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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Running Title: GSK3 mediates NP1 expression and neuronal death

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Abbreviations:

IGF-1, insulin-like growth factor 1; NP1, neuronal pentraxin 1; AKT, protein kinase B; Narp, neuronal actitivity-related pentraxin; PI-3-K, phosphatidylinositol 3-kinase; NGF, nerve growth factor; JNK, c-jun N-terminal kinase; GSK3, glycogen synthase kinase 3.

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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 N-terminal kinase (JNK) offer transitory neuroprotection from the cell death evoked by non-depolarizing concentrations of potassium. However, neither of these neuroprotective treatments prevent the overexpression of NP1 upon potassium depletion, indicating that non-depolarizing 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 non-depolarizing 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 pathways.

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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 hours if serum is removed and the concentration of K^+ is kept below depolarizing levels. In 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); 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 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). While 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 in addition to activate survival signals it also supresses death signals. However, the intracellular signaling pathways that activate

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apoptosis by potassium deprivation may vary between different cell types (Ham *et al.*, 2000).

In sympathetic neurons, the activation of the c-jun N-terminal kinase (JNK) pathway appears to be necessary and sufficient to induce apoptosis upon nerve growth factor (NGF) 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 activate JNK signaling, offers sympathetic neurons long term protection against cell death evoked by NGF 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 non-depolarizing [K^+], cerebellar granule cells increase the levels of Neuronal Pentraxin 1 (NP1) protein prior to 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- to 6- fold 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 non-depolarizing conditions (DeGregorio-Rocasolano *et al.*, 2001).

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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 (Narp; also called neuronal pentraxin 2 - NP2), encodes a series of coiled-coil domains that appear to be essential for homo-multimerization (O'Brien *et al.*, 2002). The carboxy-terminal half encodes a calcium dependent lectin-binding domain (Emsley *et al.*, 1994;Tsui *et al.*, 1996). Since NP1 mediates neuronal death evoked by non-depolarizing conditions in cerebellar granule cells, while Narp 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.

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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 DnaseI, and plated in poly-L-Lysine (100µg/ml) coated dishes at a density of $3x10^5$ cells/cm^2 in basal Eagle's medium (BME) supplemented with: 10% heat-inactivated foetal bovine serum, 0.1 mg/ml gentamicin, 2 mM L-glutamine and 25 mM KCl. Cytosine-D-Arabinofuranoside (10µM) was added to the culture medium 24 hours after plating to prevent the replication of non-neuronal cells. The cultures were maintained at 37º 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 D.C., U.S.A., 1996).

Induction of neuronal death by potassium depletion and neuroprotective treatments.

After 8 days in culture, the medium in which cerebellar granule cells were 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 media. The inhibitor of the JNK signaling pathway, CEP-11004-2, was kindly provided

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by Cephalon (West Chester, Pennsylvania, USA). Stock solutions of CEP-11004-2 (4 mM) were prepared in dimethylsulphoxide (DMSO), stored at –20ºC and a working 40 μM solution of CEP11004-2 was prepared in 1% bovine serum albumin (BSA)/BME on the day of experiment. Cells were preincubated with 400 nM CEP11004-2 for 4 hours at 37ºC before potassium depletion. The Glycogen synthase kinase 3 (GSK3) inhibitor, SB-415286, was kindly provided by GlaxoSmithKline (Stevenage, UK), from which stock solutions (20 mM) were prepared in DMSO and stored at –20ºC. The inhibitor of p38 MAP kinase (MAPK) phosphorylation SB-203580 was from Calbiochem (Darmstadt, Germany), and stock solutions (10 mM) of this drug were prepared in DMSO, stored at –20ºC, 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 LY-294002 was from Sigma (Madrid, Spain), and 10 mM stock solutions of LY-294002 were prepared in DMSO and stored at –20ºC.

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) with 530 nm excitation (25-nm band pass) and 645 nm (40-nm band pass) emission filters. The percentage of non-viable cells was measured using a modification of the method described by Rudolph et al. (Rudolph *et al.*, 1997). Baseline fluorescence F_1 was measured 1 hour 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 minutes with 500 μM digitonin at 37ºC to obtain the

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maximum fluorescence corresponding to 100% of cell death (*Fmax*). The percentage of cell death was calculated as follows: % cell death = 100 X $(F_n - F_1)/(F_{max}-F_1)$, where F_n is the fluorescence at any given time. Cells were kept in the incubator between measurements.

SDS-PAGE 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, 0.01 % bromophenol blue) and sonicated briefly. The homogenate was boiled and stored at –20ºC before separting the proteins by 10% SDS-polyacrylamide gel electrophoresis and transferring them to Hybond^{TM}Ecl^{TM} nitrocellulose membranes (Amersham Biosciences, Germany). The membranes were preincubated with 5 % non-fat dry milk (NFM) in Tris-buffered saline before immunostaining. For specific immunodetection of the NP1 protein, a mouse anti-rat NP1 monoclonal antibody (Transduction Laboratories, Los Angeles, CA) was diluted 1:1500 in a solution containing 3 % BSA in Tris-buffered saline (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 (Thr 183/Tyr 185), rabbit anti-p38 MAP Kinase, rabbit anti-Phospho- p38-MAP Kinase (Thr 180/Tyr 182) antisera, all of them from Cell Signaling (Beverly, MA, USA); the mouse monoclonal anti-GSK3β antibody and the phospho-specific antibody anti-GSK3β phosphorylated on tyrosine 216 from BD Biosciences (Pharmingen); the goat polyclonal anti-β–Catenin antiserum from Santa Cruz Biotechnology. Peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG from Cell Signaling or donkey anti-goat from Jackson Immunoresearch (West Grove, PA, USA) were used as secondary antibodies in a solution of 5% NFM in TBS with 0.1 % Tween 20. In all

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experiments a rabbit anti-actin antibody (Sigma) was used to control for the amount of protein loaded. Immunolabelled proteins were visualized using an enhanced chemiluminiscence detection system (Immun-Star^{TM}HRP Substrate Kit, Bio-Rad, Madrid, Spain), and the intensity of the bands was quantified with a Fluor-S^{TM} MultiImager (Bio-Rad, Madrid, Spain). In the case of phospho-p38 MAP Kinase, the bands were visualized on film and quantification was performed using Kodak DS1 computer software. Densitometric values of the immunoreactive bands representing NP1 and non-phosphorylated proteins were normalized to the values of the corresponding actin bands.

GSK-3β immunoprecipitation and activity assay.

The activity of GSK-3β 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 23G syringe, incubated for 10 min on ice and centrifuged at 20,800xg for 15 min. The protein concentration was determined using the BCA protein assay kit (Pierce). Each sample (100 μg protein) was cleared with 40 μl of protein G-Sepharose beads for 90 min at 4ºC, before incubating with 1.2 μg of mouse anti-GSK3β antibody overnight at 4ºC. This procedure allowed a complete immunoprecipation 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 $MgCl_2$, 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

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μM ATP, 1.4 μCi of [γ-^{32}P] ATP, and 100 μM phosphoglycogen synthase peptide-2 (YRRAAVPPSPSLSRHSSPHQSEDEEE; Upstate Biotechnology, Inc., Lake Placid, NY). Kinase buffer without peptide was used as a control. The samples were incubated for 30 min at 30ºC, placed on ice for 2 min before centrifuging for 3 min at 1800xg. The reaction supernatants were spotted onto 1 cm x 2 cm P81 filter paper (3 spots of 5 μl each; Upstate) and the filters were washed 4 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.

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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 ten-fold (from 6±1% to 50±3%) in cultures of cerebellar granule cells within 24 hrs. This neurotoxicity was partially counteracted by IGF-1 (50ng/ml), recovering to 17±2%. The neuroprotective effect of IGF-1 was completely blocked by co-incubation with the PI-3 kinase inhibitor LY-294002 (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 threefold increase in the protein levels of NP1 within four hours (Fig. 1C). However, neither IGF-1 (50 ng/ml) nor LY-294002 significantly modified these levels (Fig. 1B), nor was the accumulation of NP1 affected by the combined treatment of IGF-1 and LY-294002 (Fig. 1C). Hence, the activation of the PI-3 kinase pathway by IGF-1 promotes survival but does not appear to regulate NP1 expression. Since NP1 mediates cell death following K^+ depletion, we hypothesized that treatments that do not affect the overexpression of NP1 should provide only transient neuroprotection, while 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 hours after the beginning of treatment, the survival promoting effects of IGF-1 were

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reduced by 48% (Fig 1 D). To check whether this reduction of the neuroprotective effect of IGF-1 was due to degradation of IGF-1 over time, we re-added IGF-1 (50 ng/ml) 24 hours after the first treatment. The results we obtained with re-addition of IGF-1 were the same we observed with only one addition, indicating that the reduction in the neuroprotective effect of IGF-1 was not due to instability of the growth factor in the culture medium (results 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 hours of potassium deprivation (Fig 2 A), 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 three hours after depletion (Fig 2 B).

Potassium depletion increases p38 MAP kinase phosphorylation but blocking this effect neither reduces NP1 overexpression nor cell death.

Recent reports suggest that p38 MAP kinase is involved in the death of cerebellar granule cells upon potassium depletion (Yamagishi *et al.*, 2001). Hence, we investigated whether p38 MAP kinase phosphorylation might also influence NP1 overexpression. In the absence of serum, exposing cerebellar granule cells to non-depolarizing concentrations of potassium produced a marked increase in p38 MAP kinase phosphorylation. This effect was observed both 1 h and 4 h after removing

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potassium (Fig. 3A) and was completely blocked by 1 μM SB-203580, an inhibitor of p38 MAP kinase phosphorylation (Fig 3B). However, the presence of SB-203580 did not modify either the levels of cell death or NP1 overexpression (Figs. 3C and D). We concluded that phosphorylation of p38 MAP kinase is not required for the neurotoxic effects of potassium depletion. At concentrations greater than 1 μM, SB-203580 also inhibits JNK phosphorylation and has a neuroprotective effect (Coffey *et al.*, 2002). Nevertheless, SB-203580 did not modify the overexpression of NP1 evoked by potassium depletion even at high concentrations (results 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;Harris *et al.*, 2002b). 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 reduced JNK phosphorylation (Fig. 4A) but after 24h, it also completely blocked cerebellar granule cell death induced by potassium depletion (EC_{50} = 35±3nM 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).

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SB-415286, 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 SB-415286 blocks GSK3 activity and does not significantly alter the activity of 24 different serine/threonine and tyrosine protein kinases including c-Jun N-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 due to an interaction with the JNK pathway we studied the effect of a neuroprotective concentration of SB-415286 on JNK phosphorylation. We found that treatment of cerebellar granule cells with 30 μM SB-415286 does not significantly modify either basal or low potassium evoked JNK phosphorylation (results not shown). We next examined the time course of neuroprotection by SB-415286 and studied the effects of GSK3 activity on the overexpression of NP1. Incubation of cerebellar granule cells with SB-415286 (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 SB-415286 abolished cerebellar granule cell death 24h after potassium depletion, with an EC_{50} = 16±0.1 nM and E_{max}=99±1%, (Fig. 5B). As with the neuroprotection afforded through IGF-1 and the JNK inhibitor, SB-415286 associated neuroprotection diminished over time and was reduced to 30% of the cells after 72 hours (Fig 5D). However, in contrast to IGF1 and CEP-11004-2, SB-415286 completely blocked NP1 overexpression, both 4h and 6 h after potassium deprivation (Fig. 5C).

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Depleting potassium increases GSK3β activity and Tyr216 phosphorylation of GSK3β in cerebellar granule cells.

Since the GSK3 inhibitor SB-415286 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 the observed after 1 h (Fig 6A). The removal of serum also significantly increased GSK3β activity after 30 mins, however it returned to control values by 1 h after serum removal (results not shown).

Activation of GSK3β requires tyrosine phosphorylation on Y-216. Pro-apoptotic stimuli such as staurosporine augment GSK3β activity by increasing Y-216 phosphorylation (Hughes *et al.*, 1993;Bhat *et al.*, 2000). The depletion of potassium, but not serum deprivation, significantly increased Y216 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).

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Simultaneous pharmacological reduction of JNK and GSK3 activities provides long-term protection against death at non-depolarizing 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 SB-415286, permitted cerebellar granule cells to survive in the absence of serum and potassium for up to 72 hours after the beginning of treatment. The combined pharmacological reduction of JNK and GSK3 activities sustained long term survival in a way that was undistinguishable 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.

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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 Y216 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 neurotrophin signaling pathways that suppress the apoptotic program through both transcription-dependent and independent mechanisms; 2) by blocking the signaling pathways that trigger the apoptotic gene expression program. Whether either of these two different 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 four days 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 (Vaillant *et al.*, 1999)(Miller *et al.*, 1997;Dudek *et al.*, 1997;Crowder and Freeman, 1998;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 pro-survival 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 MAP kinase 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 MAP kinase p38, because pharmacological inhibition of p38 phosphorylation neither reduces cell death nor impairs NP1 overexpression induced by non-depolarizing 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 (Ki 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 SB-415286, a selective small molecule inhibitor of GSK3 (Coghlan *et al.*, 2000), completely blocked overexpression of NP1 in non-depolarizing conditions and promoted neuroprotection against apoptosis. These effects of SB-415286 were comparable to 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 hours in non-depolarizing conditions (Fig 5B & D). However, the neuroprotective effect of inhibiting GSK3 was also transient and was reduced to only 30% of the cells within 72 hours (Fig 5D). These results indicate that, besides activating the JNK pathway, non-depolarizing concentrations of potassium also 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 because it inhibits a pro-survival 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, non-depolarizing concentrations of potassium increased GSK3β activity in cerebellar granule cells after 1h. Moreover, potassium depletion increased the phosphorylation of GSK3β on Y-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 pro-apoptotic stimuli increase GSK3β activity by

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increasing Y-216 phosphorylation (Hughes *et al.*, 1993;Bhat *et al.*, 2000). In addition, our results are consistent with studies showing that GSK3β is pro-apoptotic (Pap and Cooper, 1998); (Bijur *et al.*, 2000;Bijur and Jope, 2001;Bijur and Jope, 2003), and indicate that GSK3β contributes to cerebellar granule cell death via potassium deprivation through a pro-apoptotic 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 regulatable 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 it 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 non-depolarizing potassium conditions suggests that pro-apoptotic 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 non-depolarizing conditions, sustaining survival in a way that is not significantly different to survival sustained by high

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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 SB-415286 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 Y216 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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Footnotes

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Figure Legends

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 LY-294002 (30 μM) blocks this neuroprotective effect. Values are mean ± S.E. of three independent experiments. *, Significantly different from S^-K^+. $p< 0.05$, Student's *t* test. ***B***, representative western blot showing that IGF-1 and LY-294002 do not affect the increase of NP1 levels evoked by potassium depletion. ***C***, quantitative analysis of the effects of IGF-1 and LY-294002 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. *, Significantly different from *high* K^+. $p< 0.05$. ***D***, time course of neuroprotection by IGF-1.Values are mean ± S.E. of three independent experiments. *, Significantly different from *high* K^+. $p< 0.05$.

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

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independent experiments. *, Significantly different from *high* K^+. $p < 0.05$. ***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. *, Significantly different from *high* K^+; ***#***, significantly different from *high* and *low* K^+. $p < 0.05$.

FIG.3. **Potassium depletion increases p38 MAPK phosphorylation but SB-203580 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 SB-203580. ***A***, western blot showing the effect of SB-203580 on p38 MAPK phosphorylation. Cells were maintained in high or low K^+, with or without SB-203580 and total protein was extracted 1 and 4 hours after treatment. Western blots were incubated with rabbit anti-phospho-p38-MAP Kinase (Thr180/Tyr182) antiserum (1:1000). ***B***, quantitative analysis of the effects of SB-203580 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. *, Significantly different from *high* K^+. $p < 0.05$. ***C***, SB-203580 (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. *, Significantly different from *high* K^+. $p < 0.05$, Student's *t* test. ***D***, SB-203580 (1 μM) does not modify NP1

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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. *, Significantly different from *high* K^+. $p< 0.05$.

FIG.4. **CEP-11004-2 reduces JNK phosphorylation, 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 hours at 37ºC. 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 hours after treatment. Membranes were incubated with rabbit anti-phospho-SAPK / JNK (Thr 183/Tyr 185) 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. *, Significantly different from *high* K^+; $p< 0.05$, 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. *, Significantly different from *high* K^+. $p < 0.05$, t-test.

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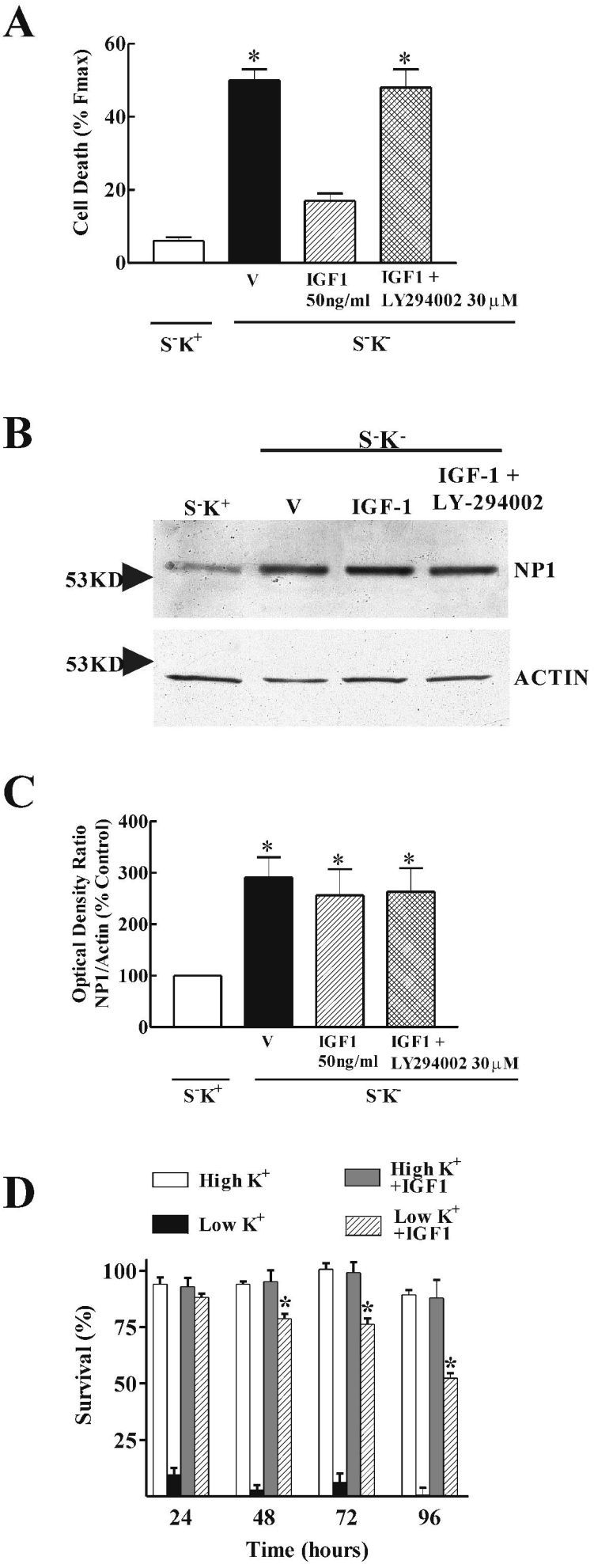
FIG.5. **SB-415286, a GSK-3 inhibitor, prevents cell death and decreases the expression of NP1 evoked by potassium depletion.** ***A***, western blot showing that SB-415286 effectively decreases GSK3 activity because it increases β-catenin levels. Cells were treated with high K^+ or low K^+ in the presence or absence of 30 μM SB-415286 and protein extraction was performed 4 hours after treatment. Membranes were incubated with goat anti β-catenin antiserum (1:500). ***B***, survival-promoting activity of SB-415286 24 h after potassium deprivation. Cells were incubated with high or low potassium in the presence or absence of increasing concentrations of SB-415286. ***C***, quantitative analysis of the effect of 30 μM SB-415286 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 SB-415286. 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. *, Significantly different from *high* K^+. $p< 0.05$.

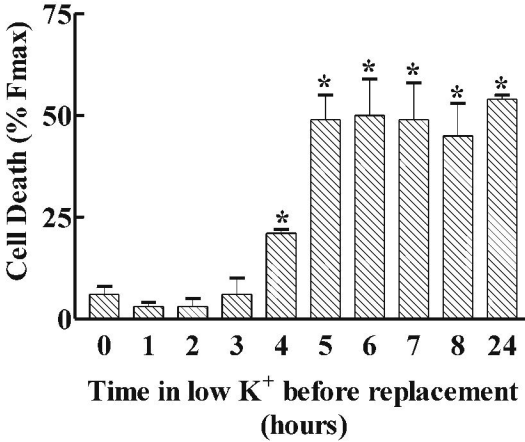
FIG.6. **Potassium depletion increases GSK3β activity and GSK3β phosphorylation on Y216.** ***A,*** The effect of potassium depletion on GSK3β activity was studied in cerebellar granule cells incubated with high or low K^+. Protein extracts were obtained at the times indicated, GSK3β was immunoprecipitated and its activity 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. *, Significantly different from *high* K^+. $p< 0.05$. ***B***, quantitative analysis of the phosphorylation of GSK3β on Y216 induced by K^+ depletion. Protein extracts were obtained at the times after K^+ withdrawal indicated. Membranes were incubated with mouse anti-GSK3β (pY216) phospho-specific antibody (1:1000). The intensity of the bands was determined by densitometric analysis of at least three independent experiments. *, Significantly different from *high* K^+. $p< 0.05$. ***C.*** Influence of IGF-1 (50ng/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.*** *, Significantly different from *high* K^+. $p< 0.05$.

FIG.7. **Combined treatment with SB-415286 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 SB-415286 and 400 nM CEP-11004-2. The cells were incubated at 37ºC 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. #, Significantly different from *high* K^+. $p< 0.05$.



A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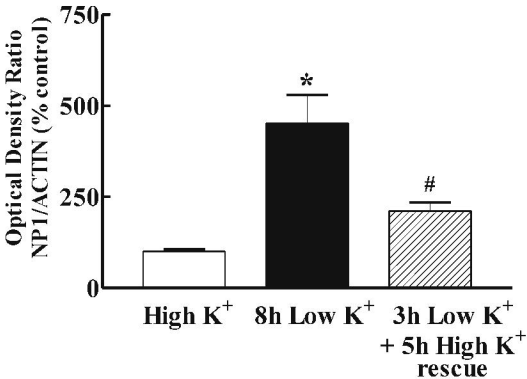
50
25
0
0 1 2 3 4 5 6 7 8 24
Time in low K+ before replacement (hours)

B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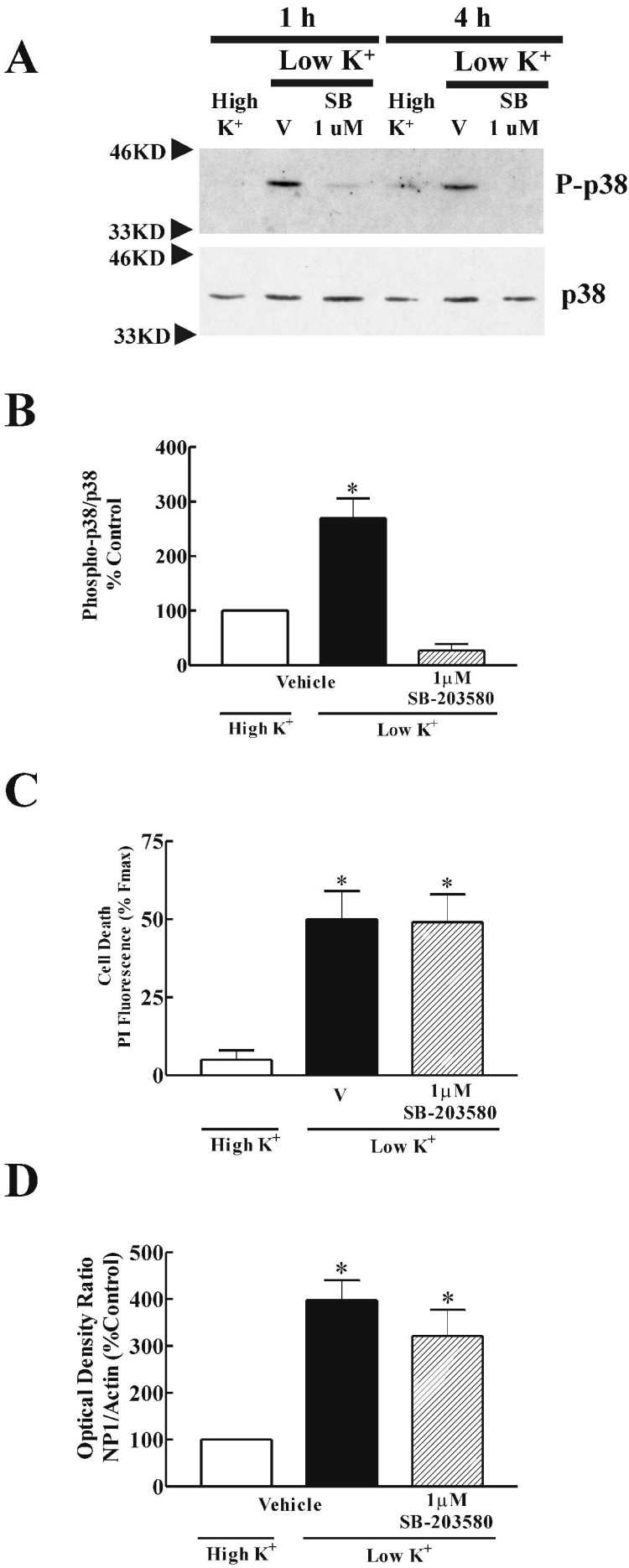
A
1 h
4 h
Low K+
Low K+
High K+
V
SB 1 uM
High K+
V
SB 1 uM
46KD
33KD
46KD
33KD
P-p38
p38
B
Phospho-p38/p38 % Control
400
300
200
100
0
*
Vehicle
1μM SB-203580
High K+
Low K+
C
Cell Death PI Fluorescence (% Fmax)
75
50
25
0
*
*
V
1μM SB-203580
High K+
Low K+
D
Optical Density Ratio NP1/Actin (%Control)
500
400
300
200
100
0
*
*
Vehicle
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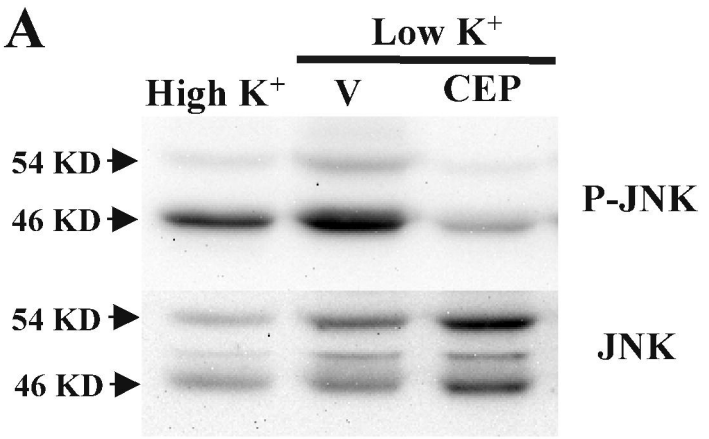
High K+
Low K+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CEP
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P-JNK
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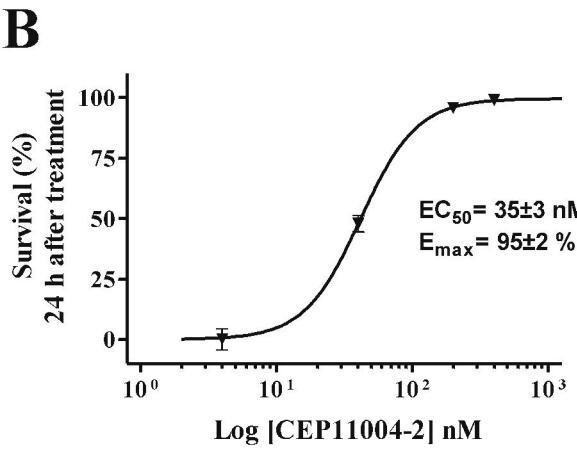
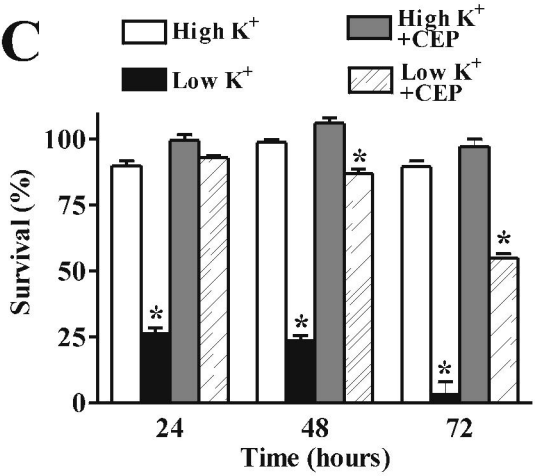
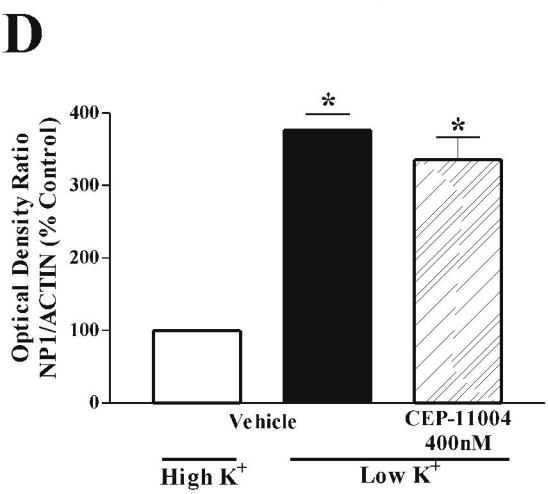
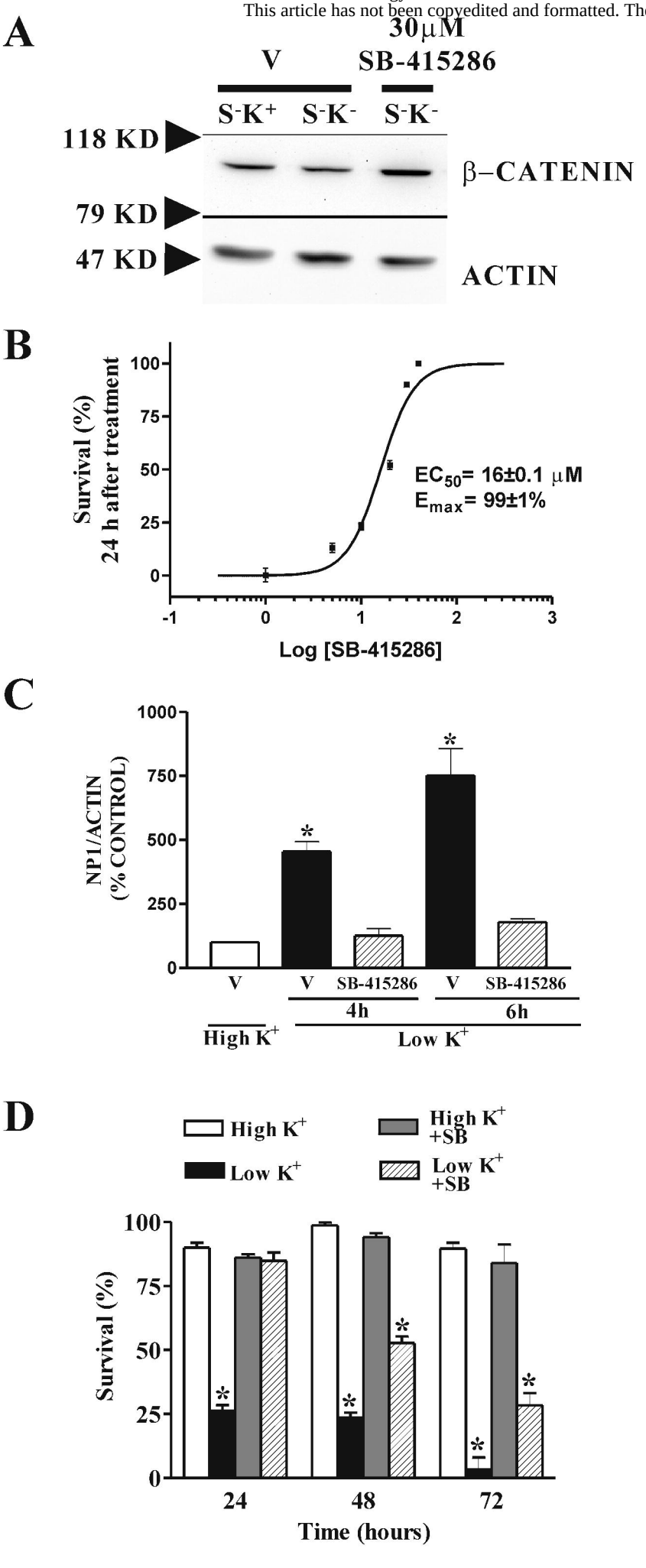
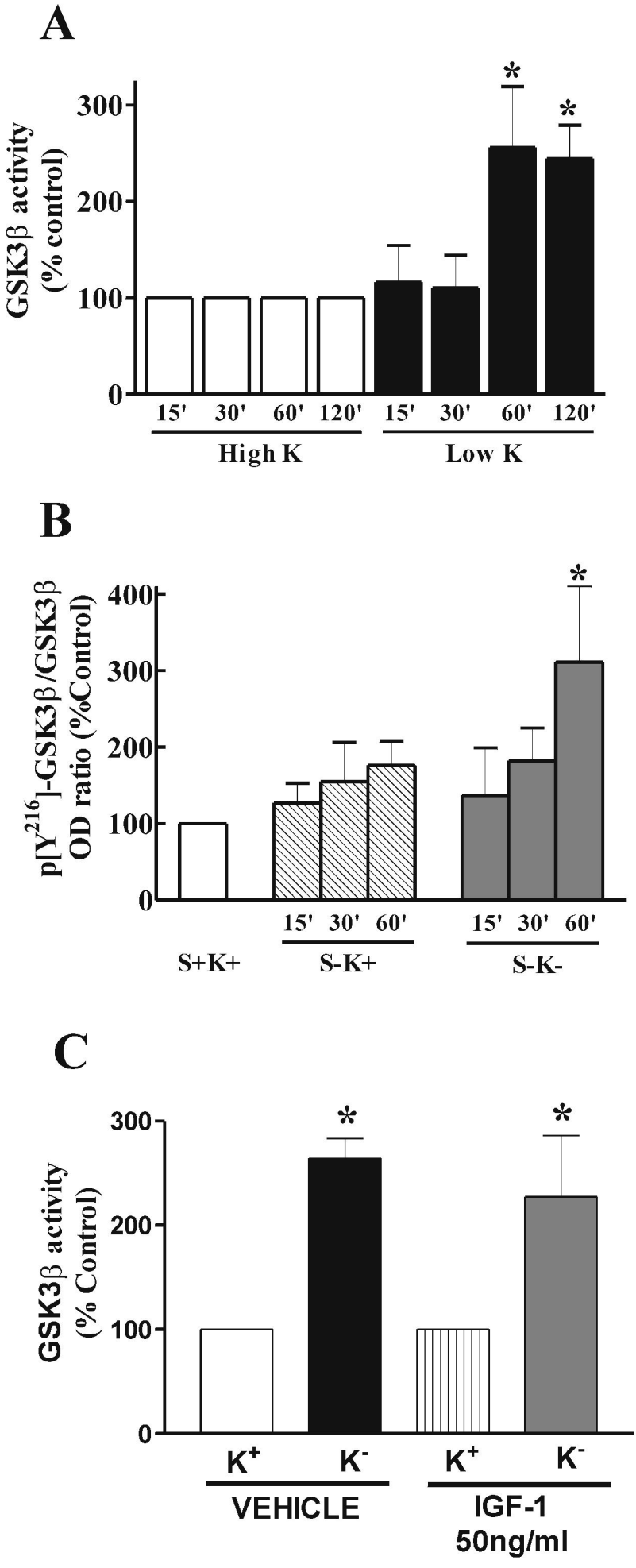
46 KD
JNK

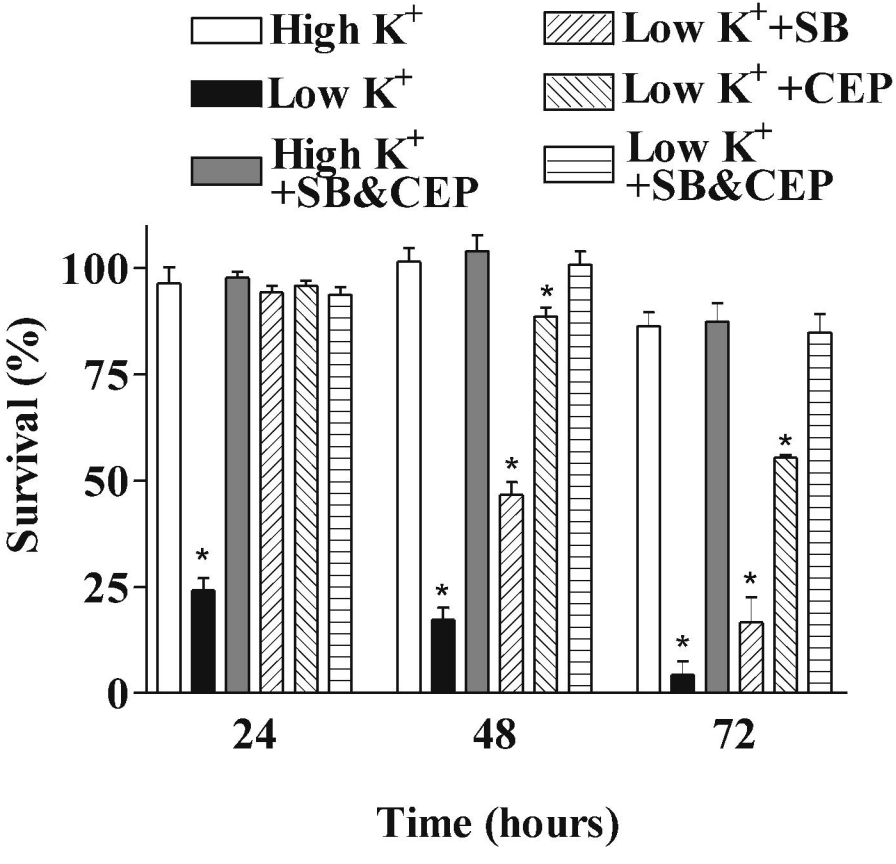

B
Survival (%)
24 h after treatment
100
75
50
25
0
EC50= 35±3 nM
Emax= 95±2 %
10 0
10 1
10 2
10 3
Log [CEP11004-2] nM


C
High K+
High K+ +CEP
Low K+
Low K+ +CEP
Survival (%)
100
75
50
25
0
*
24
48
72
Time (hours)


D
Optical Density Ratio
NP1/ACTIN (% Control)
400
300
200
100
0
*
Vehicle
CEP-11004
400nM
High K+
Low K+




A
GSK3β activity
(% control)
300
200
100
0
*
*
15' 30' 60' 120' 15' 30' 60' 120'
High K
Low K
B
p[Y216]-GSK3β/GSK3β
OD ratio (%Control)
400
300
200
100
0
*
15' 30' 60' 15' 30' 60'
S+K+
S-K+
S-K-
C
GSK3β activity
(% Control)
300
200
100
0
*
*
K+ K- K+ K-
VEHICLE
IGF-1
50ng/ml


High K+
Low K+
High K+ +SB&CEP
Low K+ +SB
Low K+ +CEP
Low K+ +SB&CEP
Survival (%)
100
75
50
25
0
24
48
72
Time (hours)