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N-Methyl-D-aspartate Blocks Activation of JNK and Mitochondrial Apoptotic Pathway Induced by Potassium Deprivation in Cerebellar Granule Cells*

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During the postnatal development of cerebellum, lack of excitatory innervation from the mossy fibers results in cerebellar granule cell (CGC) apoptosis during the migration of the cells toward the internal granule cell layer. Accordingly, CGCs die by apoptosis when cultured in physiological KCl concentrations (5 mM; K5), and they survive in the presence of depolarizing conditions such as high KCl concentration (25 mM; K25) or *N*-methyl-D-aspartate (NMDA). We have recently shown that NMDA is able to exert a long lasting neuroprotective effect when added to immature (2 days *in vitro*) CGC cultures by inhibition of caspase-3 activity. Here we show that NMDA- and K25-mediated neuroprotection is associated with an increase in the levels of Bcl-2, an inhibition of K5-mediated increase in Bax, and the inhibition of the release of apoptogenic factors from mitochondria such as Smac/DIABLO and cytochrome *c*. Moreover, we have shown that similar effects are observed when c-Jun N-terminal kinases (JNKs) are inhibited and that treatment of CGC cultures with NMDA blocks K5-mediated JNK activation. These results allow us to postulate that the inhibition of JNK-mediated release of apoptogenic factors from mitochondria is involved in the NMDA protection from K5-mediated apoptosis of CGCs.

Cerebellar granule cells (CGCs),[5] which migrate from the external to the internal granule cell layer during the postnatal development of cerebellum, will die by apoptosis if they do not receive trophic support from glutamatergic mossy fibers (1, 2). *In vivo* apoptotic death could be mimicked *in vitro* by culturing CGCs in a low potassium (5 mM; K5) medium (3). By contrast, CGCs survive when cultured in the presence of depolarizing concentrations of KCl (25 mM; K25) or the glutamate receptor agonist *N*-methyl-D-aspartate (NMDA), mainly by a calcium-dependent mechanism (3, 4). The signaling pathways leading to CGC apoptosis by KCl deprivation and the mechanisms involved in neuroprotection by depolarization or extracellular factors are still poorly known.

Mitochondria plays a pivotal role in many types of apoptotic responses (5, 6). The loss of mitochondrial transmembrane potential ($\Delta\Psi$m) and the permeabilization of the outer mitochondrial membrane allow the release of apoptogenic factors (7–9). Accordingly, the release of cytochrome *c* and apoptosis-inducing factor from mitochondria into the cytosol of KCl-deprived CGCs has been described (10–12). Release of these apoptogenic proteins is reported to be controlled by members of the Bcl-2 family (13). It is known that these cells are dependent upon Bax to execute cell death mediated by KCl deprivation (14), although a decrease in Bax expression has also been reported (11). Moreover, it is not clear whether the expression of the antiapoptotic protein Bcl-2 changes in KCl-deprived CGCs (11, 15).

c-Jun N-terminal kinases (JNKs) are widely believed to play an important role in cellular apoptosis (16). Changes in mitochondrial transmembrane potential and subsequent caspase-3 activation observed in KCl-deprived CGCs are preceded by an increase in active c-Jun (17). Although c-Jun is a substrate of JNKs, the participation of these kinases in KCl deprivation-mediated apoptosis of CGCs remains unclear. Pharmacological inhibition of JNKs seems to reduce apoptosis in CGC cultures (18, 19). However, no increase in overall JNK activity was detected in KCl-deprived CGCs cultures (17), in contrast with the increase observed when CGCs apoptosis was triggered by other stimuli (20, 21).

We have recently shown that NMDA protects CGCs from K5-mediated apoptosis by blocking the increase in caspase-3 activity caused by KCl deprivation (22). However, it is not known whether the NMDA antiapoptotic effect is affecting the release of proapoptotic proteins from mitochondria or the activation of JNK kinases. In this study, we show that the antiapoptotic effect of NMDA is mediated by inhibition of K5-mediated JNKs phosphorylation, an increase in Bcl-2 levels, a decrease in Bax levels, and the inhibition of Smac/DIABLO release to cytosol.

EXPERIMENTAL PROCEDURES

Chemicals—Basal Eagle's medium, L-glutamine, and penicillin/streptomycin were from PAN Biotech Inc. DAKO® fluorescent mounting medium was obtained from DAKO Corp. The ECL™ Western blotting detection reagent and Hybond-C extra nitrocellulose was purchased from Amersham Biosciences. The caspase-9 fluorometric kit was from R&D Systems, and the caspase-3 fluorometric kit was from Promega. Cleaved caspase-9 antibody was from Cell Signaling. Bax, Bcl-2, caspase-3, JNK2, phospho-c-Jun, Smac, and anti-goat IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse β-tubulin monoclonal antibody and X-linked IAP (XIAP) antibody were from BD Biosciences, whereas c-Jun antibody was purchased from Oncogene Science. Cytochrome *c* antibody was from Pharmingen,

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[5] The abbreviations used are: CGC, cerebellar granule cell; JNK, c-Jun N-terminal kinase; DIV, days *in vitro*; IAP, inhibitor of apoptosis protein; XIAP, X-linked IAP; K5, 5 mM KCl; K25, 25 mM KCl; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; Ψm, mitochondrial transmembrane potential.

active JNK antibody was from Promega, and Hsp60 antibody was from Stressgen. Anti-rabbit and anti-mouse IgG were purchased from Transduction Laboratories. Fluorescein isothiocyanate-conjugated AffiniPure F(ab′)$_2$ fragment goat anti-rabbit IgG was from Jackson Immunoresearch. Hoechst 33258 and JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) were obtained from Molecular Probes, Inc. (Eugene, OR). All other chemicals were obtained from Sigma or Calbiochem.

Cell Culture—Granule cell cultures were prepared from dissociated cerebella of 8-day-old Wistar rats as previously described by Balazs *et al.* (4). Cells were plated (8×10^5 cells/cm^2) in basal medium Eagle containing 5 mM (K5) or 25 mM (K25) KCl supplemented with 10% heat-inactivated fetal calf serum, 0.6% glucose, 2 mM L-glutamine, 25,000 units of penicillin, and 25 mg of streptomycin. 10 μM cytosine-β-D-arabinofuranoside was added to the cultures 20 h after plating to prevent the proliferation of nonneuronal cells. Neurons were plated on 24-well plates for measurement of cell viability and mitochondrial membrane potential, on 100-mm culture dishes for RNA extraction, on 60-mm culture dishes for measurement of caspase activity, and on 35-mm culture dishes for Western blotting analysis, immunocytochemistry, and observation of mitochondrial activity. We added 100 μM of NMDA or 20 mM KCl at 2 DIV to K5-containing culture medium to promote the survival of CGCs. In some experiments, the SP600125 was added 30 min before the addition of NMDA or KCl. The procedures followed were in accordance with guidelines of the Comissió d'Ètica en l'Experimentació Animal i Humana of the Universitat Autònoma de Barcelona and according to recommendations of the French Ethical Committee.

Cell Viability—Quantification of neuronal survival was assessed by a fluorescein diacetate assay. At 7 DIV, cells were washed once with phosphate-buffered saline (PBS) and then incubated for 15 min with 7.5 μg/ml fluorescein diacetate, rinsed with PBS, and lysed with a Tris-HCl/SDS solution. Fluorescence intensity was measured with an FL600 fluorescence microplate reader (Bio-Tek Instruments). Data are mean ± S.E. of values obtained from four independent experiments performed in triplicate.

Subcellular Fractionation—Cerebellar granule cells were washed once in PBS and harvested in isotonic buffer (250 mM sucrose, 1 mM EDTA, and 10 mM HEPES, pH 7.4) supplemented with 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. After a brief sonication, samples were transferred to 1.5-ml tubes and centrifuged at 900 × *g* for 10 min at 4 °C to eliminate nuclei and unbroken cells. Supernatant was then centrifuged at 9,500 × *g* for 15 min at 4 °C to obtain the heavy membrane pellet enriched for mitochondria, and the resulting supernatant was stored as the cytosolic fraction.

Western Blotting—Cells were washed once with PBS, and total cellular proteins were extracted by incubating neurons in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl, and 10 mM EDTA. The homogenate was centrifuged at 20,000 × *g* for 15 min at 4 °C, and the proteins contained in the supernatant were precipitated by the addition of ice-cold 10% trichloroacetic acid overnight. Protein extract was centrifuged at 15,000 × *g* for 15 min at 4 °C and washed three times with alcohol/ether (30:70). The pellet was denatured in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 140 mM β-mercaptoethanol, and 0.1% (w/v) bromphenol blue at 100 °C for 5 min. Proteins were resolved on 10% SDS-PAGE for detection of phospho-JNK, JNK, phospho-c-Jun, c-Jun, Smac, and XIAP. 12% SDS-polyacrylamide gels were used for detection of Bax, Bcl-2, caspase-3, and cytochrome *c*. After separation, proteins were transferred to a nitrocellulose membrane. The membranes were washed with TBS-Tween (Tris-buffered saline with 0.1% of Tween) and incubated with blocking buffer (5% bovine serum albumin in TBS-Tween) for 1 h at room temperature to block nonspecific binding. The blots were washed and incubated overnight at 4 °C with the primary antibodies diluted (1:500 for Bax, Bcl-2, caspase-3, cytochrome *c*, phospho-c-Jun, Smac, and JNK; 1:1000 for β-tubulin, Hsp60, c-Jun, and XIAP; 1:5000 for phospho-JNK) in blocking buffer. Blots were then washed with TBS-Tween and incubated for 1 h at room temperature with an anti-rabbit IgG polyclonal antibody for detection of Bax, Bcl-2, caspase-3, phospho-c-Jun, phospho-JNK, and JNK; anti-mouse IgG polyclonal antibody for detection of β-tubulin, c-Jun, cytochrome *c*, XIAP, and Hsp60; and anti-goat IgG antibody for detection of Smac. All secondary antibodies were diluted in blocking buffer at 1:1000. Immunoreactive bands were visualized using an enhanced chemiluminescence method and quantified by a computer-assisted densitometer. Bax, Bcl-2, and XIAP levels were normalized with their corresponding β-tubulin counterparts and represented as a percentage *versus* control (100%). Phospho-JNK and phospho-c-Jun levels were normalized with total JNK or c-Jun and also represented as percentage *versus* control. Cytochrome *c* and Smac/DIABLO levels were represented as ratios between the levels in mitochondria *versus* cytosol. Data are always expressed as the mean ± S.E. of values obtained at least in three independent experiments performed in duplicate.

Immunoprecipitation—Cells were washed twice with PBS and lysed in a Triton X-100-based lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1% Triton X-100, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride). Cell lysates containing 1 mg of protein were incubated with 1 μg of mouse anti-XIAP antibody at 4 °C overnight, and the immune complex was sequestered by the addition of protein G-Sepharose (Amersham Biosciences), followed by incubation for 1 h at 4 °C. The resulting immobilized immune complex was pelleted by centrifugation at 300 rpm for 2 min at 4 °C and washed three times with lysis buffer. The pellet was denatured in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 33 mM dithiothreitol, and 0.1% (w/v) bromphenol blue at 95 °C for 5 min. Proteins were resolved on 12% SDS-PAGE for detection of XIAP and caspase-3 by Western blotting as previously indicated.

Immunocytochemistry—Neurons were incubated from 1 to 12 h in the absence or presence of NMDA (100 μM) or K25. Then CGC cultures were washed with PBS and fixed with 4% paraformaldehyde (w/v in PBS) for 10 min at room temperature. Afterward, they were washed twice with PBS, blocked with 5% fetal calf serum and 3% bovine serum albumin in PBS (blocking buffer), and then incubated overnight at 4 °C with antibody against the active form of caspase-9 (1:100 dilution in blocking buffer). Cultures were then thoroughly washed with PBS and incubated with a fluorescein isothiocyanate-conjugated secondary antibody (1:500 dilution) in blocking buffer for 1 h at room temperature. After several washes, the wells and gasket were removed from the slides and mounted under glass coverslips with DAKO. Fluorescence was visualized under a LEICA microscope. Three independent experiments were performed in duplicate.

Caspase Activity—CGCs were incubated for different times in the absence or presence of NMDA (100 μM) or K25. Cells were washed twice with PBS at 37 °C, resuspended in PBS at 4 °C, and harvested by centrifugation at 350 × *g* for 9 min at 4 °C. Treatment of cellular pellet and incubation with caspase-9 pseudosubstrate LEHD-7-amido-4-(trifluoromethyl) coumarin or caspase-3 pseudosubstrate benzyloxycarbonyl-DEVD-R110 were done according to the protocol suggested by the manufacturer. Fluorescence intensity was measured with the microplate reader (Bio-Tek FL600). Data are mean ± S.E. of the values obtained in four independent experiments performed in triplicate.

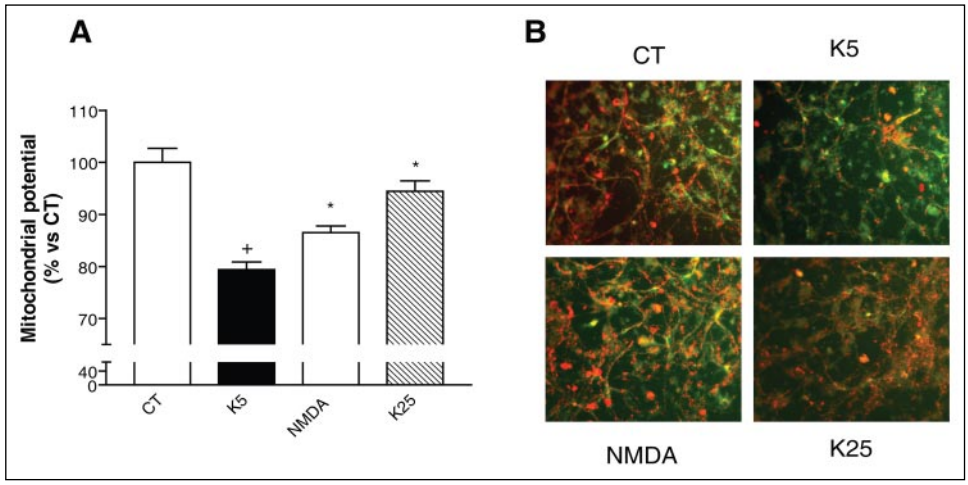

FIGURE 1. **NMDA and K25 are able to revert K5-mediated loss of mitochondrial transmembrane potential.** Neurons were plated in a K5-containing culture medium as described under "Experimental Procedures," with the exception of control (*CT*), where cells were plated in a K25-containing culture medium. At 2 DIV, NMDA (100 μM) or K25 were added for 12 h. *A*, mitochondrial transmembrane potential was assessed by JC-1 probe as indicated under "Experimental Procedures." Results are shown as percentage *versus* control and represent the mean ± S.E. of three independent experiments performed in triplicate. *B*, photomicrographs of representative fields from cultures treated as indicated. +, $p < 0.05$ *versus* control; *, $p < 0.05$ *versus* K5.

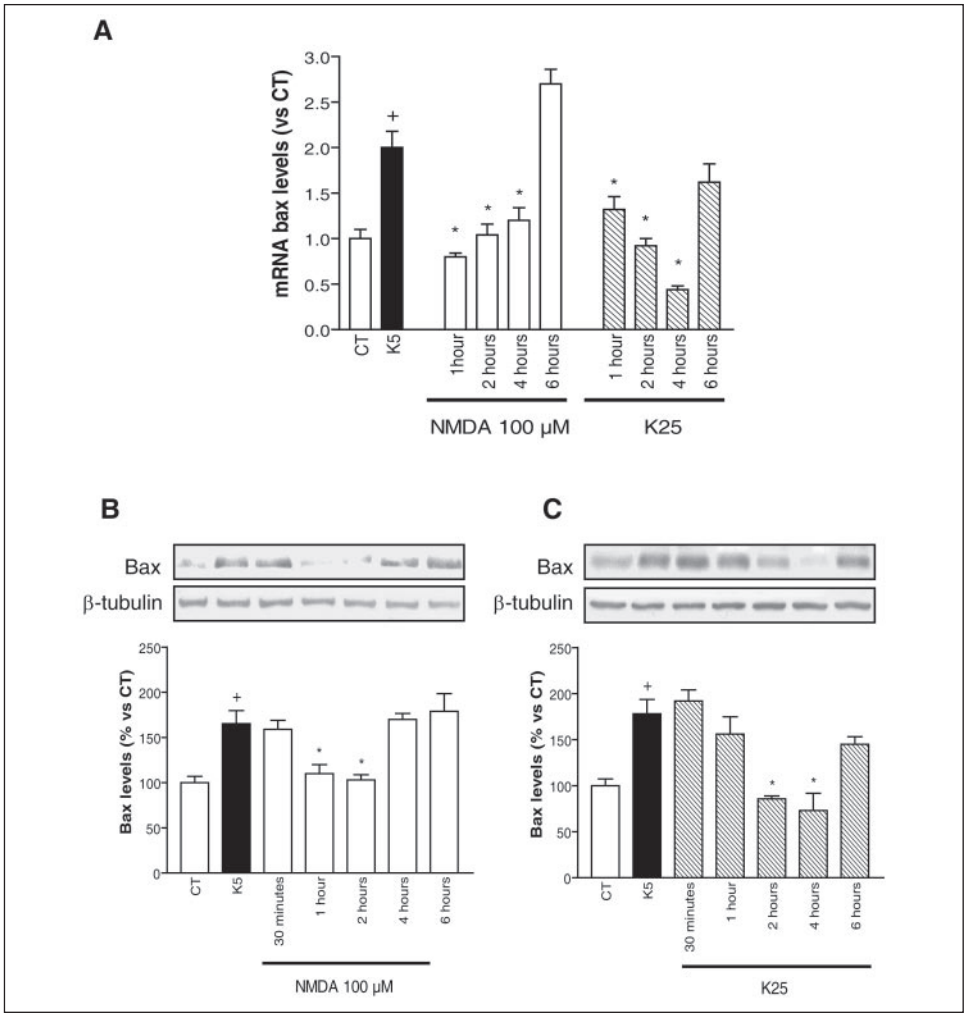

FIGURE 2. **NMDA and K25 block K5-mediated increase in mRNA and protein levels of Bax.** *A*, cells cultured in K5 were treated with NMDA (100 μM) or K25 at 2 DIV from 1 to 6 h with the exception of controls (*CT*), where cells were plated in K25. The amount of *bax* mRNA was determined by RT-PCR as described under "Experimental Procedures." Data are expressed in arbitrary units *versus* control and represent the mean ± S.E. of values obtained in four independent experiments. *B* and *C*, protein levels of Bax and β-tubulin were determined by Western blotting in cultures grown in K5 and treated with NMDA (100 μM) or K25 at 2 DIV from 30 min to 6 h. Representative Western blots are shown. Control cultures were grown in K25. Values represent the ratio between Bax and β-tubulin levels and are given as percentage *versus* control. Results are the mean ± S.E. of values obtained in five independent experiments performed in duplicate. +, $p < 0.05$ *versus* control; *, $p < 0.05$ *versus* K5.

Measurement of Mitochondrial Transmembrane Potential—Mitochondrial transmembrane potential (Ψm) was analyzed using the carbocyanine dye, JC1. When membrane potential is intact, the dye JC-1 can enter into mitochondria, where it concentrates, accumulates, and aggregates, producing an intense red emission. In cells where the mitochondrial membrane potential is altered, the monomeric form of JC-1 remains cytosolic, and it emits a green signal. At 2 DIV, cultured CGCs were treated for 6–12 h in the absence or presence of NMDA (100 μM) or K25 and in the absence or presence of SP600125 (added 30 min before the treatment with NMDA or K25). The cells were rinsed with PBS at 37 °C, incubated with 7.5 μg/ml JC-1 for 15 min, and washed twice with PBS. The ratio of fluorescence emissions at 590 nm (red) *versus* 530 nm (green) was measured with the microplate reader (Bio-Tek FL600). This ratio represents the amount of aggregates (red) divided by the amount of monomeric JC-1 molecules (green) and is an index of the mitochon-

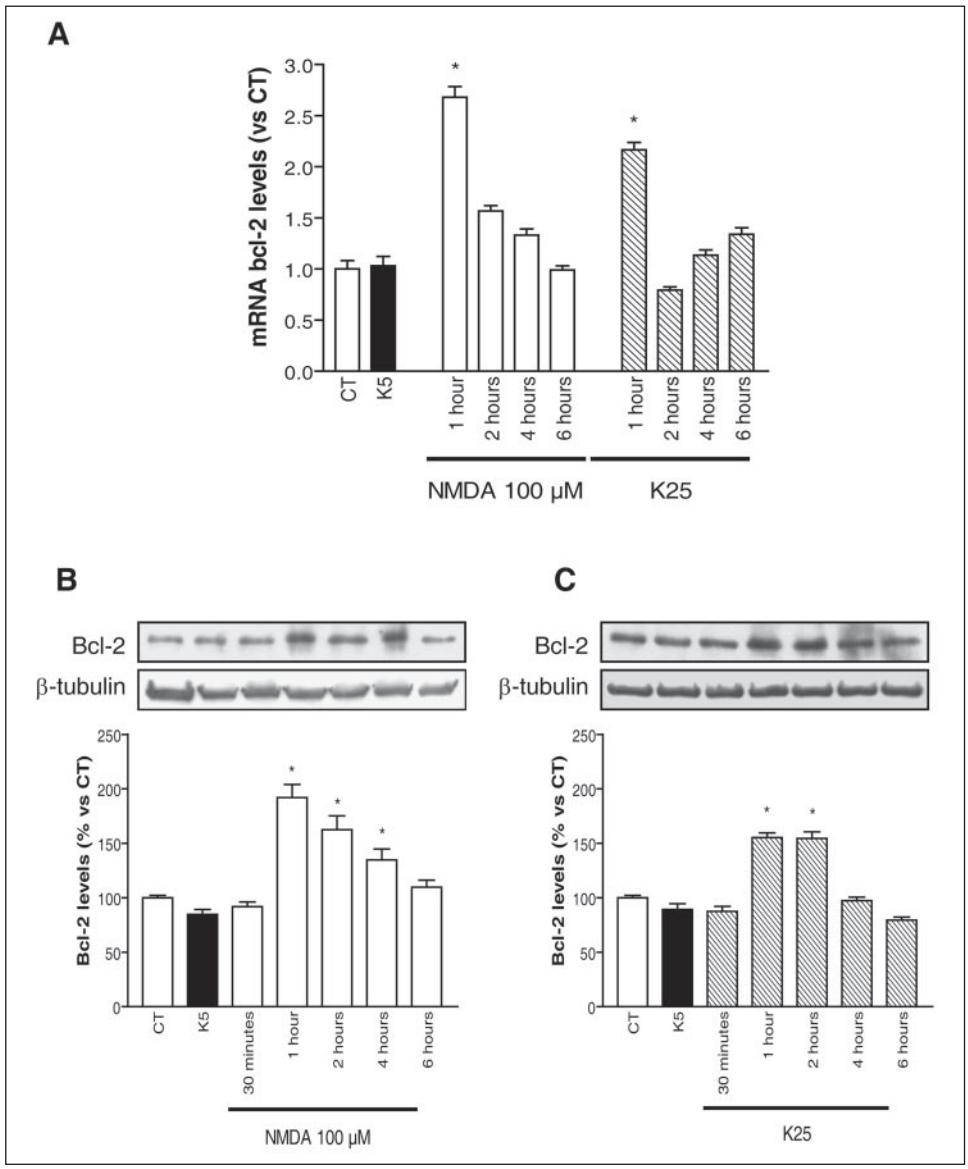

FIGURE 3. **NMDA and K25 increase the levels of Bcl-2 mRNA and protein.** *A*, the level of Bcl-2 mRNA was determined by RT-PCR in CGCs cultured in K5 and exposed to NMDA (100 μM) or K25 at 2 DIV from 1 to 6 h, with the exception of controls (*CT*), where CGCs were plated in K25. Results represent arbitrary units *versus* controls and are the mean ± S.E. of four independent experiments. *B* and *C*, Neurons grown in K5 were treated with NMDA (100 μM) or K25 at 2 DIV from 30 min to 6 h. Protein levels of Bcl-2 and β-tubulin were determined by Western blotting. Cultures grown in K25 were used as control cultures. Representative Western blots are shown. Data represent the ratio between Bcl-2 and β-tubulin levels. Results are shown as percentage *versus* controls and are the mean ± S.E. of values obtained in three independent experiments performed in duplicate. The *asterisks* indicate significant difference from K5 (*, $p < 0.05$).

drial transmembrane potential. Data represent arbitrary units and were expressed as the mean ± S.E. of values obtained in three independent experiments performed in triplicate. Changes in mitochondrial membrane potential were also monitored by epifluorescence under a Leica microscope.

RT-PCR—Cells were harvested for total RNA using the RNAeasy Mini kit from Qiagen following the manufacturer's instructions. Contaminating DNA was removed by treatment with the RNase-free DNase set from Qiagen. Total RNA (1 μg) was reverse transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen).

To study *bax* and *bcl-2* RNA levels, real time quantitative PCR analyses were performed in real time using 1× Mastermix (Applied Biosystems) containing preset concentrations of dNTPs, $MgCl_2$, and the SYBR green reporter dye along with 100 nM forward and reverse primers and either 100 nM SYBR green reporter dye, using the TaqMan 7000 sequence detection system (Applied Biosystems). RNA levels were deduced by comparison of cDNA-generated signals in samples with signals generated by a standard curve constructed with known amounts of cDNA and internally corrected with the 18 S cDNA amplification. The rat *Bax* primer set was as follows: forward primer, 5′-GACAACAACATGGMGCT-3′; reverse primer, 5′ AGCCCATGATGGTTCTGATC-3′. The human *Bcl-2* primer set was as follows: forward primer, 5′- CCGCATCAGGAAGGCTAGAG-3′; reverse primer, 5′-CTGGGACACAGGCAGGTTCT. The human *18S* primer set was as follows: forward primer, 5′- GTGGAGCGATTTGTCTGGTT-3′; reverse primer, 5′-CGCTGAGCCAGTCAGTGTAG-3′. Data represent -fold changes *versus* K5 and represent the mean ± S.E. of values obtained in four independent experiments.

XIAP mRNA and rRNA 18 S levels were determined by RT-PCR, and the products were electrophoresed on 1% agarose gels and stained with ethidium bromide. DNA size markers (Invitrogen) were run in parallel to validate the predicted sizes of the amplified bands (795 bp for XIAP and 350 bp for 18 S). The PCR products were normalized to the amplified 18 S, the internal reference gene. The rat *cdc-RIAP3* primer set was as follows: forward primer, 5′-TGAAGAAGCCAGACCGAAGA-3′; reverse primer, 5′-TGACTTGACTCATCCTGCGA-3′. The rat *18S* primer set was as follows: forward primer, 5′-TCAAGAACGAAAGTCGGAGG-3′; reverse primer, 5′-GGACATCTAAGGGCATCACA-3′. Gene expression was visualized in three independent experiments.

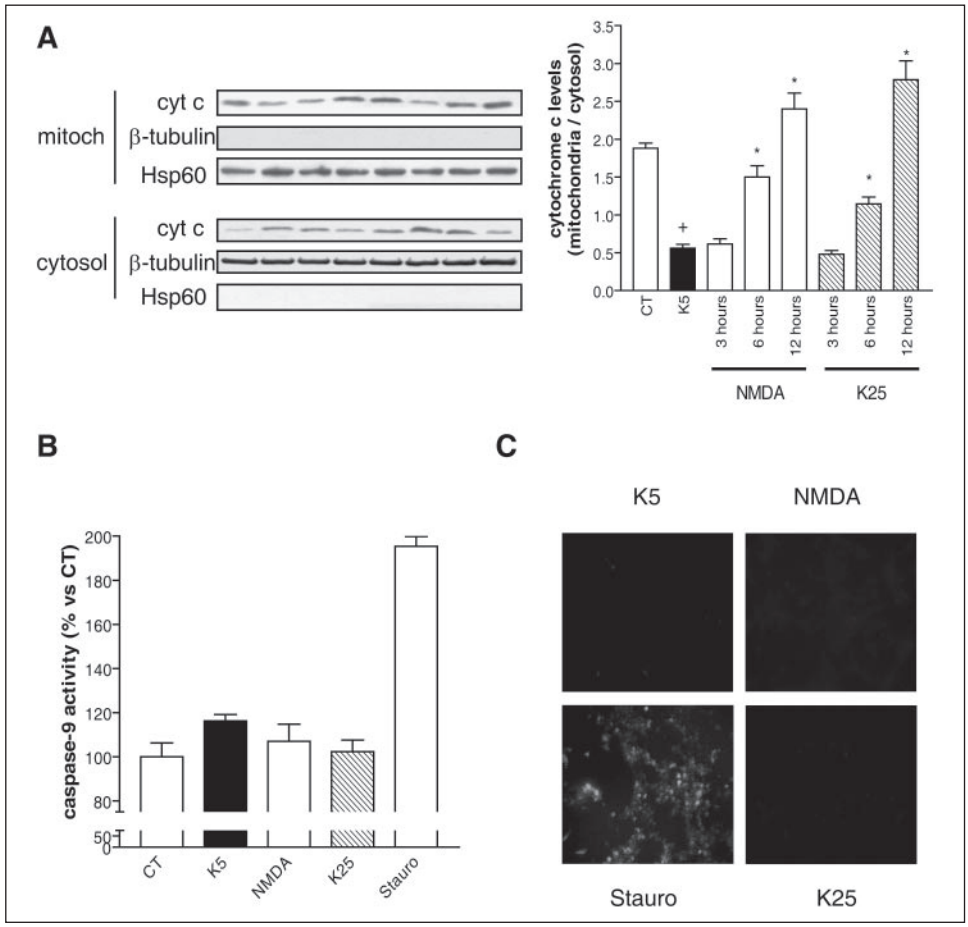

FIGURE 4. **NMDA and K25 prevent cytochrome *c* release induced by K5 but have no effect on caspase-9 activation.** Cells were plated in K5 with the exception of controls (*CT*), where cells were plated in K25. Cultured cells plated in K5 were exposed to NMDA (100 μM) and K25 at 2 DIV for 3–12 h. *A*, subcellular fractionation of cytochrome *c* was visualized by Western blot as indicated under "Experimental Procedures." Results are shown as ratio between levels of cytochrome *c* in mitochondria *versus* levels of cytochrome *c* in cytosol. Equal protein loading in cytosol was verified by analysis of β-tubulin levels. Hsp60 was used to monitor the absence of mitochondrial contamination in the cytosolic fraction and to verify equal protein loading in mitochondrial fraction. Representative Western blots of each antibody are also shown. Data represent arbitrary units and are expressed as mean ± S.E. of values obtained from four independent experiments performed in triplicate. +, $p < 0.05$ *versus* controls; *, $p < 0.05$ *versus* K5. *B*, effect of NMDA and K25 on caspase-9 activity. The caspase-9 substrate (LEHD-AFC) was used to measure caspase-9 activity as indicated under "Experimental Procedures." Results are shown as percentage *versus* controls and are mean ± S.E. of values obtained in four independent experiments performed in triplicate. *C*, immunocytochemistry of cleaved caspase-9 was assessed in cultured cells exposed to NMDA (100 μM), K25, staurosporine (1 μM; *Stauro*) for 12 h at 2 DIV and in cells plated in K5.

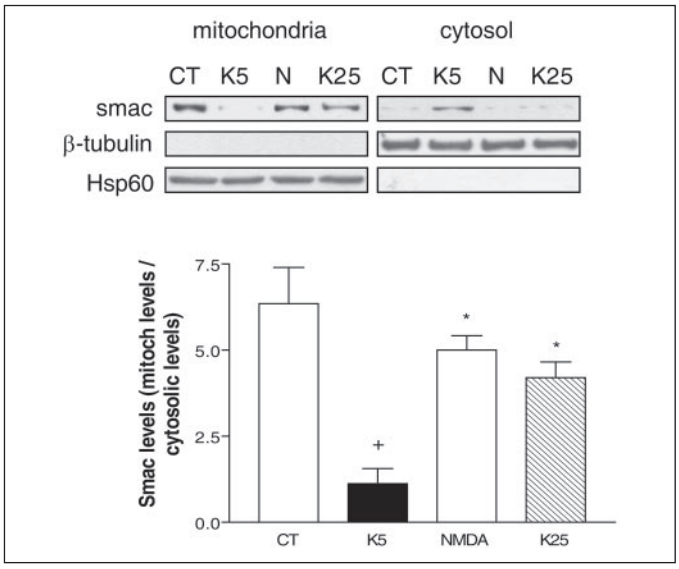

FIGURE 5. **NMDA and K25 inhibit K5-mediated release of Smac/DIABLO (*smac*) from mitochondria.** Neurons were plated in K5 or K25 (control). At 2 DIV, neurons grown in K5 were treated with NMDA (100 μM) or K25 from 3 to 12 h. Subcellular localization of Smac was determined by Western blotting after 12 h, and protein levels were quantified by computer-assisted densitometry. β-Tubulin levels were analyzed to confirm equal protein loading in cytosol. Hsp60 was used to monitor the absence of mitochondrial contamination in the cytosolic fraction and to verify equal protein loading in mitochondrial fraction. Representative Western blots are shown. Results are indicated as the ratio between Smac levels in mitochondria *versus* Smac levels in cytosol and are the mean ± S.E. of six independent experiments performed in duplicate. +, $p < 0.05$ *versus* controls (*CT*); *, $p < 0.05$ *versus* K5.

Statistical Analysis—Statistical significance was determined by one-way analysis of variance followed by Tukey's multiple comparison test. A value of $p < 0.05$ was accepted as denoting statistical significance.

RESULTS

NMDA and K25 Reduce the K5-mediated Loss of Mitochondrial Activity in Cerebellar Granule Cells—Previous results in our laboratory have indicated that a 24-h exposure to NMDA or K25 was able to exert a long lasting neuroprotective effect on immature CGCs (2 DIV) when cultured in K5 (22). Since it has been described that potassium deprivation of mature CGCs cultures induced a reduction in $\Delta\Psi$m (10, 15), we decided to explore the effect of NMDA or K25 on $\Delta\Psi$m in immature CGC cultures by using the JC-1 red/green ratio. CGCs cultured in K5 presented a 20% reduction in $\Delta\Psi$m *versus* control cells (cells grown in K25 from seeding). Treatment at 2 DIV with NMDA for 12 h reduced by 50% the loss of mitochondrial activity caused by K5. Moreover, treatment with K25 at 2 DIV for 12 h was able to prevent completely the K5-mediated reduction of $\Delta\Psi$m (Fig. 1).

NMDA and K25 Prevent the K5-mediated Induction of bax and Increase the Expression of bcl-2—Two members of the Bcl-2 family seem to have an important role in controlling the state of $\Delta\Psi$m and the decision to enter apoptosis: Bax, a proapoptotic member of the Bcl-2 family, and Bcl-2, an antiapoptotic member. These two Bcl-2 family members modulate apoptotic cell death through Bax homodimers (induction of apoptosis) or Bax-Bcl-2 heterodimers (antiapoptotic role). In this context, we wanted to explore whether the prevention of K5-mediated reduction of $\Delta\Psi$m and K5-mediated apoptosis by K25 and NMDA was correlated with changes in Bax and Bcl-2 levels. For this

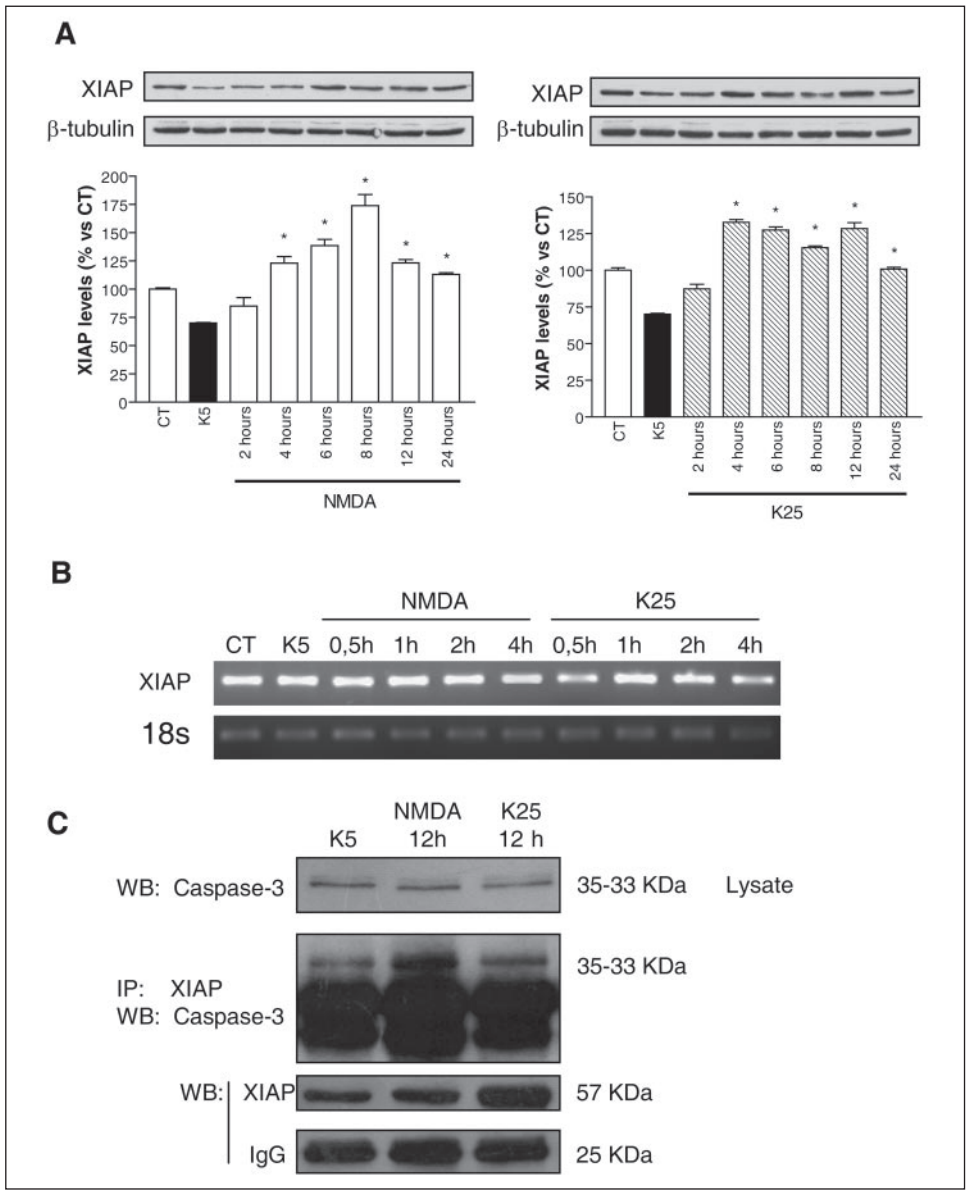

FIGURE 6. **NMDA and K25 increase the levels of XIAP protein and the binding of caspase-3 to XIAP.** Neurons were plated in K5 or K25 (control; *CT*). *A*, At 2 DIV, neurons grown in K5 were treated with NMDA (100 μM) or K25 from 2 to 24 h. Protein levels of XIAP and β-tubulin were determined by Western blotting and quantified by computer-assisted densitometry. Representative Western blots are shown. Results are indicated as the ratio between XIAP and β-tubulin levels. Data are shown as percentage *versus* controls and are the mean ± S.E. of four independent experiments performed in triplicate. *, $p < 0.05$ *versus* K5. *B*, cells cultured in K5 were treated with NMDA (100 μM) or K25 at 2 DIV from 30 min to 4 h, with the exception of controls. XIAP and 18 S mRNA were amplified by RT-PCR and analyzed in agarose gels as described under "Experimental Procedures." Data are representative of three independent experiments. *C*, At 2 DIV, neurons grown in K5 were treated for 12 h with NMDA (100 μM) or K25. Then cells were lysed, and the interaction between caspase-3 and XIAP was determined by immunoprecipitation (*IP*) and immunoblotting (*WB*) as indicated under "Experimental Procedures." The *lower panels* show the amount of XIAP and IgG present after the immunoprecipitation.

purpose, we studied both *bax* and *bcl-2* mRNA expression levels by RT-PCR and protein levels by Western blot in each condition.

CGCs cultured in K5 medium present a 2-fold increase in *bax* mRNA at 2 DIV when compared with control cultures (*CT*; cells grown in K25; Fig. 2*A*). By contrast, no differences were observed in *bcl-2* mRNA levels (Fig. 3*A*). Similar results were observed when protein levels were determined by Western blotting (Figs. 2*B* and 3*B*). The addition of NMDA at 2 DIV was able to block K5-mediated increase of *bax* mRNA levels in a time-dependent manner. A complete inhibition of K5-mediated *bax* mRNA level increase was observed until 4 h after NMDA treatment (Fig. 2*A*). No differences in *bax* mRNA levels with K5 cultures were observed after 6 h (Fig. 2*A* and data not show). The reduction in *bax* mRNA by NMDA was also accompanied by a reduction in Bax protein levels (Fig. 2*B*). Similarly, K25 was also able to block K5-mediated increase in *bax* mRNA and protein levels (Fig. 2*C*).

Next, we analyzed whether NMDA or K25 could change *bcl-2* mRNA and protein levels. Both NMDA and K25 were able to increase *bcl-2* mRNA levels (2.5- and 2-fold respectively) but only during the first 1 h of treatment (Fig. 3*A*). Accordingly, an increase in Bcl-2 protein levels by NMDA or K25 (92 and 55%, respectively, *versus* CGCs cultured in K5 for 1 h) was observed by Western blotting.

NMDA and K25 Block the K5-mediated Release of the Mitochondrial Apoptotic Factors Cytochrome c and Smac/DIABLO—Caspase activation during apoptosis is often controlled by the release of mitochondrial proapoptotic factors, such as cytochrome *c* or Smac/DIABLO. Since previous work in our laboratory (22) has shown that NMDA and K25 are able to reduce K5-mediated caspase-3 activation, we investigated whether this effect could be related to the modulation of cytochrome *c* or Smac/DIABLO release from mitochondria. For this purpose, we quantified the expression level of both proteins by Western blot in mitochondria and cytosolic fraction obtained from K5-, K25-, or NMDA-treated CGC cultures. The purity of the mitochondrial fractions was confirmed by reprobing the blots with an antibody against mitochondria-specific Hsp60 protein (Fig. 4*A*).

CGCs cultured in K5 for 2 DIV presented lower levels of cytochrome *c* in the mitochondria than in the cytosol. The observed mitochondria/cytosol ratio was around 0.5 in K5 cultures, which is 4 times lower that the observed ratio in control cultures (Fig. 4*A*). However, when NMDA

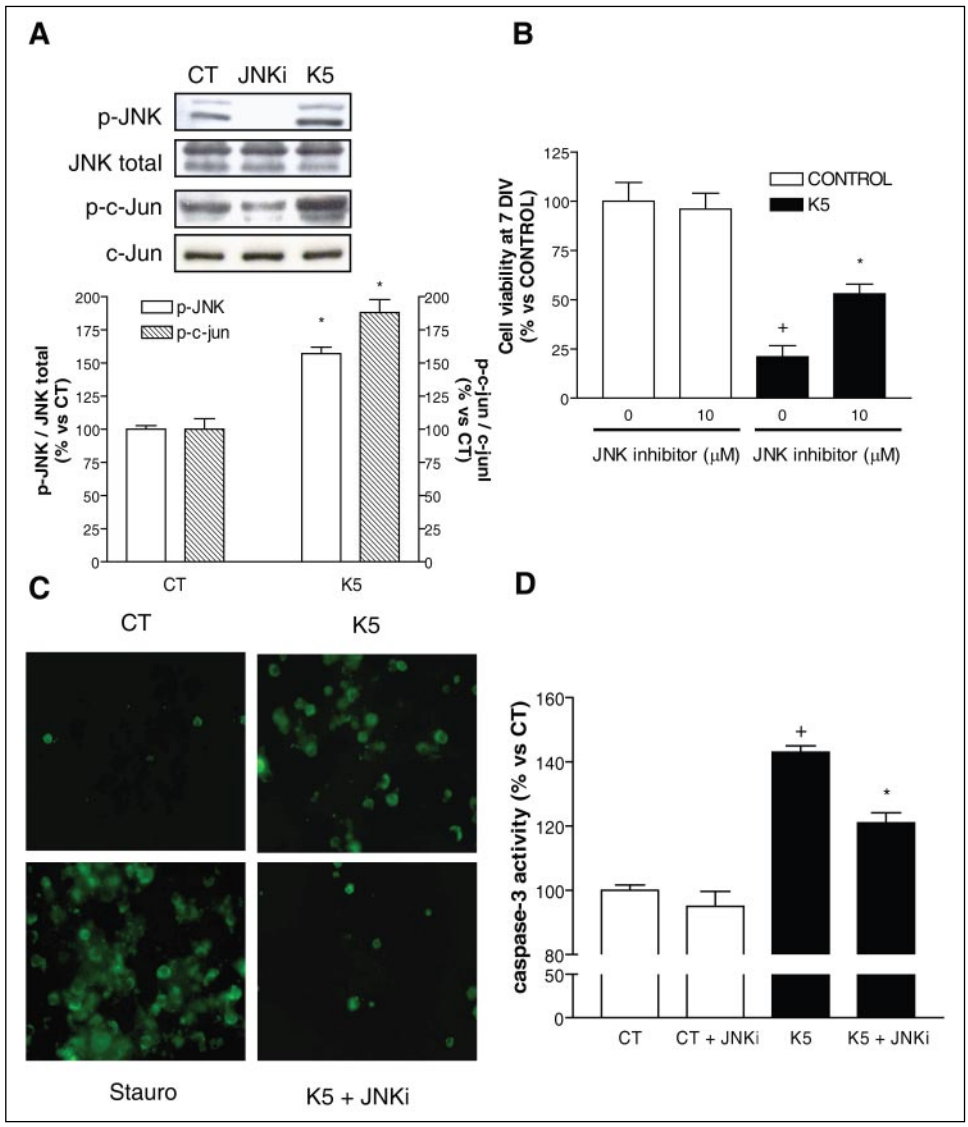

FIGURE 7. **The JNK pathway is activated in CGCs grown in K5 and plays a role in neuronal death induced by K5.** SP600125 (10 μM; *JNKi*) was added to cells cultured in K5 and K25 (Control; *CT*) at 2 DIV. *A*, cells grown in K5 showed higher phospho-JNK and phospho-c-Jun levels than cells grown in K25. The addition of SP600125 blocks K5-mediated phosphorylation of JNK and c-Jun. Representative Western blot of phospho-JNK, total JNK, phospho-c-Jun, and c-Jun was done 4 h after the addition of SP600125. *, $p < 0.05$ *versus* control cultures. *B*, SP600125 reduces neuronal death in K5 cultures. Cell viability was measured by a fluorescein diacetate assay at 7 DIV, as indicated under "Experimental Procedures." Data are shown as percentage *versus* controls and are the mean ± S.E. of values obtained in four independent experiments performed in triplicate. +, $p < 0.05$ *versus* control cultures; *, $p < 0.05$ *versus* K5 cultures. *C*, the addition of SP600125 prevents K5-mediated caspase-3 activation. Cleaved caspase-3 was determined by immunohistochemistry 12 h after the JNK inhibitor addition. *D*, caspase-3 activity was determined 12 h after the addition of SP600125 by a fluorometric assay using Ac-DEVD-7-amido-4-methylcoumarin as substrate. The values are given as percentage activity *versus* control and are the mean ± S.E. of three independent experiments performed in triplicate. +, $p < 0.05$ *versus* control cultures; *, $p < 0.05$ *versus* K5 cultures.

or K25 was added at 2 DIV, an inhibition of cytochrome *c* release from mitochondria was observed after 6 h. It is well known that cytosolic cytochrome *c* can interact with Apaf-1 (apoptosis protease-activating factor-1) and procaspase-9 to form a complex, called apoptosome, that activates caspase-9 (23). Accordingly, we decided to check whether the release of mitochondrial cytochrome *c* in K5 CGCs cultures was associated with caspase-9 activation. Surprisingly, neither the determination of caspase-9 activity nor the use of an antibody against the active fragment of caspase-9 indicated that caspase-9 was active in K5-cultured CGCs (Fig. 4*B*). No activation was also observed in NMDA- and K25-treated cultures. By contrast, staurosporine was able to activate caspase-9, as previously described (24).

On the other hand, we have also observed by Western blot the presence of the mitochondrial protein Smac/DIABLO in the cytosol of K5-cultured CGCs at 2 DIV. By contrast, no Smac/DIABLO was detected in the cytosol of control cultures (Fig. 5). Accordingly, the observed ratio between mitochondria and cytosol was 6.4 in control cultures and 1.1 in K5-cultured CGCs (Fig. 5). The presence of Smac/DIABLO in the cytosol of K5 cultures was abolished after 6 h of treatment with NMDA or K25 (Fig. 5 and data not shown). Incubation with NMDA or K25 for shorter times did not prevent the K5-mediated release of Smac/DIABLO (data not shown).

NMDA and K25 Increase the Levels of X-linked IAP and Its Interaction with Caspase-3—Cytosolic Smac/DIABLO promotes caspases activation by inhibiting the interaction between the caspases and the IAPs (24). Since NMDA blocks K5-mediated release of Smac/DIABLO, we wanted to explore the eventual role of XIAP function in NMDA-mediated inhibition of caspase-3 (22). CGCs cultured in K5 for 2 DIV, which we have shown that release Smac/DIABLO from mitochondria (Fig. 5), presented lower levels of XIAP than neurons grown in K25 (*CT*; Fig. 6*A*). The addition of NMDA at 2 DIV induced an increase in XIAP levels, which was already evident at 4 h (Fig. 6*A*). Maximal increase in XIAP levels was observed 8 h after the NMDA addition (250% *versus* K5). Similar results were observed in K25-treated cultures (Fig. 6*A*). This increase in XIAP protein levels was not due to an increase of XIAP mRNA (Fig. 6*B*).

The decrease in cytosolic Smac/DIABLO and the increase in XIAP levels observed in NMDA-treated CGCs cultures should increase the interaction between XIAP and caspase-3 and the subsequent inhibition of the protease. This possibility was tested by immunoprecipitation with XIAP and immunoblotting against caspase-3 12 h after the NMDA addition. We decided to choose this time, since in a previous report, we have described that NMDA blocked K5-mediated caspase-3 activation 12 h after its addition (22). As shown in Fig. 6*C*, we observed an increase

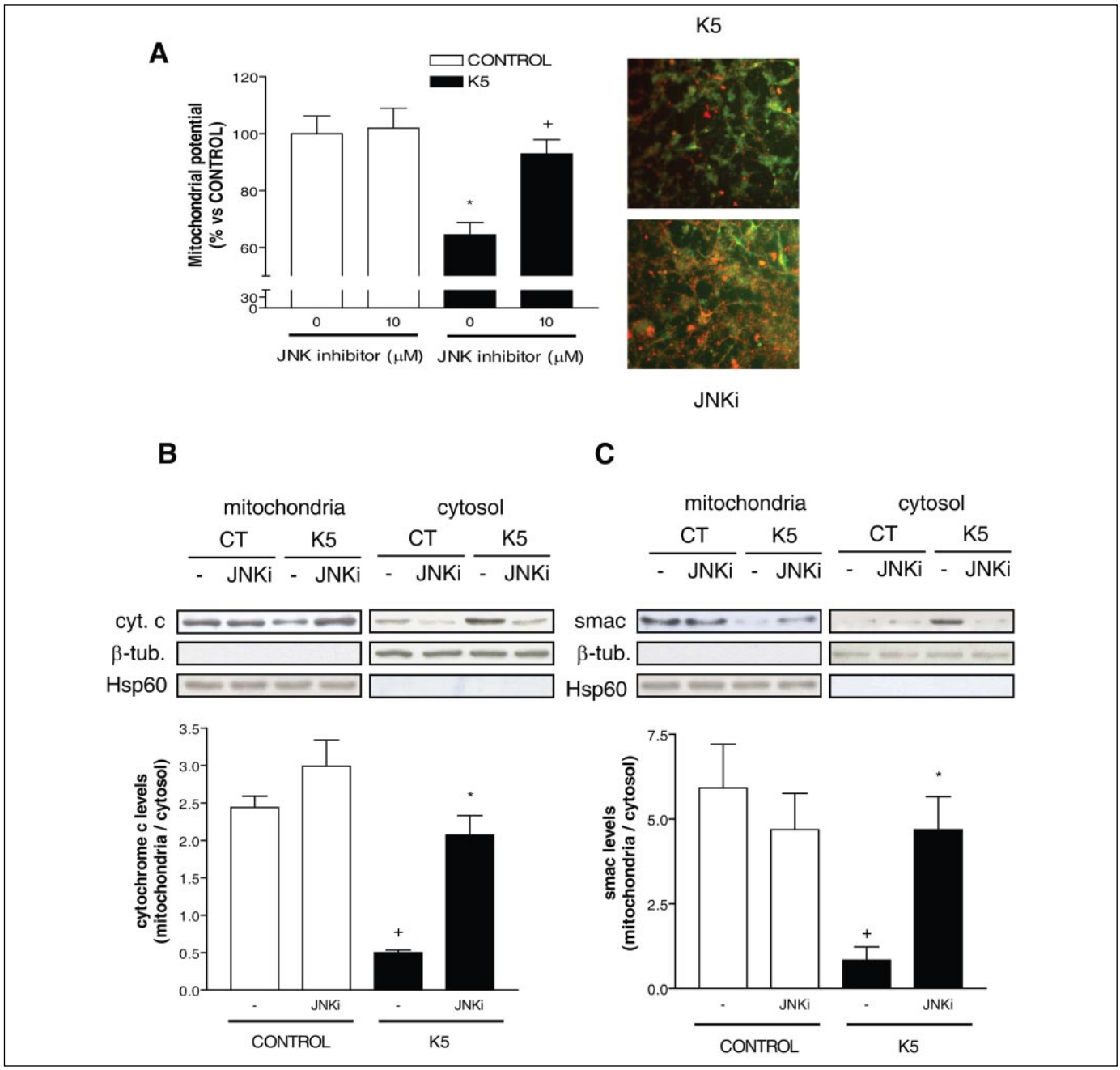

FIGURE 8. **JNK pathway seems to be involved in K5-mediated loss of mitochondrial transmembrane potential and the release of apoptogenic factors from mitochondria.** Neurons were plated in K5, with the exception of control (*CT*), where cells were plated in K25. After 2 DIV, SP600125 (10 μM) was added for 12 h. *A*, JNK inhibitor was able to block K5-mediated loss of mitochondrial transmembrane potential. Mitochondrial transmembrane potential was assessed by using JC-1 as indicated under "Experimental Procedures." Results are shown as percentage *versus* controls and represent the mean ± S.E. of three independent experiments performed in triplicate. Photomicrographs of representative fields from cultures grown in K5 with or without SP600125 are shown. *, $p < 0.05$ *versus* controls; +, $p < 0.05$ *versus* K5. *B* and *C*, JNK inhibition reduces cytochrome *c* and Smac/DIABLO (*smac*) release from mitochondria in cultures grown in K5. Subcellular fractionation was performed as indicated under "Experimental Procedures," and protein levels were determined by Western blotting. Representative Western blots of mitochondrial and cytosolic levels of cytochrome *c* (*B*), Smac (*C*), β-tubulin, and Hsp60 are shown. β-Tubulin levels were analyzed to confirm equal protein loading in cytosol. Hsp60 was used to monitor the absence of mitochondrial contamination in the cytosolic fraction and to verify equal protein loading in mitochondrial fraction. Protein levels were quantified by computer-assisted densitometry. Results are indicated as the ratio between cytochrome *c* levels in mitochondria *versus* cytochrome *c* levels in cytosol (*B*) and as the ratio between Smac levels in mitochondria *versus* Smac levels in cytosol (*C*). Values are the mean ± S.E. of five independent experiments performed in duplicate. +, $p < 0.05$ *versus* controls; *, $p < 0.05$ *versus* K5.

in the binding of caspase-3 to XIAP in NMDA-treated cultures, confirming that NMDA modulation of cytosolic Smac/DIABLO and XIAP levels is related to its inhibitory effect on caspase-3.

Inhibition of JNK Activity Reduces K5-mediated Apoptosis in Immature CGC Cultures—The activation of JNKs has been reported to be implicated in the process leading to potassium deprivation-dependent apoptosis of mature CGCs in culture (17). In this context, we wanted to address the relationship that exists between JNK activation and K5-mediated caspase-3 activation and apoptosis and whether the NMDA-mediated neuroprotection observed in immature CGCs cultures was associated with JNK activity modulation.

As expected, CGCs cultured in K5 medium showed an increase in the levels of phospho-JNK when compared with control cultures (50% increase after 2 DIV; Fig. 7*A*). As expected, the K5-mediated increase in

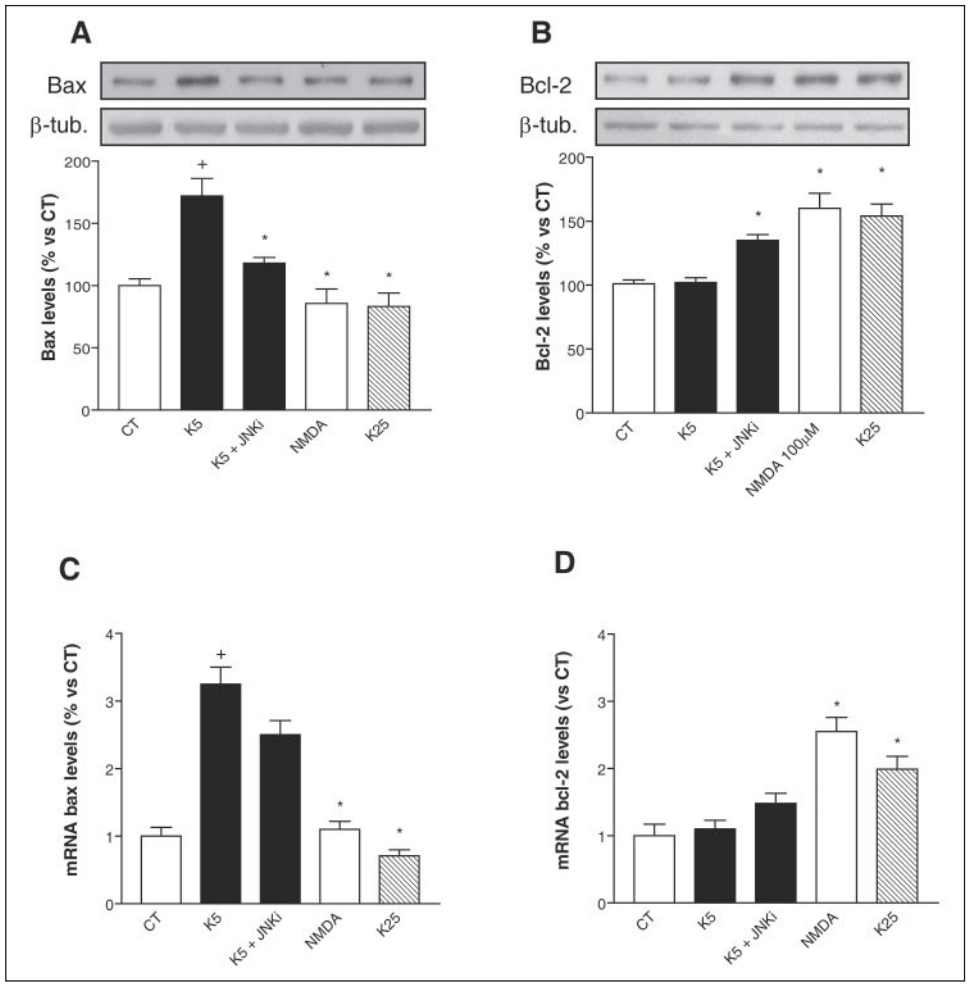

FIGURE 9. **JNK pathway modulates Bax and Bcl-2 protein levels in CGCs.** Neurons were plated in K5 or K25 (control; *CT*). At 2 DIV, neurons grown in K5 were treated with SP600125 (10 μM), NMDA (100 μM), or K25 for 1 (Bcl-2) or 2 h (Bax). *A* and *B*, the addition of JNK inhibitor prevents the increase in Bax protein by K5 and increases the Bcl-2 protein levels. Protein levels were quantified by Western blot after treatment. Representative Western blots of Bax and Bcl-2 are also shown. Data represent the ratio between Bax or Bcl-2 to β-tubulin levels. Values are given as percentage *versus* controls and are the mean ± S.E. of values obtained in five independent experiments performed in duplicate. *C* and *D*, mRNA expression levels of *bax* and *bcl-2* were unaffected in presence of the SP600125. Expression levels of *bax* mRNA and *bcl-2* mRNA were determined by RT-PCR. Data are expressed in arbitrary units *versus* controls and represent the mean ± S.E. of values obtained in four independent experiments. +, $p < 0.05$ *versus* controls; *, $p < 0.05$ *versus* K5.

phospho-JNK was correlated with an increase in the phosphorylation of the substrate c-Jun (88% *versus* control cultures; Fig. 7*A*). The addition of the SP600125 (10 μM) to 2 DIV CGCs cultures produced a dramatic inhibition of K5-mediated phosphorylation of JNK and c-Jun (Fig. 7*A*) and a 100% increase in cell viability of K5 cultures at 7 DIV (Fig. 7*B*). Moreover, the presence of SP600125 was able to dramatically reduce K5-mediated activation of caspase-3 as measured by immunocytochemistry with an antibody against cleaved caspase-3 (Fig. 7*C*). Similar results were obtained when caspase-3 activity was determined by an enzymatic assay using a specific caspase-3 substrate (Fig. 7*D*). The effect of the JNK inhibitor was dose-dependent, although concentrations above 50 μM were toxic (data not shown).

JNK Pathway Seems to Be Involved in K5-mediated Changes in Mitochondrial Activity—The decrease in cellular apoptosis observed in K5 cultures treated with SP600125 was accompanied with a recovery of $\Delta\Psi$m measured with the JC-1 probe. As indicated in Fig. 8*A*, the 40% decrease in the $\Delta\Psi$m observed in K5 cultures at 2 DIV was completely abolished in the presence of the SP600125. By contrast, the JNK inhibitor has no effect on $\Delta\Psi$m in control cultures.

Next, we studied whether the inhibition of JNK activity in K5 cultures was also correlated with changes in the release of the pro-apoptotic mitochondrial factors to the cytosol and in the expression of *bcl-2* or *bax*. As indicated above, K5 produces an increase in the cytosolic levels of cytochrome *c* and Smac/DIABLO at 2 DIV. By contrast, when the SP600125 was added to the cultures, the cytosolic levels of both protein factors were similar to those in control samples (Fig. 8*B*).

Inhibition of JNK Activity Mimics the Effect of NMDA and K25 on Bcl-2 and Bax Protein Levels in CGCs Cultured in K5—Our results suggest that the observed inhibition of K5-mediated apoptosis by NMDA is associated to an increase in Bcl-2 protein levels and an inhibition of K5-induced increase in Bax protein levels (Figs. 2 and 3). Since JNK inhibition produces similar effects to NMDA on cell viability, caspase-3 activity, release of proapoptotic factors, and $\Delta\Psi$m in CGCs cultured in K5, we explored whether this correlation was also observed when the Bax and Bcl-2 protein levels were monitored. As is shown in Fig. 9, the addition of SP600125 to K5 cultures was able to inhibit the K5-mediated increase in Bax protein levels. The SP600125 also produced an increase in Bcl-2 protein levels (35% over controls). These results were similar to those obtained with NMDA and K25 (see also Figs. 2 and 3). However, the observed changes in both protein levels were independent of changes in the expression of both genes, measured by quantitative RT-PCR (Fig. 9, *C* and *D*).

NMDA and K25 Are Able to Reduce the K5-mediated Phosphorylation of JNK—Our results suggested that similar mechanisms were involved in the antiapoptotic effect of NMDA or the SP600125 on immature CGCs cultured in K5. In this context, we wanted to assess if NMDA could block the K5-mediated increase in JNK activity (see Fig. 7*A*). When NMDA was added at 2 DIV to CGCs cultured in K5, a total inhibition of K5-mediated increase in p-JNK was observed at 2 and 4 h (Fig. 10*A*). Similar results were observed in CGCs cultures treated with K25 at 2 DIV (Fig. 10*B*). Inhibition of JNK phosphorylation by NMDA or K25 is accompanied by a decrease in phospho-c-Jun levels (Fig. 10, *C*

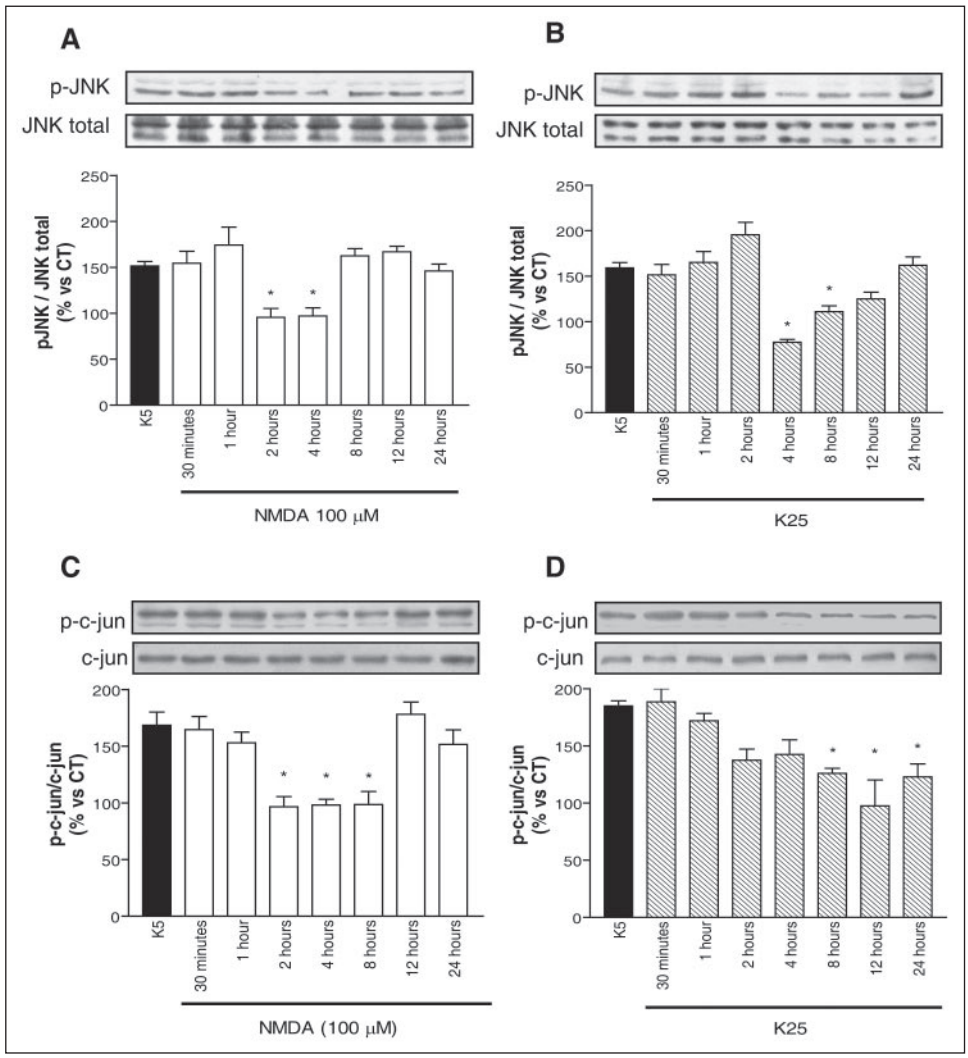

FIGURE 10. **NMDA and K25 reduce K5-mediated activation of JNK.** Cultured cells plated in K5 were exposed to NMDA (*A and C*; 100 μM) and K25 (*B* and *D*) at 2 DIV for 30 min to 24 h, and protein levels of phospho-JNK and JNK (*A* and *B*) and phospho-c-Jun and c-Jun (*C* and *D*) were analyzed by Western blot. Proteins were quantified by computer-assisted densitometry. Data represent the ratio of phospho-JNK to total JNK (*A* and *B*) and of phospho-c-Jun to c-Jun (*C* and *D*). Values are given as percentage *versus* controls (*CT*) and are the mean ± S.E. of values obtained in five independent experiments performed in duplicate. Representative Western blots of phospho-JNK (*p-JNK*), total JNK, phospho-c-Jun (*p-c-jun*), and c-Jun in cells treated with NMDA or K25 are also shown. *Asterisks* indicate significant difference from K5 (*, $p < 0.05$).

and *D*). These results support that JNK inhibition could be a crucial mechanism in NMDA and K25 neuroprotection against cellular apoptosis triggered by potassium deprivation of CGCs cultures. In accordance with that view, no additional neuroprotection was observed when SP600125 was added together with NMDA or K25 to CGCs cultured for 2 DIV in a K5-containing culture medium (data not shown).

DISCUSSION

It is widely known that the survival and differentiation of CGCs in culture require high concentrations of KCl (K25) or the presence of glutamatergic agonists such as NMDA (3, 4). It is believed that the *in vitro* effect of NMDA on CGCs cultures mimics the glutamatergic innervation that differentiating CGCs receive *in vivo* from mossy fibers (1, 2, 26). In the absence of depolarization, CGCs will die by apoptosis both *in vivo* and *in vitro* (2, 27). At present, it is poorly known which mechanisms are involved in the neuroprotective effect of NMDA or K25. Several evidences have shown that calcium influx is necessary for the antiapoptotic effect of depolarizing agents (3, 28, 29), and we have recently shown that the long lasting neuroprotective effect of NMDA on immature CGC cultures is due to an inhibition of K5-mediated caspase-3 activation (22).

Activation of caspase-3 and the apoptotic death induced by deprivation of depolarizing conditions in cultured CGCs are associated with a release of mitochondrial cytochrome *c* into the cytosol (10, 11, 15). It has been widely described, in several cell types, that cytosolic cytochrome *c* could bind to *apaf-1* and activate procaspase-9 in an ATP-dependent reaction (23). Caspase-9, in turn, activates caspase-3. Our results confirmed the release of cytochrome *c* into the cytosol in immature CGCs cultured in K5. However, the presence of cytochrome *c* in the cytosol is not accompanied by an activation of caspase-9. This is in contradiction to previous observations (11, 30) showing an activation of caspase-9 in mature CGCs cultured in K5. Thus, our results indicate that the observed activation of caspase-3 in KCl-deprived immature CGCs (22) cultures is not triggered by caspase-9 activation. What could then be the mechanism involved in K5-mediated activation of caspase-3 in immature CGC cultures? Here, we shown the release of Smac/DIABLO, another apoptogenic protein, from mitochondria to cytosol in K5-cultured immature CGCs. The presence of Smac/DIABLO in the cytosol has been described to promote caspase-3 activation by blocking the inhibitors of apoptosis proteins (IAPs) (8, 9). We believe that the release of mitochondrial Smac/DIABLO promotes caspase-3 activation by blocking the anti-caspase activity of IAPs. Furthermore, our results indicate that NMDA and K25 treatment are able to significantly reduce the release of mitochondrial apoptogenic factors. Moreover, an increase in XIAP levels was observed in NMDA- and K25-treated cultures. Accordingly, the blockade of Smac/DIABLO release together with an increase in XIAP levels could be a mechanism involved in the inhibition of caspase-3 and the antiapoptotic effect of NMDA and K25 in developing

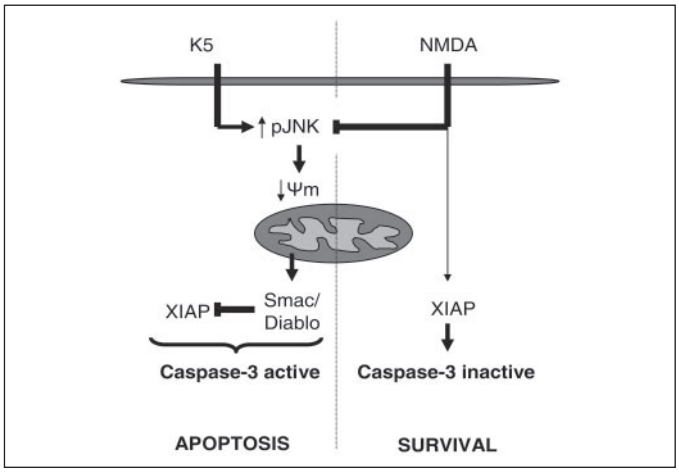

FIGURE 11. **Schematic representation of the mechanisms involved in NMDA-mediated neuroprotection of K5-mediated CGC apoptosis.** The activation of the JNK pathway observed in K5-cultured CGCs is involved in the release of Smac/DIABLO from mitochondria. Cytosolic Smac/DIABLO could then bind to XIAP and favor the activation of caspase-3. NMDA blocks the release of Smac/DIABLO by blocking K5-mediated JNK pathway activation. This blockage is accompanied by an increase of XIAP levels. Altogether, this will increase the binding of caspase-3 to XIAP, leading to the inhibition of K5-mediated caspase-3 activation.

CGCs (22). Supporting this possibility, we have observed that NMDA and K25 elicit an increase in the amount of caspase-3 bound to XIAP. On the other hand, the physiological relevance of the observed inhibition of cytochrome *c* release by NMDA and K25 is not clear, since no activation of caspase-9 is observed in K5-mediated apoptosis of immature CGCs.

Release of apoptogenic factors from mitochondria to cytosol is associated with a loss of $\Delta\Psi$m (31). Accordingly, our results have confirmed previous observations (15) showing that $\Delta\Psi$m is also reduced in K5-cultured CGCs. However, whether the release of mitochondrial factors to the cytosol is the cause or a consequence of the decrease in $\Delta\Psi$m remains to be elucidated. For example, several studies have shown that a loss in $\Delta\Psi$m precedes cytochrome *c* release (32, 33), whereas other studies have shown that cytochrome *c* release occurs prior to mitochondrial membrane potential loss (34). The present data support the latter view, since in the neuroprotective effect of NMDA (and also K25), the blockade of the release of Smac/DIABLO and cytochrome *c* is observed before the recovery of the mitochondrial membrane potential.

Several routes have been proposed to explain the release to the cytosol of mitochondrial apoptogenic factors during apoptosis. It has been reported that Bcl-2 family proteins can form mitochondrial channels that are large enough to allow the release of apoptogenic factors (35–38). By contrast, other reports suggested that the release of these mitochondrial factors might occur by a Bcl-2 family protein regulation of the mitochondrial permeability transition pore (39, 40). Besides the actual mechanism involved, it seems clear that Bcl-2 family proteins have an important role in modulating the release of apoptogenic factors from mitochondria to cytosol and cellular apoptosis (13). However, few studies have addressed the eventual role of the Bcl-2 family proteins in the apoptotic death of CGCs induced by KCl deprivation and whether the neuroprotective effect of NMDA or K25 is associated with their regulation. Some evidence suggests that Bax (a proapoptotic member of the Bcl-2 family) is involved in apoptotic death of CGCs by KCl deprivation. For example, Bax-deficient mature CGCs are resistant to K5-mediated apoptosis (14, 41). On the other hand, overexpression of Bcl-2 (an antiapoptotic member of the Bcl-2 family) protects CGCs from apoptosis (42). Accordingly, a decrease in protein levels of Bcl-2 has been reported in CGCs cultured in K5 (15). In this context, we have observed a substantial increase in mRNA and protein levels of Bax in K5-cultured immature CGCs, whereas no changes in *bcl-2* mRNA and protein levels were observed. However, when NMDA or K25 was added to immature CGCs cultures, *bcl-2* gene expression increased, whereas *bax* gene expression decreased. These changes in mRNA levels preceded the significant increase in Bcl-2 protein levels and a parallel decrease in Bax protein levels. Thus, neuroprotection by NMDA and K25 is associated with an increase in Bcl-2 and a decrease in Bax. Moreover, these changes occurred before the inhibition of the release to the cytosol of apoptogenic factors, such as Smac/DIABLO or cytochrome *c*, and the recovery of mitochondrial membrane potential.

At present, it is largely unknown which mechanisms are involved in the modulation by NMDA and K25 of the levels of Bcl-2 and Bax. There has been much evidence reporting that the JNK pathway controls the activity and expression of several members of the Bcl-2 family of proteins and the intrinsic apoptotic pathway involving the mitochondria (41, 43–45). On the other hand, several reports have suggested that the JNK pathway has an important role in K5-mediated apoptosis of CGCs. KCl deprivation produces an increase in mRNA and protein levels of c-Jun together with its phosphorylation (17, 19, 46). Surprisingly, it was also reported that JNK activity did not increase when cultured CGCs were deprived of KCl (17). By contrast, another report has suggested that KCl deprivation mobilized a specific stress-activated pool that appears to have preferential access to c-Jun (47, 48). In the present study, we have clearly shown that KCl deprivation produces an increase in JNK and c-Jun phosphorylation, and we have demonstrated that this activation of JNK signaling is triggering the up-regulation of Bax, the down-regulation of Bcl-2, the loss of mitochondrial membrane potential, the release of Smac/DIABLO and cytochrome *c* from mitochondria, and the activation of caspase-3 in K5-cultured CGCs. Moreover, pharmacological inhibition of JNK activity showed the same effects on these parameters as the addition of NMDA or K25 to CGCs cultures. Interestingly, inhibitors of JNK signaling were able to produce a long term protection of CGCs from K5-induced apoptosis, similar to the neuroprotective effect observed with NMDA (22). The possibility that neuroprotection by NMDA could be due to the inhibition of JNK signaling is supported by our results showing that NMDA (and also K25) blocks K5-mediated JNK and c-Jun phosphorylation.

In summary, we postulate that NMDA protection from KCl deprivation-induced CGC apoptotic death is mainly due to the inhibition of JNK activity that will block the release of apoptogenic proteins factors from the mitochondria (such as Smac/DIABLO). An NMDA-mediated increase in XIAP levels will also favor caspase-3 inhibition (Fig. 11).

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