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Ras Protein Activation Is a Key Event in Activity-dependent Survival of Cerebellar Granule Neurons*

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Background: Contradictory results exist on the survival role of PI3K/Akt and ERK pathways in cerebellar granule neurons.
Results: Both pathways are involved in activity-dependent survival of cerebellar granule cells when they are activated by Ras.
Conclusion: Ras is a central mediator of survival in cerebellar granule neurons.
Significance: The biological significance of PI3K/Akt and ERK pathway activation depends on which monomeric-G-protein is acting upstream.

Neuronal activity promotes the survival of cerebellar granule neurons (CGNs) during the postnatal development of cerebellum. CGNs that fail to receive excitatory inputs will die by apoptosis. This process could be mimicked in culture by exposing CGNs to either a physiological concentration of KCl (5 mM or K5) plus *N*-methyl-D-aspartate (NMDA) or to 25 mM KCl (K25). We have previously described that a 24-h exposure to NMDA (100 μM) or K25 at 2 days *in vitro* induced long term survival of CGNs in K5 conditions. Here we have studied the molecular mechanisms activated at 2 days *in vitro* in these conditions. First we showed that NMDA or K25 addition promoted a rapid stimulation of PI3K and a biphasic phosphorylation on Ser-473 of Akt, a PI3K substrate. Interestingly, we demonstrated that only the first wave of Akt phosphorylation is necessary for the NMDA- and K25-mediated survival. Additionally, we detected that both NMDA and K25 increased ERK activity with a similar time-course. Moreover, our results showed that NMDA-mediated activation of the small G-protein Ras is necessary for PI3K/Akt pathway activation, whereas Rap1 was involved in NMDA phosphorylation of ERK. On the other hand, Ras, but not Rap1, mediates K25 activation of PI3K/Akt and MEK/ERK pathways. Because neuroprotection by NMDA or K25 is mediated by Ras (and not by Rap1) activation, we propose that Ras stimulation is a crucial event in NMDA- and K25-mediated survival of CGNs through the activation of PI3K/Akt and MEK/ERK pathways.

During the development of the nervous system, apoptosis regulates both the number and connections of neuronal populations. Neuronal activity induced by extracellular stimuli has been revealed as a promoter of neuronal survival during brain development. This has been observed in several brain areas, such as thalamus and hippocampus (1–3). Interestingly, in the cerebellum NMDA[2] receptor stimulation is necessary to induce the survival and differentiation of cerebellar granule neurons (CGNs) (4). This process was described in cultured CGNs, where cells grown in physiological potassium concentrations (5 mM; K5) died by apoptosis (5), whereas CGNs grown in the presence of moderate doses of NMDA (4) or high potassium concentrations (25 mM; K25) survived. The addition of NMDA to immature CGN cultures blocks K5-mediated apoptosis, and it is believed to mimic the excitatory inputs required for the CGNs survival *in vivo* (6, 7). Altogether, NMDA- and K25-stimulated CGNs have been extensively used to study survival and apoptotic molecular signaling.

Two pathways appear to be mainly implicated in preventing the apoptotic death of several neuronal populations, including CGNs: (*a*) the phosphatidylinositol 3-kinase (PI3K)/serine-threonine kinase Akt pathway and (*b*) the extracellular signal-regulated kinase (ERK) pathway. The activation of the PI3K/Akt pathway seems to play a critical role in the neuroprotective effect of IGF-1 in CGNs (8), whereas ERK pathway activation has been reported to be a key factor in CGNs neuroprotection by BDNF or pituitary adenylate cyclase-activating peptide (9, 10). Concerning the neuroprotective effect of NMDA and KCl depolarization from apoptosis triggered by potassium deprivation, the role of these two pathways has not been well established due to contradictory results published in mature CGNs cultures. Some studies have reported the implication of the PI3K/Akt pathway in the neuroprotective effect of NMDA or K25 (11–15). However, opposite conclusions have been also reported in other studies (15–17). Regarding the role of the ERK pathway in NMDA-mediated survival, Lafon-Cazal *et al.* (15) have shown that ERK inhibition reduces the neuroprotection by NMDA exposure. On the other hand, certain studies have reported that inhibition of ERK pathway does not prevent the neuroprotective action of NMDA (12, 13). Similarly, contradic-

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[2] The abbreviations used are: NMDA, *N*-methyl-D-aspartate; CGN, cerebellar granule neuron; K5, 5 mM KCl; K25, 25 mM KCl; DIV, day(s) *in vitro*; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; CT, control; CREB, cAMP-response element-binding protein.

tory results were reported in the eventual implication of the ERK pathway in K25-mediated neuroprotection (11, 15, 18). Thus, the role of these pathways in NMDA- or K25-mediated neuroprotection in CGNs cultures is not clear yet. It seems that there are precise mechanisms regulating PI3K/Akt and ERK pathways that determine its pro-survival role in the neuroprotection mediated by NMDA receptor stimulation or KCl depolarization.

The small G proteins, Ras and Rap1, have been revealed to be crucial for the activation and regulation of PI3K/Akt and ERK pathways. Both are activated by specific guanine nucleotide exchangers that transform GDP to GTP. In PC12 cells, several extracellular stimuli, such as neurotrophins or neuropeptides, activated Ras and Rap1 (19–23). Few data exist about the activation of these proteins by NMDA or KCl stimulation. Iida *et al.* (24) show in hippocampal neurons that Ras activity is necessary for NMDA- or K25-dependent transduction signaling. In mature CGNs, both Ras and Rap1 are required to activate ERK pathway through KCl stimulation (25). However, the role of Ras and Rap1 in NMDA- and KCl-mediated survival has not been addressed yet.

We have previously described that a brief exposition to NMDA or K25 in CGNs at 2 days *in vitro* (DIV) produces a long term protection from K5-mediated apoptosis in a process that blocks K5-mediated caspase-3 activation (26). NMDA long term neuroprotective effect was blocked by PI3K inhibitors, whereas PI3K and ERK inhibitors were able to block K25-mediated neuroprotection (26). In the present study we explore the role of Ras and Rap1 in the long term NMDA and K25-mediated activation of PI3K and ERK and in the neuroprotection from K5-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Chemicals—L-Glutamine and penicillin/streptomycin were from PAN Biotech Inc. (Aidenbach, Germany). FluorSave™ Reagent was from Calbiochem. The ECL™ Western blotting detection reagent and Hybond-C Extra Nitrocellulose membranes were purchased from Amersham Biosciences. All other chemicals were obtained from Sigma.

Cell Culture and Pharmacological Treatment—Granule neurons were obtained from dissociated cerebella of 8-day-old rats as previously described by Balázs *et al.* (4). Cells were plated (8×10^5 cells/cm^2) on culture dishes coated with poly-L-lysine in Basal Medium Eagle (Sigma) containing K5 or K25 potassium (KCl) supplemented with 10% heat inactivated fetal bovine serum (Sigma), 0.6% of 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 proliferation of non-neuronal cells. At 2 DIV, we added 100 μM NMDA (Sigma) or 20 mM KCl. In some experiments specific inhibitor of PI3K (wortmannin 0.5 μM; Sigma) or specific inhibitor of MEK (PD 98059 10 μM; Sigma) was added 30 min before the addition of NMDA or KCl (unless otherwise mentioned). The addition of 50 ng/ml BDNF at 2 DIV was used as a positive control of PI3K/Akt and ERK pathways activation. The procedures followed were in accordance with the guidelines of the "Comissió d'Etica en "Experimentació Animal i Humana" from the "Universitat Autònoma de Barcelona."

Cell Viability—Neuronal viability was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma) assay. Cells were incubated with MTT (0.2 mg/ml) for 30 min at 37 °C. The blue formazan derivative was solubilized in 500 μl of dimethyl sulfoxide (DMSO), and dual wavelengths were measured at 560 and 620 nm in a Labsystems Multiskan plate reader. Data shown are the mean ± S.E. of values obtained in four independent experiments performed with triplicates.

Immunoblotting—K5-cultured cells were exposed at 2 DIV to NMDA (100 μM) and K25 from 5 min to 24 h (unless otherwise mentioned) with the exception of CT cultures, where cells were grown in K25. Then 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. Western blot was performed as described elsewhere (27). Data are displayed in arbitrary units and was expressed as the mean ± S.E. of values obtained in 3 or 4 independent experiments performed in duplicate. The following primary antibodies were used: anti-phospho-Akt (Thr-308), anti-phospho-Akt (Ser-473), anti-phospho-p44/42 map kinase (Thr-202/Tyr-204), anti-phospho-GSK-3-β (Ser-9), anti-phospho-p90Rsk (Ser-380), anti-Akt and anti-p44/42 MAP Kinase antibodies (all 1:1000, Cell Signaling Technology, Beverly, MA), and anti-Ras and anti-Rap1 (all 1:500, Santa Cruz Biotechnology, Santa Cruz, CA). Anti-rabbit and anti-mouse IgG (all 1:2000) were from BD Biosciences and were used as secondary antibodies.

Caspase-3 Activity—Caspase-3 activity was assessed at 2 DIV 12 h after the addition of NMDA or K25. Cultured CGNs were washed with PBS at 37 °C, and cells were harvested with PBS and pelleted by centrifugation (350 × *g*) at 4 °C. The cellular pellet was resuspended, and the lysates were incubated with the pseudo-substrate Z-DEVD-R110 according to the protocol recommended by the manufacturers (Promega, Madison, WI). Fluorescence intensity was measured with the Bio-Tek FL600 fluorescence microplate reader. Data are given as the mean ± S.E. of values obtained in three independent experiments performed in triplicate.

PI3K Activity—PI3K activity was determined as previously described (28). CGNs cultured in K5 were stimulated for 5 min with NMDA (100 μM) or K25, with the exception of CT cultured in K25 and then lysed in 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 20 mg/ml leupeptin. Nuclei and debris were eliminated by centrifugation. Lysates (1.5 mg of protein) were subjected to immunoprecipitation with the anti-phosphotyrosine monoclonal antibody (clone 4G10, Upstate Biotechnology, Lake Placid, NY) at 4 °C overnight. Immunocomplexes were collected with protein A-Sepharose (GE Healthcare) preconjugated with a rabbit anti-mouse IgG antibody and sequentially washed with lysis buffer, LiCl buffer (100 mM Tris-HCl, pH 7.5, 0.5 M LiCl, 1 mM EDTA, 1 mM sodium orthovanadate), and TNE buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) and then incubated with a mixture of L-α-phosphatidylinositol and L-α-phosphatidylserine (15 μg of each lipid) and 10 μCi of [γ-^{32}P]ATP.

Phosphorylated lipids were extracted and resolved by thin layer chromatography using *n*-propanol:H_2O:acetic acid (66:33:2; vol:vol:vol) as solvent. Radioactive spots were detected by exposing the plate to Kodak BIOMAX MR film.

Endogenous Ras and Rap1 GTP Loading—Ras and Rap1 GTP loading were measured by a nonradioactive method as described previously (25, 29). Briefly, after stimulation, cells were rinsed in ice-cold phosphate-buffered saline at pH 7.4 and solubilized at 4 °C for 10 min in lysis buffer (10% glycerol, 1% Nonidet P-40, 50 mM Tris, pH 7.4, 200 mM NaCl, 2.5 mM $MgCl_2$, 250 μM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 0.1 μM aprotinin, 10 mM NaF, and 1 mM Na_3VO_4). Lysates were clarified by centrifugation at 10,000 × *g* for 10 min, and supernatants were incubated with glutathione-Sepharose beads (GE Healthcare) freshly coupled to GST-Ral-GDS-RBD to isolate Rap1-GTP or to GST-Raf1-RBD to isolate Ras-GTP for 1 h at 4 °C. Precipitated protein complexes were washed three times with lysis buffer. Finally, precipitates were resuspended, and Western blotting was performed as described above.

Adenovirus Infections—AAA-Akt (S473A/T308A/K179A) adenovirus was kindly provided by Dr. R. V. Farese (University of South Florida College of Medicine, Tampa, FL), and Laz-Z adenovirus was kindly provided by Dr. Miguel Chillón (Biochemistry and Biology Molecular Department, Autonomous University of Barcelona). CGNs cultured in K5 or K25 (CT were infected at 1 DIV with different multiplicities of infection of LacZ or AAA-Akt adenoviruses. At 2 DIV, the levels of phospho-Akt in serine 473 were analyzed. To study caspase-3 activity, K5 or K25 cultured CGNs were infected at 1 DIV with a 10 multiplicity of infection of AAA-Akt or LacZ. At 2 DIV, NMDA or K25 was added in infected or not infected CGNs and 12 h after caspase-3 activity was determined in CT cells, in K5-cultured cells, and in NMDA- and K25-treated infected neurons.

Lentiviral Construct Expression and Infection—The cDNAs for Ras-N17, Ras-V12, Rap1A-N17, and Rap1-V12 were kindly provided by Dr. Pérez (Institut de Recerca de l'Hospital de la Pau i de la Santa Creu; Barcelona) and were verified by DNA sequencing. Lentivirus was generated as described previously (30) using HEK 293T cells, and viral titers were determined by measuring the levels of GFP by immunoblotting. The infection of Ras-N17 and Rap1A-N17 was performed at 1 DIV in CGNs cultured in K5 for 10 h, and then medium was replaced by the plated medium. At 2 DIV, CGNs were treated or not with NMDA (100 μM) and K25 for 30 min; subsequently CGNs were harvested and subjected to immunoblotting. The cell viability study was performed at 7 DIV. Infections of Ras-V12 and Rap1-V12 were performed at 1 DIV in CGNs cultured in K5 for 10 h, and then the medium was replaced by the plated medium. In some cases, Ras-V12-infected CGNs were co-infected with AAA-Akt or were treated at 2 DIV with wortmannin (0.5 μM). Then the cell viability was performed at 7 DIV. Infection efficiency found an average of 80%, determined by the number of GFP-positive neurons.

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 Activate PI3K/Akt Pathway in Immature Cerebellar Granule Neurons Grown in K5—Previous results in our laboratory have indicated that a 24-h exposure to NMDA or K25 was able to induce a long-lasting neuroprotective effect in immature CGNs (2 DIV) when cultured in K5. Pharmacological inhibition of PI3K blocked NMDA- and K25-mediated neuroprotection and K5-mediated activation of caspase-3 (26). We decided to examine in immature CGNs whether NMDA and K25 were able to activate the PI3K/Akt pathway. First, we analyzed the activity of PI3K. Treatment with NMDA (100 μM) or K25 at 2 DIV induced the activation of PI3K in comparison with neurons plated in K5 (Fig. 1*A*). Then, we studied Akt phosphorylation in threonine 308 and serine 473. At 2 DIV, we observed a 36% of decrease of Ser-473-Akt (Fig. 1, *B* and *C*) in neurons plated in K5 respect to control neurons (CGNs plated in K25). As expected, K5-mediated Ser-473-Akt dephosphorylation correlated with a reduction in Ser9-GSK-3β phosphorylation (Fig. 1*B*), a well known substrate of Akt, indicating the inactivation of PI3K/Akt pathway. The addition of NMDA (100 μM) or K25 at 2 DIV promoted biphasic Ser-473-Akt phosphorylation (Fig. 1, *B* and *C*). The biphasic increase was observed from 15 min until 1 h and after 12 h of NMDA stimulation. In cultures switched from K5 to K25 at 2 DIV, the first increase in phosphorylation was observed 30 min to 1 h after K25, and the second increase was observed at 24 h of K25 stimulation (Fig. 1, *B* and *C*). Both Ser-473-Akt phosphorylation by NMDA and K25 are accompanied by an induction of Ser9-GSK-3β phosphorylation (Fig. 1*B*), indicating the activation of PI3K/Akt pathway. No changes were observed in Thr-308-Akt phosphorylation in any condition (Fig. 1, *B* and *C*).

Only Early Phosphorylation of Akt Seems to Be Involved in the Neuroprotection by NMDA and K25—Next, we wanted to explore whether the early (around 30 min) and late (between 12–24 h) phases of Akt phosphorylation were needed for NMDA- or K25-mediated neuronal survival. For this purpose we added at 2 DIV wortmannin, an inhibitor of PI3K, 30 min previous to NMDA or K25 stimulation to block both phases of Akt phosphorylation or 10 h after NMDA or K25 to block only the latest phase of Akt phosphorylation (Fig. 2, *C* and *D*). Cell viability was then monitored at 7 DIV. Culture medium was replaced by K5-conditioned medium from sister cultures at 3 DIV to limit to 24 h the presence of NMDA or K25. The addition of wortmannin 30 min before NMDA treatment at 2 DIV totally blocked the NMDA neuroprotection from K5-mediated apoptosis (Fig. 2*A*). By contrast, no differences were observed in cell viability when wortmannin was added 10 h after NMDA at 2 DIV (Fig. 2*A*). Similar results were observed with K25-mediated neuroprotection (Fig. 2*B*). To confirm the implication of the PI3K/Akt pathway in the NMDA- and K25-mediated survival, we infected CGNs with AAA-Akt (S473A/T308A/K179A) adenoviruses, an adenoviral-delivered dominant negative form of Akt (Fig. 2*F*), and we analyzed caspase-3 activity at 7 DIV. The infection with AAA-Akt (S473A/T308A/K179A) adenoviruses completely abolished the inhibition by NMDA or K25 of K5-mediated caspase-3 activity (Fig. 2*E*).

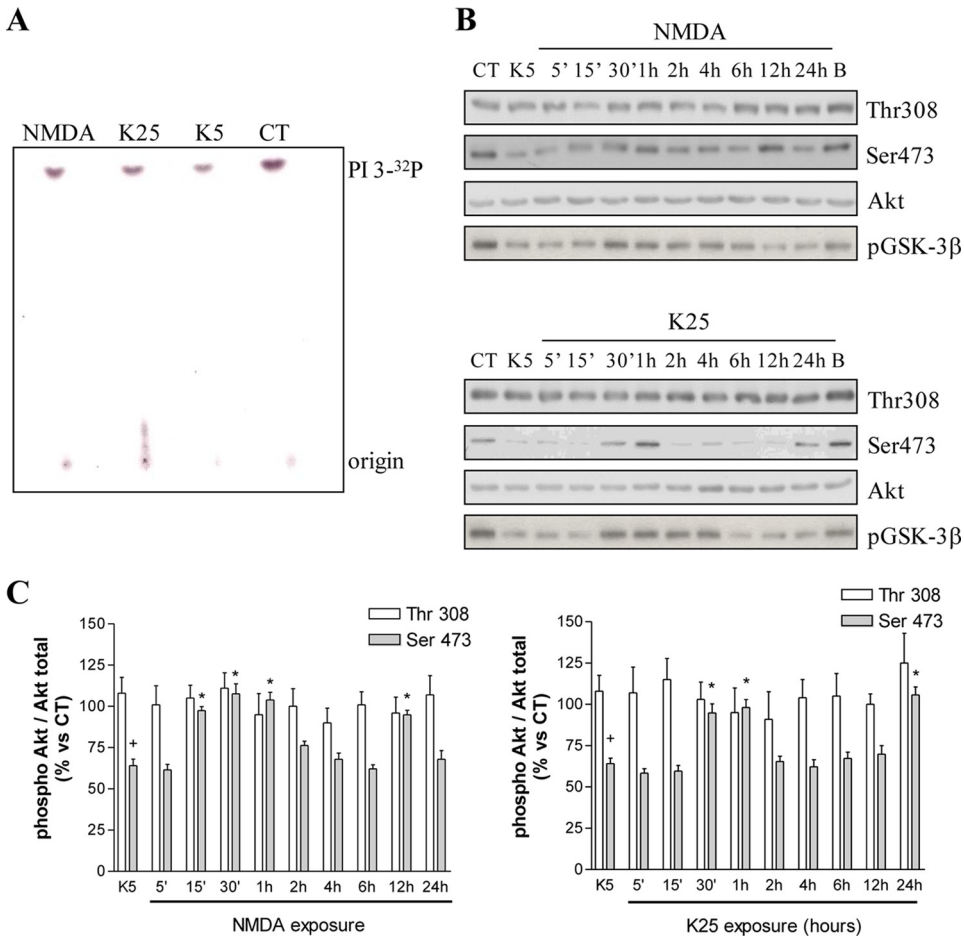

FIGURE 1. **NMDA and K25 induce a biphasic activation of PI3K/Akt pathway in immature CGNs.** Cultured cells grown in K5 were exposed at 2 DIV to NMDA (100 μM) or high potassium concentration of KCl (*K25*). CTs were plated in K25. *A*, PI3K activity was assessed 5 min after the addition of NMDA or K25 in K5-cultured CGNs as described under "Experimental Procedures." *B*, K5-cultured cells were exposed to NMDA or K25 for indicating times. The addition of BDNF (50 ng/ml; *B*) for 5 min was used as a positive control of Akt phosphorylation. Representative Western blots of phospho-Akt (Thr-308 and Ser-473), Akt, and phospho-GSK3β (Ser-9) protein levels are shown. *C*, levels of phospho-Akt (Thr-308 and Ser-473) and Akt were quantified by computer-assisted densitometry. Results were expressed as the ratio between phospho-Akt and Akt levels. Values are indicated in arbitrary units *versus* CT and are the mean ± S.E. of four independent experiments performed in duplicate. *, $p < 0.05$ *versus* K5; +, $p < 0.05$ *versus* CT. *PI*, phosphatidylinositol.

NMDA and K25 Promote ERK Pathway Activation in Immature Cerebellar Granule Neurons Grown in K5—We had shown that administration of a MEK inhibitor was able to block the K25-mediated neuroprotection, but no effect was observed on NMDA-mediated CGNs survival (26). To know whether these results were related to a differential activation of MEK/ERK pathway by NMDA or K25, we decided to study the phosphorylation of ERK during the first 24 h after NMDA and K25 exposure at 2 DIV. Surprisingly, the addition of NMDA at 2 DIV was also able to revert the reduction in ERK phosphorylation observed in K5 cultures *versus* control (50% of increase; Fig. 3*A*). NMDA reverted ERK phosphorylation levels from 5 min to 2 h after NMDA addition (Fig. 3*A*). The switch from K5 to K25 at 2 DIV was able to block the K5 reduction of ERK phosphorylation from 5 min until 2 h (Fig. 3*B*). The phosphorylation of ERK by NMDA or K25 was accompanied by an increase in the phosphorylation of Rsk, an ERK substrate, indicating the activation of MEK/ERK pathway (Fig. 3, *A* and *B*).

Differential Activation of Ras and Rap1 Small G Proteins by NMDA and K25 in Immature Cerebellar Granule Neurons—Ras and Rap1 small G proteins are involved in the activation of PI3K/Akt and MEK/ERK pathways (31). To know the eventual role of Ras and Rap1 in the NMDA- and K25-mediated long term survival effect, we studied the activation of Ras and Rap1 after NMDA treatment or K25 exposure at 2 DIV. The addition of NMDA and K25 at 2 DIV in K5-cultured CGNs induced an increase on Ras-GTP but with different kinetics. Although NMDA promoted an enhancement of Ras-GTP at 15 and 30 min (Fig. 4, *A* and *C*), K25 stimulated the presence of Ras-GTP from 30 s to 10 min (Fig. 4, *B* and *C*) after their addition. The analysis of Rap1 activation revealed that NMDA stimulation, but not switching from K5 to K25, promoted an increase in CGNs Rap1-GTP levels (Fig. 5). This increase was observed at 5 and 10 min after NMDA addition (Fig. 5, *A* and *C*).

Ras Mediates the Activation of the PI3K/Akt Pathway by NMDA and K25 in Immature Cerebellar Granule Neurons—Next we wanted to study whether Ras and/or Rap-1 was necessary for NMDA and K25-mediated activation of Akt. For this purpose we infected K5-cultured CGNs at 2 DIV with lentiviruses expressing a dominant negative form of Ras, RasN17, and a dominant negative form of Rap1, Rap1N17. Levels of phospho (Ser-473)-Akt were monitored 30 min after NMDA and K25 addition. Interestingly, we observed a blockage of NMDA- and K25-induced Akt phosphorylation in CGNs transfected with

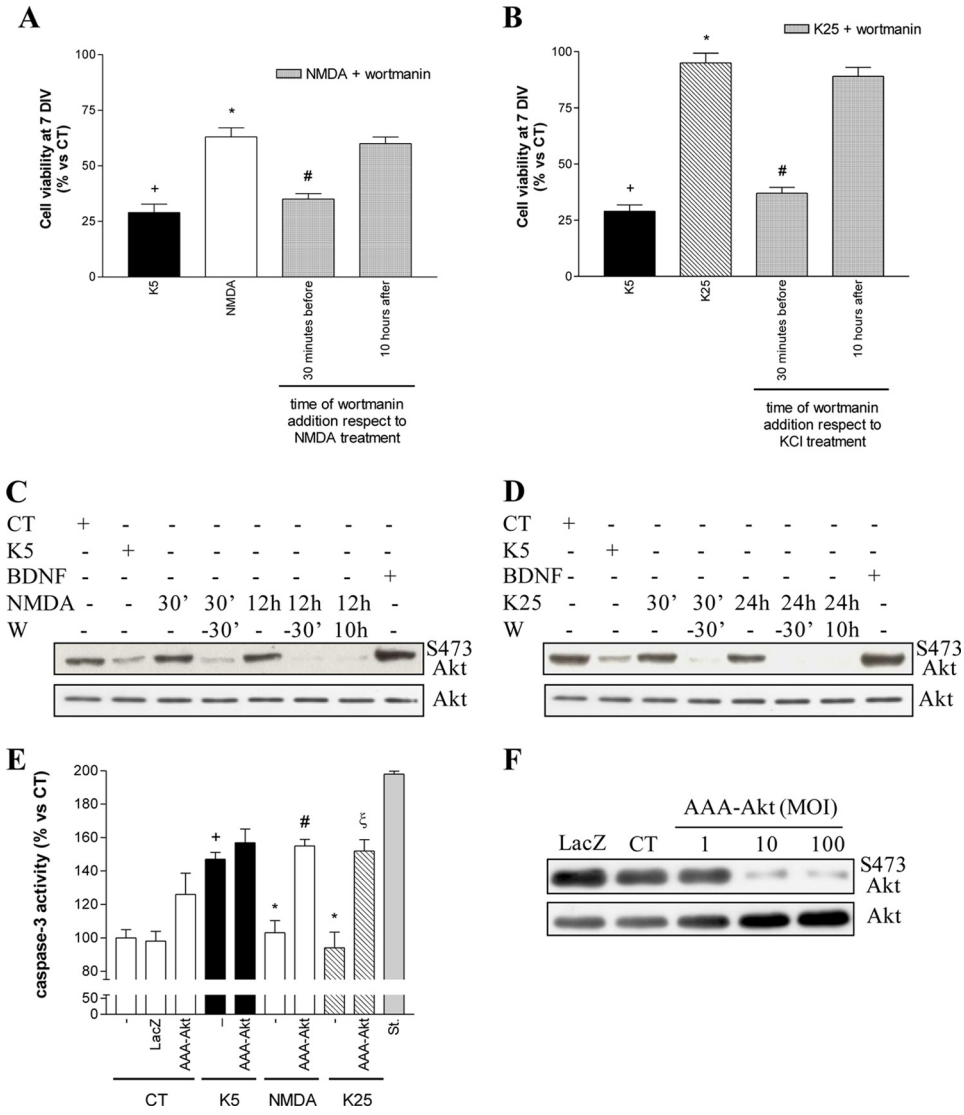

FIGURE 2. **Early, and not late, activation of PI3K/Akt pathway is involved in NMDA- and K25-mediated long term survival.** Neurons were plated in K5 and treated with NMDA (100 μM) or K25 at 2 DIV. Wortmannin (0.5 μM; inhibitor of PI3K) was added 30 min before the NMDA and K25 administration or 10 h after NMDA and K25 stimulation. Medium culture was removed at 3 DIV and replaced by conditioned medium from sister cultures. Cell viability in NMDA (*A*)- or K25 (*B*)-stimulated neurons was monitored at 7 DIV by MTT assay. Results are shown as the percentage of cell viability *versus* CT cultures (neurons plated and maintained in K25 for 7 DIV) and represent the mean ± S.E. of 4 independent experiments performed in triplicate. $^{+}$, $p < 0.05$ *versus* CT; *, $p < 0.05$ *versus* K5; #, $p < 0.05$ *versus* NMDA (*A*) or *versus* K25 (*B*). *C* and *D*, representative Western blots of phospho-Ser-473-Akt and Akt 30 min and 12 h after NMDA addition (*C*) or 30 min and 24 h after K25 treatment (*D*) with or without wortmannin (*W*). Exposition of BDNF (50 ng/ml) for 5 min was used as a positive control of Akt phosphorylation. *E*, at 1 DIV, CGNs were infected with AAA-Akt and LacZ as described under "Experimental Procedures." At 2 DIV, CGNs were treated with NMDA (100 μM) or high potassium concentration of KCl (K25), and 12 h later, caspase-3 activity was assessed. Staurosporine (1 μM) was used as a positive control of caspase-3 activation. Results are shown as percentage of caspase-3 activity *versus* CT cultures (neurons plated in K25) and represent the mean ± S.E. of three independent experiments performed in triplicate. +, $p < 0.05$ *versus* CT; *, $p < 0.05$ *versus* K5; #, $p < 0.05$ *versus* NMDA; δ, $p < 0.05$ *versus* K25. *F*, representative Western blots of phospho-Ser-473-Akt and Akt at 2 DIV in CGNs infected or not with LacZ and AAA-AKT are shown. *MOI*, multiplicity of infection.

RasN17 but not in neurons transfected with Rap1N17 (Fig. 6, *A* and *B*), indicating a role of the former in NMDA- and K25-mediated activation of Akt.

Rap1 and Ras Are Both Involved in MEK/ERK Pathway Activation by NMDA and K25—As above, we wanted to know the role of Ras and Rap-1 small G-proteins in the activation of MEK/ERK pathway by NMDA or K25. The expression of Rap1N17 in CGNs lead to the abolition of ERK phosphorylation promoted by the presence of NMDA (Fig. 6, *A* and *C*). On the other hand, Rap1N17 expression did not affect the K25-mediated phosphorylation of ERK (Fig. 6, *A* and *C*). Strikingly, the infection of CGNs with RasN17 promoted a drastic reduction in the phosphorylation of ERK by K25. Moreover, no changes were detected in phospho-ERK levels after NMDA stimulation in CGNs transfected or not with RasN17 (Fig. 6, *A* and *C*).

Activation of Ras, and Not Rap1, Mediates the Pro-survival Effect of NMDA and K25 in Immature K5-cultured CGNs—To determine the role of Ras and Rap1 in the long-lasting survival effect of NMDA and K25, we infected immature CGNs at 1 DIV with lentivirus expressing RasN17 or Rap1N17, and then we added NMDA or K25 at 2 DIV. The study of cell viability at 7 DIV revealed that the presence of RasN17 prevented the pro-survival effect of both NMDA and K25 (Fig. 7*A*), but this was not the case in CGNs expressing Rap-1N17 (Fig. 7*A*). To know

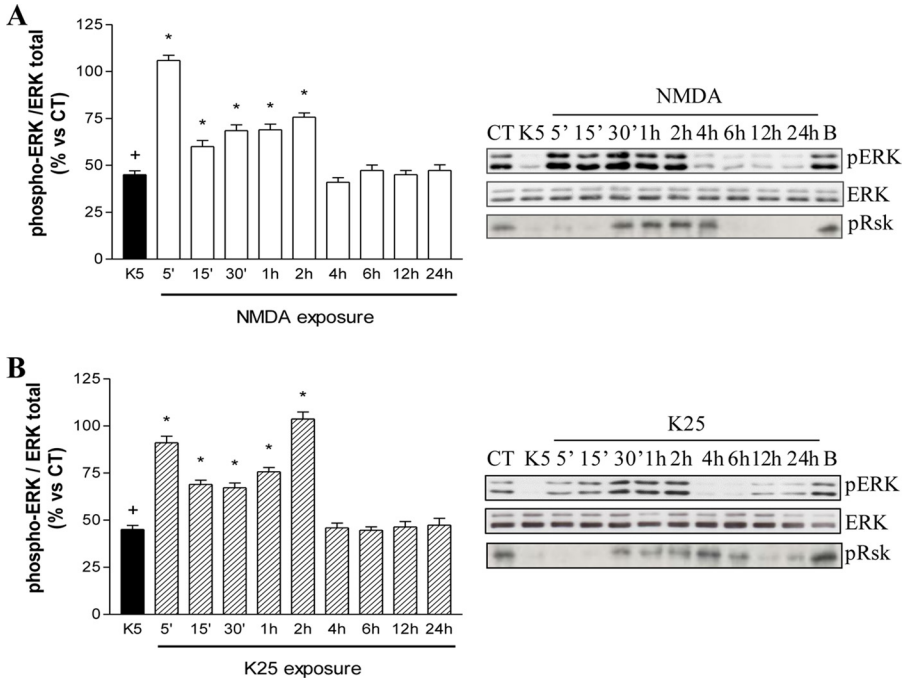

FIGURE 3. **NMDA and K25 phosphorylate ERK with a similar time-course in immature cerebellar granule neurons.** Cells were plated at K5 except control CT where cells were plated at K25. *A* and *B*, At 2 DIV, K5-cultured cells were exposed to NMDA (100 μM) or K25 for 5 min to 24 h. After treatments at the indicated times, protein levels of phospho-ERK, ERK, and phospho-Rsk (Ser-380) were analyzed by Western blot. Bands were quantified by computer-assisted densitometry, and results were expressed as the ratio between phospho-ERK and ERK levels. Values are indicated in arbitrary units *versus* CT and are the mean ± S.E. of four independent experiments performed in duplicate. $^{+}p < 0.05$ *versus* CT and $*p < 0.05$ *versus* K5. Representative Western blots of phospho-ERK, total ERK, and phospho-Rsk were done. The addition of BDNF (50 ng/ml; *B*) for 5 min was used as positive control of ERK phosphorylation.

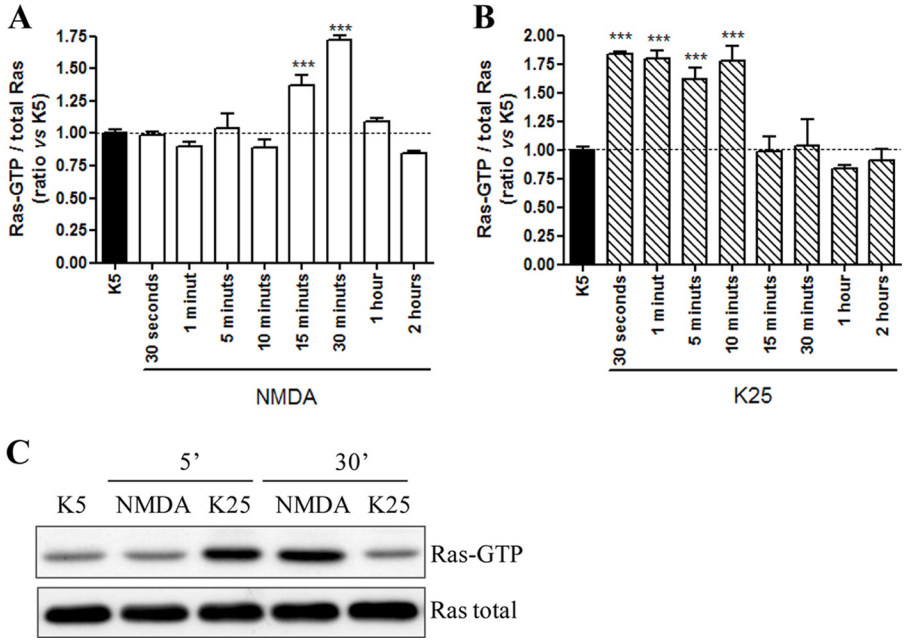

FIGURE 4. **NMDA and K25 activate Ras with a different time-course.** Cultured cells grown in K5 were exposed at 2 DIV to NMDA (100 μM) or 20 mM KCl (K25) for 30 s to 2 h. At the indicated times, the levels of Ras-GTP and total Ras were determined after NMDA (*A*) and K25 (*B*) addition as indicated under "Experimental Procedures." Ras-GTP and total Ras levels were quantified by computer-assisted densitometry, and values represent the ration between the GTP form and the total form. Values are indicated in arbitrary units *versus* K5 and are the mean ± S.E. of 4 independent experiments performed in duplicate. ***, $p < 0.001$ *versus* K5. *C*, representative Western blots of Ras-GTP and total Ras were done at 5 and 30 min after NMDA and K25 stimulation.

whether Ras was crucial for the viability of immature CGNs, we infected immature K5-cultured CGNs with a positive dominant mutant of Ras, RasV12, and we evaluated cell viability at 7 DIV. We observed a strong increase of cell viability in CGNs infected with RasV12 compared with K5-cultured CGNs (Fig. 7*B*). To know whether the PI3K/Akt is required for the survival effect of Ras, we co-infected CGNs with RasV12 and AAA-Akt or we added to the RasV12-infected CGNs the PI3K inhibitor wortmannin at 2 DIV. In both cases the inhibition of PI3K/Akt by the wortmannin or AAA-Akt drastically reduced the protective

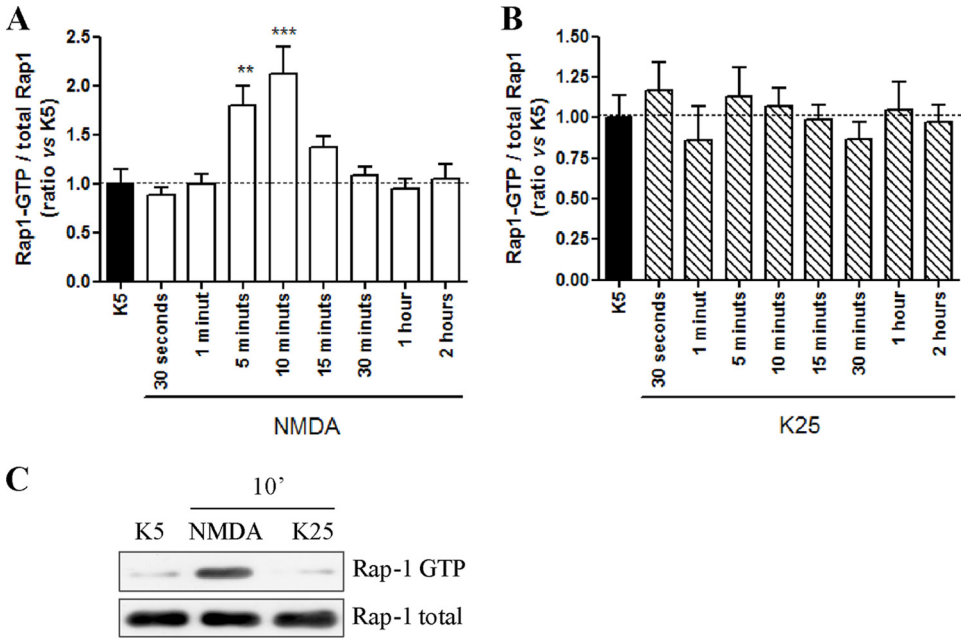

FIGURE 5. **NMDA, but not K25, activates Rap-1 in immature CGNs.** Cultured cells grown in K5 were exposed at 2 DIV to NMDA (100 μM) or high potassium concentration of KCl (K25) for 30 s to 2 h. At different times the levels of Rap-1-GTP and total Rap-1 were monitored after NMDA (*A*) and K25 (*B*) addition as indicated under "Experimental Procedures." The Rap-1-GTP and total Rap-1 levels were quantified by computer-assisted densitometry, and values represent the ration between the GTP-form and the total form. Values are indicated in arbitrary units *versus* K5 and are the mean ± S.E. of four independent experiments performed in duplicate. **, $p < 0.01$ and ***, $p < 0.001$ *versus* K5. *C*, representative Western blots of Rap-1-GTP and total Rap-1 were done at 10 min after NMDA and K25 stimulation.

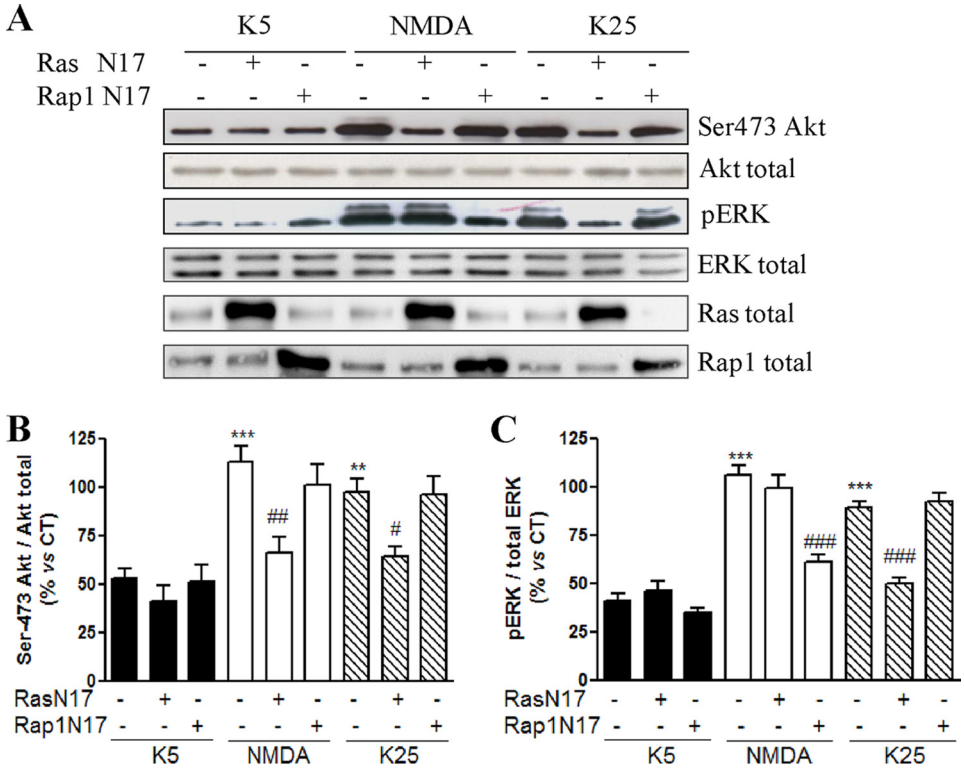

FIGURE 6. **Phosphorylation of Akt by NMDA and K25 is mediated by Ras, whereas ERK activation is differentially regulated by Rap-1 and Ras, respectively.** Cultured neurons were plated in K5 and transfected with GFP and with a negative dominant of Ras (*Ras N17*) and Rap-1 (*Rap-1 N17*) as indicated under "Experimental Procedures." At 2 DIV, cells were exposed to NMDA (100 μM) and K25 for 30 min and then were harvested to study the protein levels of phospho-Akt (Ser-473), Akt, phospho-ERK, ERK, Ras, and Rap-1 by Western blot. *A*, representative Western blots of phospho-Akt, Akt, phospho-ERK, ERK, Ras, and Rap-1 are shown. Neurons transfected with GFP are indicated as minuses. *B*, the phospho-Akt and Akt levels were quantified by computer-assisted densitometry, and values represent the ratio between the phospho-form and the total form. *C*, the phospho-ERK and ERK levels were quantified by computer-assisted densitometry, and values represent the ration between the phosphorylated form and the total form. Values are indicated in percentage *versus* CT (cells plated in K25) and are the mean ± S.E. of three independent experiments performed in duplicate. **, $p < 0.01$ and ***, $p < 0.001$ *versus* K5; and #, $p < 0.05$, ##, $p < 0.01$, and ###, $p < 0.001$ *versus* NMDA or K25.

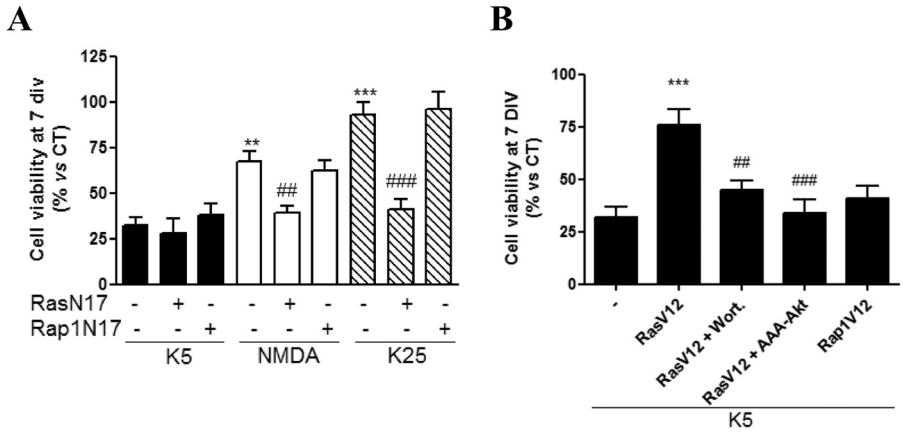

FIGURE 7. **Activation of Ras is a key event in the NMDA- and K25-mediated long term survival.** *A*, cultured neurons were plated in K5 and transfected with GFP and with a negative dominant mutant of Ras (*RasN17*) or Rap-1 (*Rap1N17*) as indicated under "Experimental Procedures." At 2 DIV, cells were exposed to NMDA (100 μM) and K25 for 24 h, and then cell viability was assessed at 7 DIV by the MTT assay. Results are shown as the percentage of cell viability *versus* CT cultures (neurons plated in K25) and represent the mean ± S.E. of four independent experiments performed in triplicate. **, $p < 0.01$ and ***, $p < 0.001$ *versus* K5; ##, $p < 0.01$ and ###, $p < 0.001$ *versus* NMDA or K25. *B*, cultured neurons were plated in K5 and transfected with GFP and with a positive dominant of Ras (*RasV12*) with RasV12 and a negative dominant of Akt (*AAA-Akt*) and with a positive dominant of Rap-1 (*Rap-1V12*) as indicated under "Experimental Procedures." In some cases, wortmannin (*Wort.*, 0.5 μM) was added at 2 DIV in RasV12-infected cultures. Cell viability was assessed at 7 DIV by the MTT assay. Results are shown as the percentage of cell viability *versus* CT cultures (neurons plated in K25) and represent the mean ± S.E. of four independent experiments performed in triplicate. ***, $p < 0.001$ *versus* K5. ##, $p < 0.01$ and ###, $p < 0.001$.

effect induced by the infection of RasV12 (Fig. 7*B*). On the other hand, the infection with a positive dominant of Rap1, Rap1V12, did not block the K5-mediated cell death at 7 DIV (Fig. 7*B*).

DISCUSSION

It is well reported that neuronal activity, mainly through glutamate receptor activation, promotes the development and survival of neurons of several brain areas. Numerous studies have shown that activation of NMDA-type of glutamate receptors or membrane-mediated depolarization by high KCl (K25) promotes the survival and differentiation of CGNs in the developing cerebellum (4, 5). We have also previously described that 24 h of exposition to NMDA or K25 is enough to support a long-lasting neuroprotective effect (26). Although it is well known that both NMDA and K25 mediate their pro-survival effect through extracellular calcium influx, contradictory results have been reported about the transduction mechanisms involved (32).

Two main pathways have been extensively involved in NMDA and K25 pro-survival effect on developing CGNs: the PI3K/Akt and MEK/ERK pathways (33–35). In the present study we have further explored the role of these pathways because their contribution to NMDA or K25 to CGNs survival is still controversial and not fully understood. For this purpose we have used immature (2 DIV) CGNs cultures because we believe that they will reflect the *in vivo* situation better than the older cultures (6–8 DIV) commonly used by others. Under these conditions both NMDA and K25 were able to activate PI3K and Akt phosphorylation. NMDA rapidly activates PI3K (5 min after NMDA addition) and Akt phosphorylation (starting 15 min after NMDA addition). This first increase in Akt phosphorylation was observed for 1 h. Afterward, Akt phosphorylation levels return to K5 conditions. Surprisingly, a second increase in Akt phosphorylation was observed 12 h after NMDA addition. K25-treated CGC cultures showed a similar time-course activation of PI3K and Akt phosphorylation. The unexpected observation that Akt was phosphorylated at two different time intervals by K25 and NMDA led us to ask whether both phosphorylation waves were necessary for the neuroprotective effects of NMDA and K25. We knew from previous reports that the presence of wortmannin (a PI3K inhibitor) before the addition of NMDA or K25 was able to significantly block their pro-survival effect (26). Here, the addition of wortmannin after the first peak of Akt phosphorylation clearly showed that the second wave of Akt phosphorylation is not involved in NMDA and K25 pro-survival effect and allow us to conclude that activation of the PI3K/Akt pathway during the first hour is necessary for NMDA and K25 pro-survival effect on developing CGNs cultures. Interestingly, we have previously demonstrated that NMDA- and K25-mediated neuroprotection was related to an inhibition of caspase-3 at 12 h (26), after the first wave of Akt activation, which it is related to survival. The involvement of this pathway in NMDA-mediated survival of CGNs was also reported previously in immature CGNs at 4 DIV (12). It has also been described that BDNF release mediates NMDA pro-survival effect on CGNs (26, 30, 36, 37) and BDNF also promote CGNs survival through activation of PI3K/Akt (13). Thus it is likely that, although NMDA receptor stimulation could activate this pathway directly (35), NMDA-mediated activation of Akt is due to BDNF-mediated stimulation of its receptor. Accordingly, we observed that NMDA failed to activate Akt when anti-BDNF was added to culture medium (data not shown). In addition, that may also explain the slightly delay in Akt activation (starting 15 min after stimulus addition) when compared with other systems. More controversial is the role of the PI3K/Akt pathway in K25-mediated survival. Our results showing that this pathway is needed for K25 pro-survival effect were also observed by others (11, 14). By contrast some reports indicate that this pathway is not related to K25-mediated neuroprotection of CGNs (15, 16). We think that this discrepancy could be due to the age of CGNs used in the studies. When

mature cultures are used, PI3K/Akt pathway do not seem to be strongly related to CGNs survival by NMDA or K25 (15–17), whereas it is essential in immature (which is more related to the *in vivo* situation) CGNs (26).

Although activation of the MEK/ERK pathway has been also extensively related to cell survival and differentiation, its role in NMDA and K25-mediated neuroprotection in CGNs cultures is still unclear. Some studies have suggested that ERK activation is an important event, whereas other reports claimed that ERK is not involved (13, 15, 18). We have previously reported that pharmacological inhibition of ERK pathway in immature CGNs cultures was able to block K25, but not NMDA, neuroprotection from K5-mediated apoptosis (26). However, in the present study both NMDA and K25 promoted a similar increase of ERK phosphorylation at 2 DIV with an identical time-course. So, it was not clear why ERK activation is not involved in NMDA-mediated neuroprotection. Some reports have suggested that ERK function depends on its intracellular location (nuclear *versus* cytosolic; Ref. 38). We were not able to see any differences in ERK subcellular localization. In all the conditions tested, ERK was always observed in the cytosol (data not shown). So what is the reason for this differential implication of MEK/ERK pathway in K25 and NMDA-mediated CGNs survival? A possibility could be a differential effect downstream ERK. One of the main cytosolic ERK substrates is p90Rsk that directly phosphorylates the cAMP-response element-binding protein (CREB), perhaps the most important transcription factor related to activity-dependent neuronal survival in general and for CGNs survival in particular (30, 39). However, we have observed that both NMDA and K25 are able to activate p90Rsk and CREB (Ref. 30 and data not shown), so we could not explain why MEK/ERK is not related to NMDA-mediated CGNs survival in terms of a differential downstream effect on p90Rsk and CREB. Next, we wonder whether the difference could be upstream MEK/ERK. It is well known that small G proteins from the Ras superfamily are closely related to activation of the MEK/ERK pathway (19). In particular, Ras has been reported to play a major role in MEK/ERK activation in CGNs (25). Our results clearly show that NMDA and K25 activate Ras in immature CGNs. However, slightly differences in the time-course are observed as Ras activation by K25 occurs much faster (30 s) than with NMDA (15 min). Moreover, the use of a dominant negative form of Ras (RasN17) indicates that Ras activity is needed for K25-mediated ERK activation. By contrast, RasN17 did not block NMDA-mediated ERK activation, suggesting that another small G protein could be involved. In fact, our results demonstrate that Rap1 is involved in NMDA-mediated ERK activation, giving further details to initials studies that suggested Rap1 was involved in NMDA signaling in neurons (40, 41). The role of Rap1 in NMDA-mediated ERK activation could explain why no differences were observed between the temporal profile of NMDA and K25-mediated ERK phosphorylation. Because Rap1 has been reported to be an important mediator in neurite outgrowth, it is tempting to speculate that Rap1 could be involved in the MEK/ERK role in neurite outgrowth and maintenance elicited by neuronal activity or other extracellular factors (42). Altogether, these results indicate that Ras activation is an event that explains the differential implication of MEK/ERK pathway in CGNs survival.

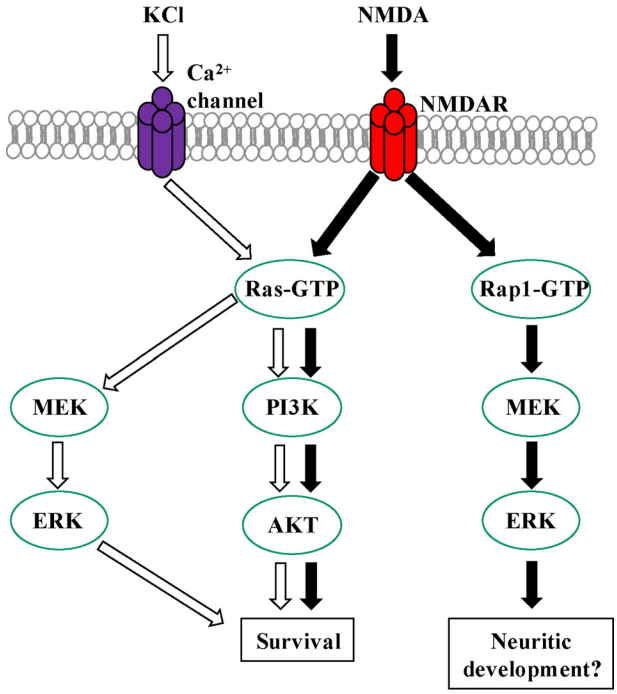

FIGURE 8. **Schematic diagram representing the role of PI3K/Akt and ERK pathways in NMDA- and K25-mediated long term survival and their regulation by Ras and Rap-1.** The diagram represents the key role of Ras activation of PI3K/Akt and ERK pathways in the NMDA- and K25-mediated long term survival effect. *Solid arrows* represent the pathways activated after NMDA addition, and *empty arrows* shown the pathways activated after KCl addition. *NMDAR*, NMDA receptor.

Ras also promotes the survival of many cell types, including neurons by activating the PI3K/Akt pathway (43–45). In the present study we have also observed that Ras mediates the NMDA and K25-dependent activation of PI3K/Akt in CGNs. Thus, our results suggest a correlation between activation of Ras and the importance of a signaling pathway in CGNs survival. Only when Ras is involved in MEK/ERK activation (K25), is this pathway involved in activity-dependent survival of CGNs. On the other hand, we have shown that Ras is involved in K25 and NMDA activation of the PI3K/Akt pathway and that this pathway is involved in their pro-survival effect (Fig. 8). The key role of Ras for activity-dependent CGNs survival was further demonstrated as we observed that RasN17 did prevent NMDA and K25-mediated survival, whereas RasV12 was able to support CGNs survival in the absence of NMDA or depolarizing conditions. This is the first report describing a crucial role of Ras in CGNs survival in NMDA and depolarizing conditions, and we suggest that the involvement of this small G protein is a key event in the neuroprotective role that PI3K/Akt and MEK/ERK pathways have on different neuronal populations. Supporting that view, activation of Ras has been observed in striatal (46), cortical (47), and hippocampal (24) neurons treated with NMDA or in depolarizing conditions.

In summary, our results indicate that the small G protein Ras seems to be a central mediator of activity-dependent CGNs survival because, besides its role in the activation of PI3K/Akt pathway, Ras also determines whether MEK/ERK pathway acti-

vation is involved in activity-dependent CGN survival. The stimulation of MEK/ERK pathway by other small G proteins, such as Rap1, is not sufficient to promote a protective action.

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Neurobiology:

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