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Glycogen Synthase Kinase 3 Activity Mediates Neuronal Pentraxin 1 Expression and Cell Death Induced by Potassium Deprivation in Cerebellar Granule Cells

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ABSTRACT

Expression of neuronal pentraxin 1 (NP1) is part of the apoptotic cell death program activated in mature cerebellar granule neurons when potassium concentrations drop below depolarizing levels. NP1 is a glycoprotein homologous to the pentraxins of the acute phase immune response, and it is involved in both synaptogenesis and synaptic remodeling. However, how it participates in the process of apoptotic neuronal death remains unclear. We have studied whether the signaling pathways known to control neuronal cell death and survival influence NP1 expression. Both activation of the phosphatidylinositol 3-kinase/Akt (PI-3-K/AKT) pathway by insulin-like growth factor I and pharmacological blockage of the stress activated c-Jun NH_2-terminal kinase (JNK) offer transitory neuroprotection from the cell death evoked by nondepolarizing concentrations of potassium. However, neither of these neuroprotective treatments prevents the overexpression of NP1 upon potassium depletion, indicating that nondepolarizing conditions activate additional cell death signaling pathways. Inhibiting the phosphorylation of the p38 mitogen-activated protein kinase without modifying JNK, neither diminishes cell death nor inhibits NP1 overexpression in nondepolarizing conditions. In contrast, impairing the activity of glycogen synthase kinase 3 (GSK3) completely blocks NP1 overexpression induced by potassium depletion and provides transient protection against cell death. Moreover, simultaneous pharmacological blockage of both JNK and GSK3 activities provides long-term protection against the cell death evoked by potassium depletion. These results show that both the JNK and GSK3 signaling pathways are the main routes by which potassium deprivation activates apoptotic cell death, and that NP1 overexpression is regulated by GSK3 activity independently of the PI-3-K/AKT or JNK pathway.

In culture, cerebellar granule cells require serum and high extracellular [K^+] to grow and differentiate (Gallo et al., 1987). Once mature, the majority of these cells die within 24 h if serum is removed and the concentration of K^+ is kept below depolarizing levels. Under these conditions, the death of the cells is morphologically apoptotic and requires the de novo synthesis of both RNA and protein (D'Mello et al., 1993; Galli et al., 1995; Nardi et al., 1997; Watson et al., 1998). Therefore, it is the de novo production of lethal proteins, rather than a reduction in the expression of survival proteins, that mediates the death of cerebellar granule cells. The intracellular signaling pathways that regulate the production of lethal proteins in neurons upon reduction of activity have not been fully characterized.

The fate of both mature and developing cells generally depends on a highly regulated balance between survival and death signals. The survival of mature cerebellar granule cells after serum and K^+ withdrawal can be maintained by several means: restoring depolarizing [K^+] (Gallo et al., 1987); adding cyclic AMP (D'Mello et al., 1993), lithium (D'Mello et al., 1994), or *N*-methyl-D-aspartate (Marini and Paul, 1992); or by exposure to growth factors such as insulin-like growth factor 1 (IGF-1; D'Mello et al., 1993; Dudek et al., 1997) and

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ABBREVIATIONS: IGF-1, insulin-like growth factor 1; AKT, protein kinase B; JNK, c-Jun NH_2-terminal kinase; NP1, neuronal pentraxin 1; DMSO, dimethyl sulfoxide; GSK3, glycogen synthase kinase 3; MAPK, mitogen-activated protein kinase; PI-3-K, phosphatidylinositol 3-kinase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; TBS, Tris-buffered saline; SAPK, stress-activated protein kinase; CEP-1347, 3,9-bis[(ethylthio)methyl]-(8*R**,9*S**,11*S**)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo(*a*,*g*)cycloocta(*cde*)trinden-1-one; CEP-11004-2, 3,9-bis-[(isopropylthio)methyl]-(8*R**,9*S**,11*S**)-(−)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo(*a*,*g*)cycloocta (*cde*)trinden-1-one; SB415286, 3-(3-chloro-4-hydroxyphenylamino)-4-(2-nitrophenyl)-1*H*-pyrrole-2,5-dione.

hepatocyte growth factor (Zhang et al., 2000). It has been established that all these different survival factors converge on the activation of the serine/threonine protein kinase B/Akt (AKT) (Crowder and Freeman, 1998; Vaillant et al., 1999; Kumari et al., 2001). Although AKT activation is fundamental to suppress apoptosis through neurotrophins, recent studies have shown that survival mediated by membrane depolarization is independent of AKT activity (Chin and D'Mello, 2004). This suggests that membrane depolarization not only activates survival signals but also suppresses death signals. However, the intracellular signaling pathways that activate apoptosis by potassium deprivation may vary between different cell types (Ham et al., 2000).

In sympathetic neurons, the activation of the c-Jun NH_2-terminal kinase (JNK) pathway seems to be necessary and sufficient to induce apoptosis upon nerve growth factor withdrawal (Estus et al., 1994; Ham et al., 1995). Accordingly, pharmacological inhibition of the JNK pathway with CEP-1347, an inhibitor of the kinases that activates JNK signaling, offers sympathetic neurons long-term protection against cell death evoked by nerve growth factor deprivation (Harris et al., 2002b). However, recent evidence has emerged that apoptosis of cerebellar granule cells evoked by K^+ deprivation may also involve other pathways. In these cells, inhibiting JNK signaling provides only transitory protection against neuronal death evoked by K^+ deprivation (Harris et al., 2002a), indicating that a JNK-independent pathway is also activated when cerebellar granule cells are deprived of K^+ (Ham et al., 2000; Harris et al., 2002a).

We have previously shown that at nondepolarizing [K^+], cerebellar granule cells increase the levels of neuronal pentraxin 1 (NP1) protein before undergoing cell death. The increase in the protein expression of NP1 can be detected immediately after potassium deprivation and peaks 4 h later at between 4- and 6-fold of the control levels. This accumulation of NP1 precedes cytoplasmic membrane damage by at least 4 h, and the maximal accumulation of NP1 protein approximately corresponds to the point at which cerebellar granule cells become committed to die. Incubation of cerebellar granule cells with antisense oligodeoxyribonucleotides directed against NP1 mRNA inhibited the increase in NP1 protein levels and attenuated neuronal death. Based on these results, we proposed that NP1 is part of the gene program that leads to apoptotic cell death in cerebellar granule cells in nondepolarizing conditions (DeGregorio-Rocasolano et al., 2001).

NP1 is a secreted glycoprotein whose expression is restricted to the nervous system (Schlimgen et al., 1995). NP1 is a member of the pentraxin family of proteins that is divided into two structural classes based on size (Goodman et al., 1996). The amino-terminal half of the long pentraxins, such as NP1, and neuronal activity-related pentraxin (also called neuronal pentraxin 2), encodes a series of coiled-coil domains that seem to be essential for homomultimerization (O'Brien et al., 2002). The carboxyl-terminal half encodes a calcium-dependent lectin-binding domain (Emsley et al., 1994; Tsui et al., 1996). Because NP1 mediates neuronal death evoked by nondepolarizing conditions in cerebellar granule cells, whereas neuronal activity-related pentraxin promotes synapse formation, we hypothesized that neuronal pentraxins constitute a genetic sensor that regulates neuronal death or survival, depending on synaptic activity (DeGregorio-Rocasolano et al., 2001). Here, we have investigated the regulation of NP1 expression by signaling pathways that mediate neuronal survival and death.

Materials and Methods

Cell Culture. Primary cultures of cerebellar granule neurons were prepared from postnatal day 7 Sprague-Dawley rat pups as described previously (DeGregorio-Rocasolano et al., 2001). Cells were dissociated in the presence of trypsin and DNase I and plated in poly-L-lysine (100 μg/ml)-coated dishes at a density of 3×10^5 cells/cm^2 in basal Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mg/ml gentamicin, 2 mM L-glutamine, and 25 mM KCl. Cytosine-D-arabinofuranoside (10 μM) was added to the culture medium 24 h after plating to prevent the replication of non-neuronal cells. The cultures were maintained at 37°C in a humidified incubator with 5% CO_2, 95% air and left undisturbed until experiments were performed 8 days after plating (8 days in vitro). All procedures involving animals and their care were approved by the ethics committee of the University of Barcelona, and they were conducted in accordance with guidelines that conform with national (Generalitat de Catalunya) and international laws and policies (Guide for the Care and Use of Laboratory Animals, National Academy Press, Washington, DC, 1996).

Induction of Neuronal Death by Potassium Depletion and Neuroprotective Treatments. After 8 days in culture, the medium in which cerebellar granule cells was grown (conditioned medium, $S_c^+K^+$) was replaced with either fresh unconditioned serum-free medium supplemented with 25 mM potassium (S^-K^+) or fresh unconditioned serum-free medium containing 5 mM potassium (S^-K^-). The drug treatments were performed at 8 days in vitro, immediately after the replacement of the medium. The inhibitor of the JNK signaling pathway, CEP-11004-2, was kindly provided by Cephalon (West Chester, PA). Stock solutions of CEP-11004-2 (4 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C, and a working 40 μM solution of CEP11004-2 was prepared in 1% bovine serum albumin/basal Eagle's medium on the day of experiment. Cells were preincubated with 400 nM CEP11004-2 for 4 h at 37°C before potassium depletion. The glycogen synthase kinase 3 (GSK3) inhibitor SB415286 was kindly provided by GlaxoSmithKline (Stevenage, UK), from which stock solutions (20 mM) were prepared in DMSO and stored at −20°C. The inhibitor of p38 mitogen-activated protein kinase (MAPK) phosphorylation SB203580 was from Calbiochem (Darmstadt, Germany), and stock solutions (10 mM) of this drug were prepared in DMSO and stored at −20°C, and a working 2.25 mM solution was prepared the day of experiment. IGF-1 (50 ng/ml) (Sigma, Madrid, Spain) was prepared in cell culture media containing 0.1% bovine serum albumin as a carrier protein. The PI-3-K inhibitor LY294002 was from Sigma (Madrid, Spain), and 10 mM stock solutions of LY294002 were prepared in DMSO and stored at −20°C.

Determination of Cell Death. Cell death was assessed using propidium iodide staining. Propidium iodide fluorescence was measured in 24-well plates using a CytoFluor 2350 scanner (Millipore Corporation, Billerica, MA) with 530-nm excitation (25-nm band pass) and 645-nm (40-nm band pass) emission filters. The percentage of nonviable cells was measured using a modification of the method described by Rudolph et al. (1997). Baseline fluorescence F_1 was measured 1 h after addition of propidium iodide (30 μM) as an index of the cell death not related to the treatment. Subsequently, fluorescence readings were taken at different times after the onset of the treatment. At the end of the experiment, the cells were permeabilized for 10 min with 500 μM digitonin at 37°C to obtain the maximum fluorescence corresponding to 100% of cell death (F_{max}). The percentage of cell death was calculated as follows: % cell death = $100 \times (F_n - F_1)/(F_{max} - F_1)$, where F_n is the fluorescence at any given time. Cells were kept in the incubator between measurements.

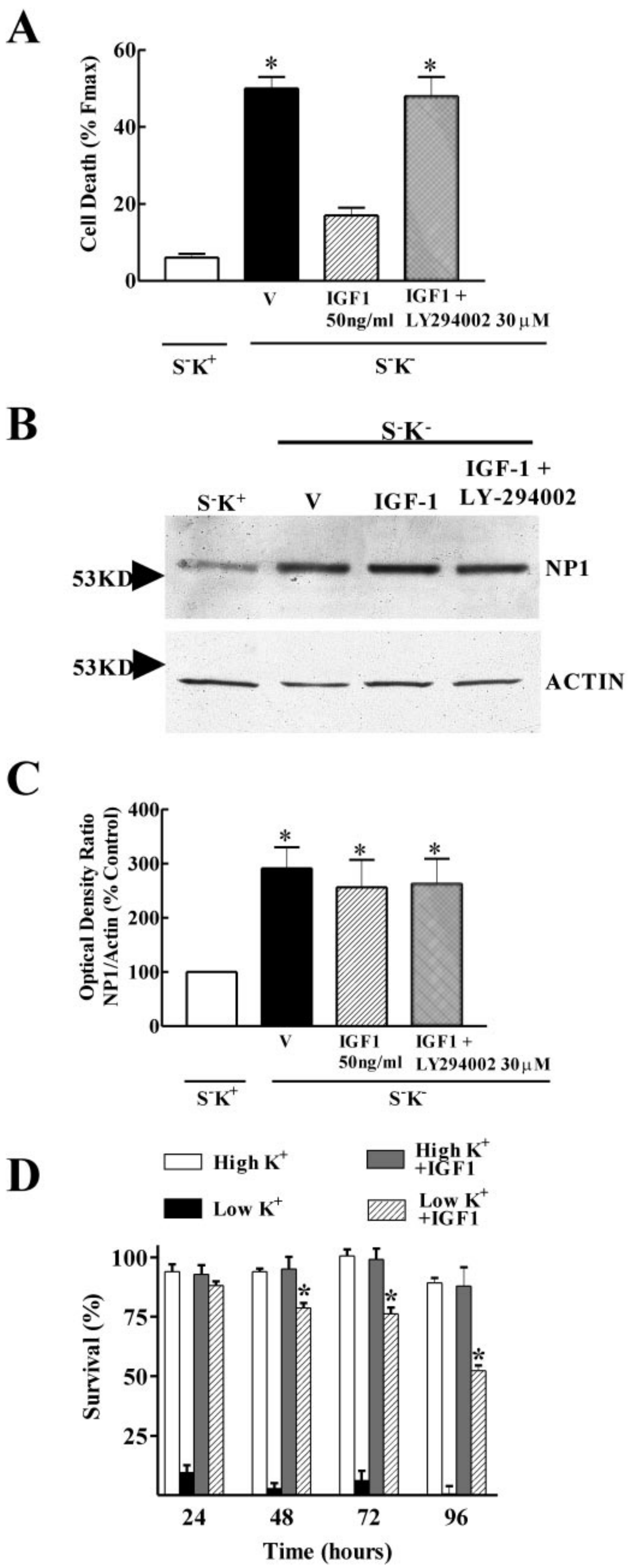

Fig. 1. IGF-1 provides transient neuroprotection in a PI-3-K-dependent manner but does not modify NP1 overexpression evoked by potassium depletion. Mature (8 days in vitro) cerebellar granule cells were incubated in high (S^-K^+) or low potassium (S^-K^-). A, IGF-1 (50 ng/ml) reduces cell death evoked by potassium depletion 24 h after treatment, and LY294002 (30 μM) blocks this neuroprotective effect. Values are mean ± S.E. of three independent experiments. *, $p < 0.05$, significantly different from S^-K^+. Student's t test. B, representative Western blot showing that IGF-1 and LY294002 do not affect the increase of NP1 levels evoked by potassium depletion. C, quantitative analysis of the effects of IGF-1 and LY294002 on the increase of NP1 protein levels evoked by potassium depletion 4 h after beginning the treatment. NP1 protein was normalized to the levels of actin expression. The intensity of the bands was determined by densitometric analysis of at least three independent experiments. The ratio of NP1 over actin intensity was expressed as percentage of control values. *, $p < 0.05$, significantly different from high K^+. D, time course of neuroprotection by IGF-1. Values are mean ± S.E. of three independent experiments. *, $p < 0.05$, significantly different from high K^+.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting. After the corresponding treatments, cells were solubilized in lysis buffer [62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% bromphenol blue] and sonicated briefly. The homogenate was boiled and stored at −20°C before separating the proteins by 10% SDS-polyacrylamide gel electrophoresis and transferring them to Hybond ECL nitrocellulose membranes (Amersham Biosciences Europe, Freiburg, Germany). The membranes were preincubated with 5% nonfat dry milk in Tris-buffered saline (TBS) before immunostaining. For specific immunodetection of the NP1 protein, a mouse anti-rat NP1 monoclonal antibody (BD Transduction Laboratories, Los Angeles, CA) was diluted 1:1500 in a solution containing 3% bovine serum albumin in TBS with 0.1% Tween 20. Immunodetection of the other proteins was also performed with the following: rabbit anti-SAPK/JNK, rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185), rabbit anti-p38 MAPK, and rabbit anti-phospho-p38-MAPK (Thr180/Tyr182) antisera, (Cell Signaling Technology Inc., Beverly, MA); mouse monoclonal anti-GSK3β antibody and the phospho-specific antibody anti-GSK3β phosphorylated on Tyr216 (BD Biosciences PharMingen, San Diego, CA); and goat polyclonal anti-β-catenin antiserum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Cell Signaling Technology Inc.) and donkey anti-goat (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies in a solution of 5% nonfat dry milk in TBS with 0.1% Tween 20. In all experiments, a rabbit anti-actin antibody (Sigma) was used to control for the amount of protein loaded. Immunolabeled proteins were visualized using an enhanced chemiluminescence detection system (Immun-Star horseradish peroxidase substrate kit; Bio-Rad, Madrid, Spain), and the intensity of the bands was quantified with a Fluor-S MultiImager (Bio-Rad). In the case of phospho-p38 MAPK, the bands were visualized on film, and quantification was performed using Kodak DS1 computer software. Densitometric values of the immunoreactive bands representing NP1 and nonphosphorylated proteins were normalized to the values of the corresponding actin bands.

GSK3β Immunoprecipitation and Activity Assay. The activity of GSK3β was essentially measured as described in Bijur et al. (2000). Cells were lysed in immunoprecipitation lysis buffer (20 mM Tris, pH 7.5, 0.2% Nonidet P-40, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 1 nM okadaic acid, 100 mM sodium fluoride, and 1 mg/ml glycogen). The cell lysates were passed through a 23-gauge syringe, incubated for 10 min on ice, and centrifuged at 20,800g for 15 min. The protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL). Each sample (100 μg of protein) was cleared with 40 μl of protein G-Sepharose beads for 90 min at 4°C, before incubating with 1.2 μg of mouse anti-GSK3β antibody overnight at 4°C. This procedure allowed a complete immunoprecipitation of GSK3β in all samples. The immune complexes were washed three times with immunoprecipitation lysis buffer and once with 20 mM

Tris, pH 7.5, 5 mM $MgCl_2$, and 1 mM dithiothreitol. Kinase activity was assayed in a total volume of 15 μl of kinase buffer containing 20 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 250 μM ATP, 1.4 μCi of [γ-^{32}P]ATP, and 100 μM phosphoglycogen synthase peptide-2 (YRRAAVPPSPSLSRHSSPHQSEDEEE; Upstate Biotechnology, Lake Placid, NY). Kinase buffer without peptide was used as a control. The samples were incubated for 30 min at 30°C and placed on ice for 2 min before centrifuging for 3 min at 1800*g*. The reaction supernatants were spotted onto 1 × 2-cm P81 filter paper (three spots of 5 μl each; Upstate Biotechnology), and the filters were washed four times in 0.5% phosphoric acid for a total time of 1 h. Then, the filters were washed with 95% ethanol for 2 min, air-dried, and counted in a liquid scintillation counter. The efficiency of GSK3β immunoprecipitation was examined by immunoblotting for GSK3β. The value of GSK3β activity from each sample was normalized with the corresponding optical density value of the GSK3β assayed.

Statistical Analysis. Results are expressed as mean ± S.E. of at least three separate experiments. The statistical significance of the differences was examined using independent *t* tests or using one-way analysis of variance when required.

Results

IGF-1 Does Not Influence the Overexpression of NP1 Evoked by Potassium Depletion and Provides Transient Protection against Cell Death. We have been interested in the mechanisms by which depletion of potassium to concentrations below depolarizing levels provokes cell death in certain neurons. In the absence of serum, the depletion of potassium increased the extent of cell death nearly 10-fold (from 6 ± 1 to 50 ± 3%) in cultures of cerebellar granule cells within 24 h. This neurotoxicity was partially counteracted by IGF-1 (50 ng/ml), recovering to 17 ± 2%. The neuroprotective effect of IGF-1 was completely blocked by coincubation with the PI-3 kinase inhibitor LY294002 (30 μM; Fig. 1A), confirming that PI-3-K activity is necessary for IGF-1 to promote survival (Dudek et al., 1997).

We investigated whether IGF-1 might also influence the overexpression of NP1 that occurs before cell death in non-depolarizing conditions (DeGregorio-Rocasolano et al., 2001). As expected, potassium depletion in cultures of cerebellar granule cells induced a 3-fold increase in the protein levels of NP1 within 4 h (Fig. 1C). However, neither IGF-1 (50 ng/ml) nor LY294002 significantly modified these levels (Fig. 1B), nor was the accumulation of NP1 affected by the combined treatment of IGF-1 and LY294002 (Fig. 1C). Hence, the activation of the PI-3 kinase pathway by IGF-1 promotes survival but does not seem to regulate NP1 expression. Because NP1 mediates cell death after K^+ depletion, we hypothesized that treatments that do not affect the overexpression of NP1 should provide only transient neuroprotection, whereas treatments that provide long-term survival are likely to reduce NP1 overexpression. This hypothesis gathered support from the fact that treatment with IGF-1 (50 ng/ml) completely blocked neuronal death evoked by potassium depletion over 24 h, but at 96 h after the beginning of treatment, the survival-promoting effects of IGF-1 were reduced by 48% (Fig. 1D). To check whether this reduction of the neuroprotective effect of IGF-1 was caused by degradation of IGF-1 over time, we readded IGF-1 (50 ng/ml) 24 h after the first treatment. The results we obtained with readdition of IGF-1 were the same that we observed with only one addition, indicating that the reduction in the neuroprotective effect of IGF-1 was not caused by instability of the growth factor in the culture medium (data not shown). Hence, the neuroprotection afforded by IGF-1 was transient and diminished over time.

In contrast, restoring potassium concentrations to depolarizing levels sustained the long-term survival of cerebellar granule cells (Fig. 1D). The granule cells could be rescued from death if depolarizing concentrations of potassium were re-established within the 4 h of potassium deprivation (Fig. 2A), indicating that the mechanisms that induce cell death are irreversibly activated after such a period. Rescue from cell death by recovering a depolarizing potassium concentration was associated with a reversion of the NP1 overexpression induced by potassium deprivation. Indeed, the overexpression of NP1 was antagonized when potassium was replaced 3 h after depletion (Fig. 2B).

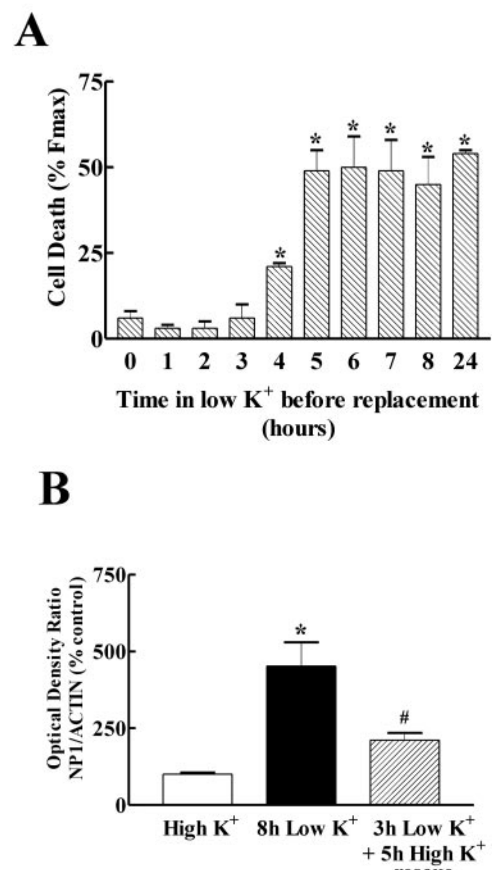

Fig. 2. Rescue from cell death by potassium replenishment is associated with a reduction of NP1 overexpression. A, mature cerebellar granule cells were incubated in serum-free medium with 30 mM potassium (high K^+) or serum-free medium with 5 mM potassium (low K^+). Cells incubated in low K^+ were supplemented with 25 mM K^+ at different times after the initiation of potassium deprivation. Cell death was assessed by propidium iodide fluorescence after 24 h and expressed as a percentage of the maximum cell death obtained with digitonin. Values are mean ± S.E. of three independent experiments. *, $p < 0.05$, significantly different from high K^+. B, effect of K^+ replacement on the increase in NP1 protein levels evoked by low potassium. Cells were incubated in high or low K^+ medium. Three hours after treatment with low potassium, high potassium was replaced, and proteins were extracted 5 h later. NP1 protein levels were normalized to the actin levels. The intensity of the bands was determined by densitometric analysis, and the ratio of NP1 over actin was expressed as a percentage of the control values of at least three independent experiments. *, $p < 0.05$, significantly different from high K^+; #, $p < 0.05$, significantly different from high and low K^+.

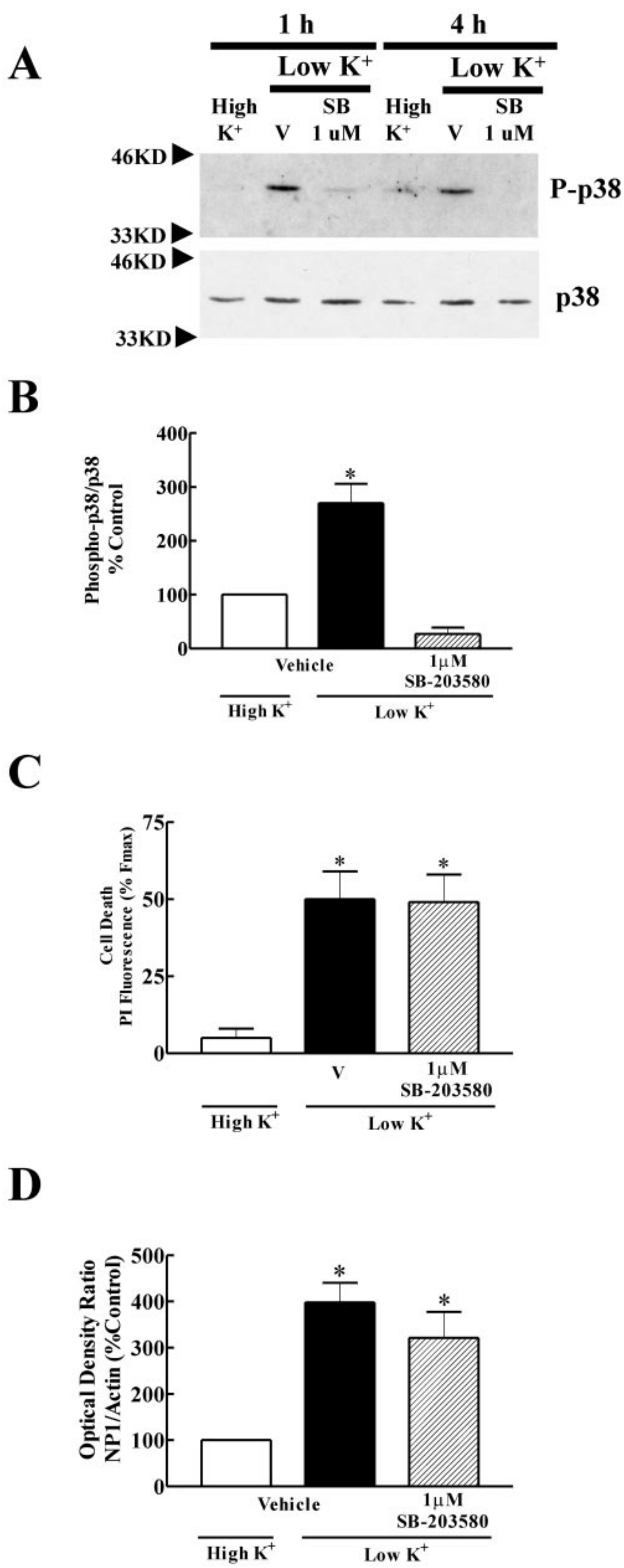

Fig. 3. Potassium depletion increases p38 MAPK phosphorylation, but SB203580 does not block cell death or NP1 overexpression. Mature (8 days in vitro) cerebellar granule cells were incubated in high (S^-K^+) or low potassium (S^-K^-) in the presence or absence of 1 μM SB203580. A, Western blot showing the effect of SB203580 on p38 MAPK phosphorylation. Cells were maintained in high or low K^+, with or without SB203580, and total protein was extracted 1 and 4 h after treatment. Western blots were incubated with rabbit anti-phospho-p38-MAPK (Thr180/Tyr182) antiserum (1:1000). B, quantitative analysis of the effects of SB203580 on p38 MAPK phosphorylation 1 h after potassium deprivation. Phospho-p38 MAPK levels were normalized to p38. The autoradiographic signal intensities were determined by densitometric analysis of three independent experiments. *, $p < 0.05$, significantly different from high K^+. C, SB203580 (1 μM) does not modify cell death evoked by potassium depletion. Cell death was assessed by propidium iodide fluorescence after 24 h. Values are mean ± S.E. of three independent experiments. *, $p < 0.05$, significantly different from high K^+. Student's t test. D, SB203580 (1 μM) does not modify NP1 protein levels evoked by low potassium 4 h after the beginning of treatment. NP1 levels were normalized to actin levels and the intensity of the bands was determined by densitometric analysis of three independent experiments. *, $p < 0.05$, significantly different from high K^+.

Potassium Depletion Increases p38 MAPK Phosphorylation, but Blocking This Effect Neither Reduces NP1 Overexpression nor Cell Death. Recent reports suggest that p38 MAPK is involved in the death of cerebellar granule cells upon potassium depletion (Yamagishi et al., 2001). Hence, we investigated whether p38 MAPK phosphorylation might also influence NP1 overexpression. In the absence of serum, exposing cerebellar granule cells to nondepolarizing concentrations of potassium produced a marked increase in p38 MAPK phosphorylation. This effect was observed both 1 and 4 h after removing potassium (Fig. 3A) and was completely blocked by 1 μM SB203580, an inhibitor of p38 MAPK phosphorylation (Fig. 3B). However, the presence of SB203580 did not modify either the levels of cell death or NP1 overexpression (Fig. 3, C and D). We concluded that phosphorylation of p38 MAPK is not required for the neurotoxic effects of potassium depletion. At concentrations greater than 1 μM, SB203580 also inhibits JNK phosphorylation and has a neuroprotective effect (Coffey et al., 2002). Nevertheless, SB203580 did not modify the overexpression of NP1 evoked by potassium depletion even at high concentrations (data not shown).

CEP-11004-02, a Mixed Lineage Jun Kinase Inhibitor, Decreases Neuronal Death but Not NP1 Overexpression. The reduction of c-Jun phosphorylation decreases apoptotic neuronal cell death in sympathetic neurons and cerebellar granule cells (Harris et al., 2002a,b). Thus, we studied what effect pharmacological inhibition of JNK pathway might have on cerebellar granule cell death and NP1 expression evoked by potassium depletion. Not only did the mixed lineage Jun kinase inhibitor CEP-11004-02 markedly reduce JNK phosphorylation (Fig. 4A) but also, after 24 h, it completely blocked cerebellar granule cell death induced by potassium depletion (EC_{50} = 35 ± 3 nM and E_{max} = 95 ± 2%; Fig. 4B). However, the neuroprotection afforded by CEP-11004-2 was transient and was reduced to approximately 50% of the cells by 72 h after potassium depletion (Harris et al., 2002a; Fig. 4C). Despite this transient neuroprotective activity, CEP-11004-02 did not significantly modify the overexpression of NP1 evoked by potassium depletion (Fig. 4D).

SB415286, an Inhibitor of GSK3 Activity, Provides Transient Protection against Cell Death and Blocks NP1 Overexpression Evoked by Low Potassium. Selective inhibitors of GSK3 have been shown to protect cerebellar granule neurons from death evoked by potassium depletion (Cross et al., 2001). Recent studies have shown that the anilinomaleimide SB415286 blocks GSK3 activity and does not significantly alter the activity of 24 different serine/threonine and tyrosine protein kinases, including c-Jun NH_2-

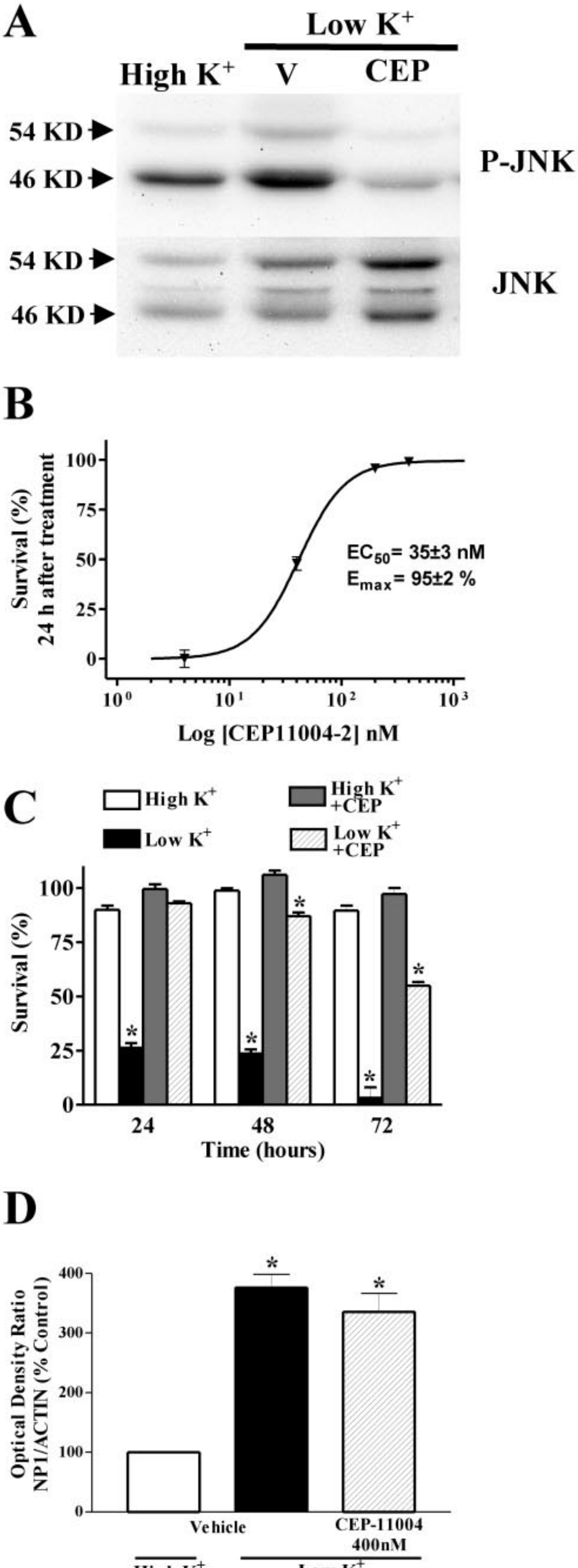

Fig. 4. CEP-11004-2 reduces JNK phosphorylation and provides transient neuroprotection but does not modify NP1 protein levels induced by potassium depletion. A, Western blot showing the effect of CEP-11004-2 on JNK phosphorylation. Cells were preincubated with 400 nM CEP11004-2 for 4 h at 37°C. Then, the cells were treated with high K^+ or low K^+ in the presence or absence of 400 nM CEP-11004-2, and protein extraction was performed 4 h after treatment. Membranes were incubated with rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) antiserum (1:1000). B, survival-promoting activity of CEP-11004-2 24 h after potassium deprivation. Cerebellar granule cells were exposed to high or low potassium in the presence or absence of increasing concentrations of CEP-11004-2. C, time course of neuroprotection by CEP-11004-2. Cell death was assessed by propidium iodide fluorescence at the times indicated after initiating the treatment and expressed as a percentage of survival. Survival of the cultures maintained in high K^+ without serum at the beginning of treatment was taken as 100%. Values are mean ± S.E. of three independent experiments. *, $p < 0.05$, significantly different from high K^+, t test. D, quantitative analysis of the effects of 400 nM CEP-11004-2 on NP1 protein levels evoked by low potassium 4 h after the beginning of treatment. NP1 levels were normalized to actin levels in three independent experiments. *, $p < 0.05$, significantly different from high K^+, t test.

terminal kinase at the concentration range used in our studies (10–30 μM) (Coghlan et al., 2000). However, to rule out the possibility that the neuroprotective effect of GSK3 inhibitors might be the result of an interaction with the JNK pathway, we studied the effect of a neuroprotective concentration of SB415286 on JNK phosphorylation. We found that treatment of cerebellar granule cells with 30 μM SB415286 does not significantly modify either basal or low potassium-evoked JNK phosphorylation (data not shown). We next examined the time course of neuroprotection by SB415286 and studied the effects of GSK3 activity on the overexpression of NP1. Incubation of cerebellar granule cells with SB415286 (30 μM) increased the levels of β-catenin, a substrate that is targeted for degradation after phosphorylation by GSK3, indicating that GSK3 activity was efficiently impaired in this system (Fig. 5A). Moreover, inhibiting GSK3 activity with SB415286 abolished cerebellar granule cell death 24 h after potassium depletion, with an EC_{50} = 16 ± 0.1 μM and E_{max} = 99 ± 1% (Fig. 5B). As with the neuroprotection afforded through IGF-1 and the JNK inhibitor, SB415286-associated neuroprotection diminished over time and was reduced to 30% of the cells after 72 h (Fig. 5D). However, in contrast to IGF-1 and CEP-11004-2, SB415286 completely blocked NP1 overexpression, both 4 and 6 h after potassium deprivation (Fig. 5C).

Depleting Potassium Increases GSK3β Activity and Tyr216 Phosphorylation of GSK3β in Cerebellar Granule Cells. Because the GSK3 inhibitor SB415286 completely blocked NP1 overexpression, depleting potassium may produce an increase in GSK3 activity. We measured the activity of purified GSK3β at several time points after serum/potassium deprivation. The depletion of extracellular potassium produced a marked increase (156%) in the activity of GSK3β 1 h after the beginning of treatment (Fig. 6A). This effect of low potassium was sustained and the increase of GSK3β activity by low potassium 2 h after the beginning of treatment was not significantly different from that observed after 1 h (Fig. 6A). The removal of serum also significantly increased GSK3β activity after 30 min; however, it returned to control values by 1 h after serum removal (data not shown).

Activation of GSK3β requires tyrosine phosphorylation on Tyr216. Proapoptotic stimuli such as staurosporine augment GSK3β activity by increasing Tyr-216 phosphorylation (Hughes et al., 1993; Bhat et al., 2000). The depletion of potassium, but not serum deprivation, significantly in-

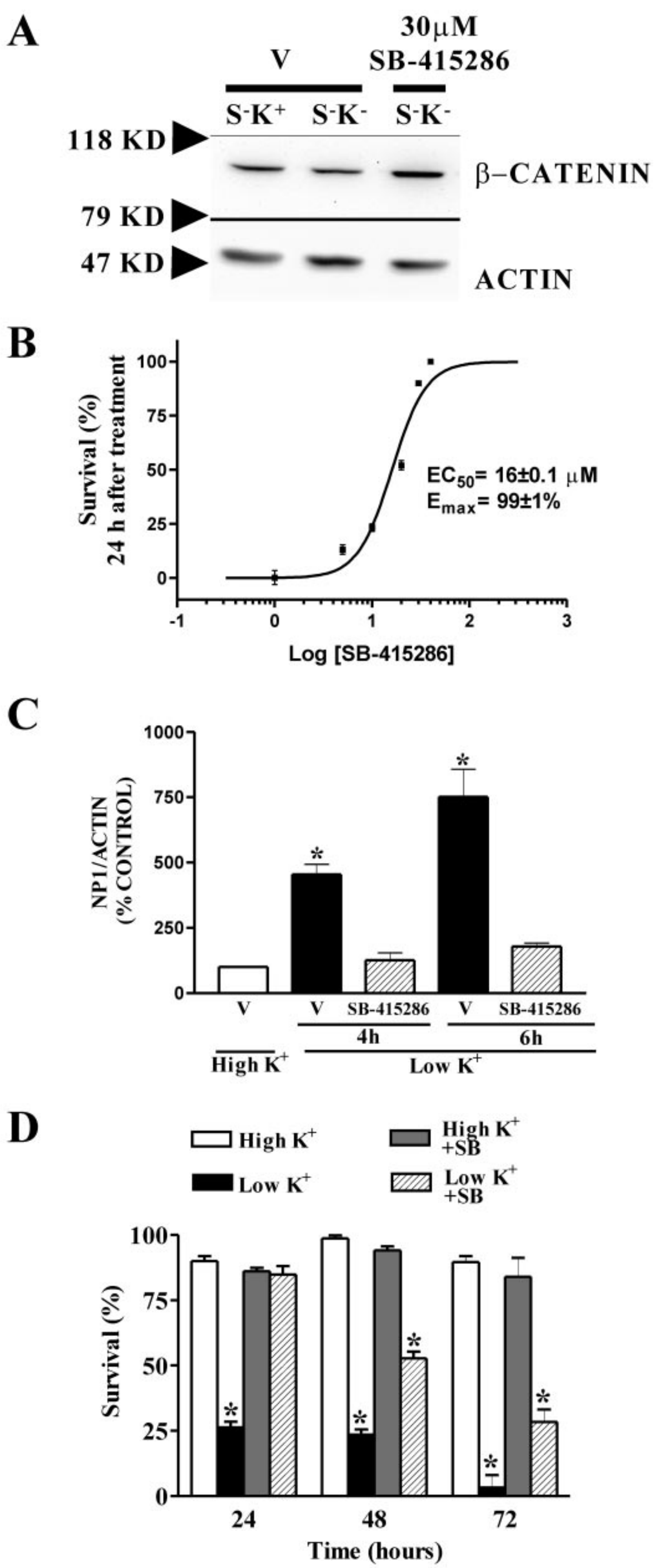

Fig. 5. SB415286, a GSK3 inhibitor, prevents cell death and decreases the expression of NP1 evoked by potassium depletion. A, Western blot showing that SB415286 effectively decreases GSK3 activity because it increases β-catenin levels. Cells were treated with high K^+ or low K^+ in the presence or absence of 30 μM SB415286, and protein extraction was performed 4 h after treatment. Membranes were incubated with goat anti β-catenin antiserum (1:500). B, survival-promoting activity of SB415286 24 h after potassium deprivation. Cells were incubated with high or low potassium in the presence or absence of increasing concentrations of SB415286. C, quantitative analysis of the effect of 30 μM SB415286 on NP1 protein levels evoked by low potassium 4 h after the beginning of treatment. NP1 levels were normalized to the levels of actin and the intensity of the bands was determined by densitometric analysis of at least three independent experiments. * Significantly different from high K^+. $p < 0.05$. D, time course of neuroprotection by SB415286. Cell death was assessed by propidium iodide fluorescence at the times indicated after initiation of treatment and expressed as a percentage of survival. Survival of cultures maintained in high K^+ without serum at the beginning of treatment is taken as 100%. Values are mean ± S.E. of at least three independent experiments. *, $p < 0.05$, significantly different from high K^+.

creased Tyr-216 phosphorylation of GSK3β 1 h after the beginning of treatment in cerebellar granule cells (Fig. 6B).

Next, we investigated the influence of inhibitory phosphorylation of GSK3 and studied the effect of IGF-1 on the increase in GSK3β activity evoked by potassium deprivation. We found that a neuroprotective concentration of IGF-1 (50 ng/ml) does not significantly modify the increase in GSK3β activity evoked by 2 h of treatment with low potassium (Fig. 6C).

Simultaneous Pharmacological Reduction of JNK and GSK3 Activities Provides Long-Term Protection against Death at Nondepolarizing Concentrations of Potassium. We further studied the contribution of JNK and GSK3 activities on neuronal death evoked by potassium depletion, by simultaneously inhibiting the activity of these two enzymes to determine whether this provided long-term neuroprotection. Simultaneous inhibition of JNK activity with CEP-11004-2, and of GSK3 activity with SB415286, permitted cerebellar granule cells to survive in the absence of serum and potassium for up to 72 h after the beginning of treatment. The combined pharmacological reduction of JNK and GSK3 activities sustained long-term survival in a way that was indistinguishable from the long-term survival afforded by depolarizing levels of potassium (Fig. 7). This indicates that the signaling pathways associated with JNK and GSK3 activities are the major contributors to cell death by low potassium.

Discussion

The main objective of this study was to examine whether signaling pathways known to mediate neuronal survival and death also regulate the expression of NP1. The experiments presented here show that GSK3 activity, but not JNK, p38, or PI-3-K activities, regulates NP1 expression. We also show for the first time that depriving cerebellar granule cells of potassium increases the Tyr-216 phosphorylation and activity of GSK3β and that pharmacological inhibition of the activity of this enzyme completely blocks NP1 overexpression, retarding apoptosis.

In agreement with earlier results, potassium depletion produces a marked increase in NP1 protein levels in cerebellar granule neurons. This increase precedes the earliest morphological signs of apoptosis and is part of the gene expression program induced by potassium depletion that leads to neuronal death in cerebellar granule cells (DeGregorio-Rocasolano et al., 2001). It is widely accepted that the cell fate of mature neurons depends on the balance between survival and death signals. Thus, preventing apoptotic cell death may be achieved by two different routes: 1) by activation of neu-

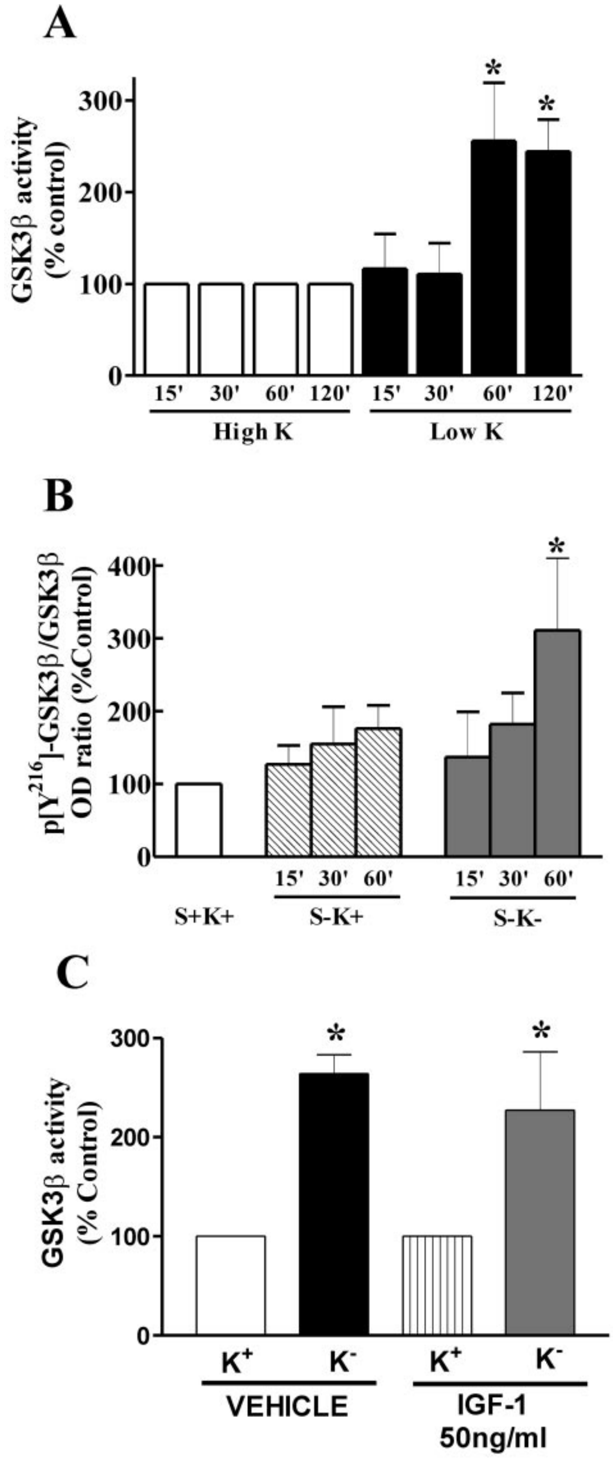

Fig. 6. Potassium depletion increases GSK3β activity and GSK3β phosphorylation on Tyr-216. A, effect of potassium depletion on GSK3β activity was studied in cerebellar granule cells incubated with high or low K^+. Protein extracts were obtained at the times indicated, GSK3β was immunoprecipitated, and its activity was assayed. Values are dpm of [γ-^{32}P] incorporated to phosphoglycogen synthase peptide-2 normalized with the densitometric value of the corresponding immunoprecipitated GSK3β. The ratio of dpm over GSK3β immunoreactivity was expressed as percentage of control. Values are mean ± S.E. of at least three independent experiments. *, $p < 0.05$, significantly different from high K^+. B, quantitative analysis of the phosphorylation of GSK3β on Tyr-216 induced by K^+ depletion. Protein extracts were obtained at the times after K^+ withdrawal indicated. Membranes were incubated with mouse anti-GSK3β (pTyr-216) phosphospecific antibody (1:1000). The intensity of the bands was determined by densitometric analysis of at least three independent experiments. *, $p < 0.05$, significantly different from high K^+. C, influence of IGF-1 (50 ng/ml) on the increase of GSK3β activity evoked by low potassium. Protein extracts were obtained 2 h after K^+ withdrawal. Immunoprecipitation and assay was performed as in A. *, $p < 0.05$, significantly different from high K^+.

rotrophin signaling pathways that suppress the apoptotic program through both transcription-dependent and -independent mechanisms; and 2) by blocking the signaling pathways that trigger the apoptotic gene expression program. Whether either of these two neuroprotective routes is sufficient to sustain long-term survival is unclear and may depend on the cell type. We examined the ability of these neuroprotective strategies to suppress NP1 overexpression and provide long-term protection against the cerebellar granule cell death evoked by potassium depletion.

The PI-3-K/AKT signaling cascade is an important pathway in mediating neuronal survival. Thus, activating this pathway with IGF-1 protects cerebellar granule cells from cell death evoked by potassium depletion for up to 24 h. However, the neuroprotective effect of IGF-1 is transient and decreases to approximately 50% after 4 days. In contrast, replacement of potassium after potassium deprivation sustains the survival of the majority of cerebellar granule cells for at least 4 d in the absence of serum. It is now widely accepted that to sustain survival, IGF-1 and potassium depolarization converge by activating the serine/threonine protein kinase AKT through different signaling mechanisms (Dudek et al., 1997; Miller et al., 1997; Crowder and Freeman, 1998; Vaillant et al., 1999; Kumari et al., 2001). However, the finding that depolarizing potassium concentrations are capable of sustaining the survival of cerebellar granule cells for a longer period than IGF-1 indicates that membrane depolarization has other effects in addition to activating AKT. In support of this hypothesis, and despite its neuroprotective effect, IGF-1 did not modify the overexpression of NP1 induced by potassium depletion. In contrast, potassium replacement rescued cells from death and suppressed the increase of NP1 expression induced by prior potassium deprivation. Our interpretation of these results is that serum/potassium deprivation triggers the apoptotic cerebellar granule cell death program by simultaneously suppressing survival signals and activating death signaling pathways. Exposure to IGF-1 is neuroprotective because it restores prosurvival signaling, but such neuroprotection is transient because it fails to suppress the death signaling pathway that induces overexpression of NP1. In contrast, our results indicate that, in addition to activating survival signaling, the replacement of potassium suppresses the death signal that triggers overexpression of NP1.

To identify the mechanisms responsible for the increased expression of NP1, we examined the effects of inhibiting the activity of death signal transduction pathways previously shown to be involved in apoptosis related to potassium deprivation, such as the JNK and p38 MAPK pathways. Pharmacological inhibition of JNK signaling did not significantly alter NP1 overexpression. Moreover, CEP-11004-02 provided only short-term protection against cerebellar granule cell death induced by potassium deprivation, providing further evidence that potassium depletion activates an additional cell death signaling pathway (Harris et al., 2002a). Such a pathway is not associated with the activation of the MAPK p38, because pharmacological inhibition of p38 phosphorylation neither reduces cell death nor impairs NP1 overexpression induced by nondepolarizing conditions. Consequently, these results indicate that in cerebellar granule cells, potassium depletion activates a cell death signaling pathway independent of JNK that induces expression of NP1 before apoptotic death.

We have previously shown that lithium decreases expression of NP1 before reducing cell death evoked by low [K^+] (DeGregorio-Rocasolano et al., 2001). Lithium acts on multiple biochemical mechanisms, but one of them is to inhibit GSK3 activity. Lithium is an ATP noncompetitive inhibitor of GSK3β activity (K_i of 1–2 mM) (Klein and Melton, 1996), and it also has the ability to increase the inhibitory phosphorylation of the enzyme (Chuang et al., 2002). There is now increasing evidence indicating that lithium's neuroprotective effects are mediated by its action on reducing GSK3 activity (Jope, 2003). Thus, based on our previous results with lithium as well as on recent findings that GSK3 activity contributes to apoptotic signal transduction (Grimes and Jope, 2001), we examined the influence of GSK3 on NP1 expression and cell death evoked by potassium deprivation. Pharmacological inhibition of GSK3 activity with SB415286, a selective small molecule inhibitor of GSK3 (Coghlan et al., 2000), completely blocked overexpression of NP1 in nondepolarizing conditions and promoted neuroprotection against apoptosis. These effects of SB415286 were comparable with those we previously observed after treatment with a less selective inhibitor of GSK3 activity such as lithium and provide further evidence showing that GSK3 activity regulates expression of NP1 and cell death evoked by low potassium.

In agreement with previous results (Cross et al., 2001), inhibition of GSK3 activity completely blocked cell death for 24 h in nondepolarizing conditions (Fig. 5, B and D). However, the neuroprotective effect of inhibiting GSK3 was also transient and was reduced to only 30% of the cells within 72 h

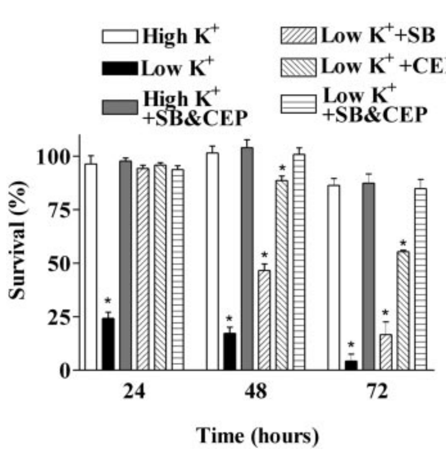

Fig. 7. Combined treatment with SB415286 and CEP-11004-2 provides long-term protection against cell death evoked by low potassium. Cerebellar granule cells were treated with high and low potassium in the absence of serum, both in the presence or absence of 30 μM SB415286 and 400 nM CEP-11004-2. The cells were incubated at 37°C for different times up to 72 h, and cell death was assessed by propidium iodide fluorescence at the times indicated after initiation of treatment and expressed as a percentage of survival. Survival of cultures maintained in high K^+ without serum at the beginning of treatment is taken as 100%. Values are mean ± S.E. of at least three independent experiments. #, $p < 0.05$, significantly different from high K^+.

(Fig. 5D). These results indicate that, besides activating the JNK pathway, nondepolarizing concentrations of potassium activate another cell death signaling cascade associated with an increase in GSK3 activity. The question remains whether potassium deprivation increases GSK3 activity through the activation of a cell death signal or by inhibition of a prosurvival pathway. GSK3 activity can be inhibited by serine phosphorylation through survival signaling cascades and activated by tyrosine phosphorylation by apoptotic stimuli. Thus, it is possible that potassium depletion activates GSK3 by reducing the inhibitory serine phosphorylation. However, the fact that the activation of the PI-3-K/AKT pathway by IGF-1 inhibits GSK3 by phosphorylating this serine residue but does not modify NP1 overexpression induced by potassium deprivation provides strong evidence against such possibility. Therefore, our results suggested that potassium depletion augments GSK3 activity through a mechanism that is independent of PI-3-K/AKT kinase activity and that involves activation of GSK3 rather than a reduction of its inhibition.

In support of this interpretation, nondepolarizing concentrations of potassium increased GSK3β activity in cerebellar granule cells after 1 h. Moreover, potassium depletion increased the phosphorylation of GSK3β on Tyr-216 in a similar time course to the effect observed on the increase of GSK3β activity. These results are in line with recent studies showing that several proapoptotic stimuli increase GSK3β activity by increasing Tyr-216 phosphorylation (Hughes et al., 1993; Bhat et al., 2000). In addition, our results are consistent with studies showing that GSK3β is proapoptotic (Pap and Cooper, 1998; Bijur et al., 2000; Bijur and Jope, 2001, 2003) and indicate that GSK3β contributes to cerebellar granule cell death via potassium deprivation through a proapoptotic signal transduction cascade that involves the regulation of NP1 expression.

Furthermore, we found that a neuroprotective concentration of IGF-1 does not significantly modify the increase in GSK3β activity evoked by low potassium. This indicates that reduction of neuronal activity activates a pool of GSK3β that is not regulated by neurotrophic factor-induced inhibitory phosphorylation. This finding provides further evidence to support our interpretation that potassium depletion augments GSK3 activity through a mechanism that is independent of PI-3-K/AKT kinase activity and that involves activation of GSK3 rather than a reduction of its inhibition.

The observation that pharmacological inhibition of either JNK or GSK3 activity provides only transient protection against cerebellar granule cell death in nondepolarizing potassium conditions suggests that proapoptotic signaling cascades that involve GSK3 and JNK activities independently contribute to the death of cerebellar granule cells. Thus, our results show that, in cerebellar granule cells, potassium deprivation activates two death signaling pathways that act in concert: the JNK pathway and another pathway involving GSK3 activity and NP1 overexpression. Treatments that block just one of these pathways provide only short-term neuroprotection. Interestingly, simultaneous pharmacological blockage of both JNK and GSK3 activities offers long-term protection against cell death evoked by nondepolarizing conditions, sustaining survival in a way that is not significantly different to survival sustained by high potassium. The long-term survival afforded by the combined treatment with GSK3 and JNK inhibitors argues against the possibility that the transient neuroprotective effect of each of these drugs when administered alone is the result of drug breakdown. On the other hand, the fact that CEP-11004-2 and SB415286 sustained long-term survival when administered simultaneously, in a manner indistinguishable from the survival sustained by replenishing potassium, indicates that the cell death signaling pathways that increase JNK and GSK3 activities are the major contributors to cell death by low potassium.

In summary, the results presented here show that potassium deprivation increases GSK3β phosphorylation on Tyr-216 and that overexpression of NP1 is regulated by GSK3 activity independently of PI-3-K/AKT or JNK. In addition, simultaneous pharmacological blockage of both JNK and GSK3 activity provides long-term protection against cell death evoked by potassium deprivation, indicating that the JNK and GSK3 cell death signaling pathways are the major contributors to apoptosis induced by potassium deprivation in cerebellar granule cells.

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