.M201014200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 51 references, 30 of which can be accessed free at
http://www.jbc.org/content/277/23/20717.full.html#ref-list-1