

c-JUN N-TERMINAL KINASES/c-JUN AND p38 PATHWAYS COOPERATE IN CERAMIDE-INDUCED NEURONAL APOPTOSIS

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Abstract—Understanding the regulation of the apoptotic program in neurons by intracellular pathways is currently a subject of great interest. Recent results suggest that c-Jun N-terminal kinases (JNK), mitogen-activated protein kinases and the transcription factor c-Jun are important regulators of this cell death program in post-mitotic neurons following survival-factor withdrawal. Our study demonstrates that ceramide levels increase upon survival-factor withdrawal in primary cultured cortical neurons. Furthermore, survival-factor withdrawal or addition of exogenous c₂-ceramide induces JNK pathway activation in these cells. Western blot analyses of JNK and c-Jun using phospho-specific antibodies reveal that JNK and subsequent c-Jun phosphorylation occur hours before the initiation of apoptosis, reflected morphologically by neurite retraction and fragmentation, cell-body shrinkage and chromatin fragmentation. Immunocytochemistry using the same antibodies shows that phospho-JNK are localized in the neurites of control neurons and translocate to the nucleus where phospho-c-Jun concurrently appears upon ceramide-induced apoptosis. To determine if ceramide-induced c-Jun activation is responsible for the induction of the apoptotic program, we performed transient transfections of a dominant negative form of c-Jun, truncated in its transactivation region. Our results show that DNc-Jun partially protects cortical neurons from ceramide-induced apoptosis. Treatment of dominant negative c-Jun-expressing neurons with the pharmacological inhibitor of p38 kinase, SB203580, completely blocked neuronal death. Thus our data show that p38 and JNK/c-Jun pathways cooperate to induce neuronal apoptosis. © 2003 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: primary cultures, SAPK, serum withdrawal, neuronal death.

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Abbreviations: Ara-C, cytosine arabinoside; BSA, bovine serum albumin; DNc-Jun, dominant negative form of c-Jun; FCS, fetal calf serum; GFP, green fluorescent protein; HS, horse serum; JNK, c-Jun N-terminal kinases; MAP kinase, mitogen-activated protein kinase; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclo-hepten-5,10-iminemaleate; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PC12, pheochromocytoma 12; P-c-Jun, phospho-c-Jun; P-JNK, phospho-c-Jun N-terminal kinases; SB p38 inhibitor, SB203580; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Many recent studies have sought to identify the key components of the cell-death machinery in neurons and to understand how the cell-death program is regulated by intracellular signaling pathways activated by death factors, binding to specific cell-surface receptors. Recent results suggest that mitogen-activated protein kinase (MAP), c-Jun N-terminal kinases (JNK) and p38 are important regulators of the cell death program in post-mitotic neurons following survival-factor withdrawal (Mielke and Herdegen, 2000). Experiments with sympathetic neurons cultured *in vitro*, as well as with cerebellar granule neurons and differentiated pheochromocytoma 12 (PC12) cells, have demonstrated that JNK/c-Jun and p38 signaling can promote apoptosis following survival-factor withdrawal. Overexpression of dominant negative components of the JNK or the p38 pathway reverses apoptosis induced by trophic-factor withdrawal in PC12 cells (Xia et al., 1995).

One major target of the JNK pathway is the transcription factor c-Jun, which is regulated at both transcriptional and post-transcriptional levels by activated JNK (Davis, 2000). C-Jun may play a role in a wide variety of phenomena including survival, differentiation, and neuronal regeneration (Herdegen et al., 1997). Recent evidence, wherein blocking c-Jun function has protected neurons from apoptosis, both *in vitro* (Estus et al., 1994; Ham et al., 1995) and *in vivo* (Behrens et al., 1999; Crocker et al., 2001), has specifically implicated c-Jun in apoptosis.

Recent studies in cultured cortical neurons have demonstrated that increased ceramide levels, a second messenger generated intracellularly by the sphingomyelinase-mediated cleavage of sphingomyelin, activate MAP kinase JNK and p38, subsequent to induction of apoptosis (Willaime et al., 2001). Ceramide generation has been reported as a mechanism for induction of apoptosis in response to a variety of lethal agents such as tumor necrosis factor, Fas-ligand, ionizing radiation, ultraviolet-C, heat shock, oxidative stress and growth factor withdrawal in non-neuronal cells (Mathias et al., 1998). Its implication in apoptotic processes is strongly supported by experiments on lymphoblasts from Niemann-Pick patients expressing inactive sphingomyelinase or cultured fibroblasts from acid sphingomyelinase knockout mice. In both situations cells failed to respond to ionizing radiation-induced apoptosis (Santana et al., 1996; Lozano et al., 2001). These defects, as well as ceramide production, can be reversed when acid sphingomyelinase activity is restored, suggesting that ceramide is a central molecule in the apoptotic machinery.

Previous studies using *in vivo* and *in vitro* models of neuronal death also implicate ceramide in the apoptotic process. Firstly, sphingomyelinase activity is strongly in-

creased during developmental neuronal death in rat brain (Spence and Burgess, 1978). Secondly, increased ceramide levels are observed during apoptosis induced by growth factor withdrawal in PC12 cells (Lambeng et al., 1999) and after lethal ischemia in the gerbil hippocampus (Nakane et al., 2000). Furthermore, apoptosis can be induced by exogenous ceramide treatment of PC12 cells (France-Lanord et al., 1997; Hartfield et al., 1997), mesencephalic (Brugg et al., 1996), hippocampal (Mitoma et al., 1998) or cortical neurons (Willaime et al., 2001).

The objective of this study was to evaluate the role of the signaling cascade downstream ceramide after survival-factor withdrawal in cortical neurons. Using a dominant negative transfection approach in combination with a pharmacological inhibitor, we analyze herein the contribution of JNK/c-Jun and p38 pathways in ceramide-induced neuronal cell death.

EXPERIMENTAL PROCEDURES

Materials

C₂-ceramide (*N*-acetyl sphingosine, Biomol Research Laboratory, Plymouth Meeting, PA, USA) was prepared as a 25 mM stock in ethanol and incubated in the culture media as described. The specificity of c₂-ceramide was evaluated by comparing its effects with those of c₂-dihydro-ceramide (Biomol Research Laboratory, Plymouth Meeting, PA, USA), an analog lacking the 4–5 *trans* double bond in the sphingosine moiety of c₂-ceramide, which has been shown to be incapable of activating the sphingomyelin pathway in lymphocytes (Bielawska et al., 1993; Obeid et al., 1993) and neurons (Brugg et al., 1996).

Primary cultures of mouse cortex

Embryos were removed at day 14 from timed-pregnant Swiss mice (Janvier, Le Genest-St-Isle, France). The cortex was dissected, mechanically dissociated and plated on polyethyleneimine (1 mg/ml) coated culture wells, in Eagle's basal medium (Eurobio, Les Ulis, France), supplemented with 5% horse serum (HS; Eurobio) and 2.5% fetal calf serum (FCS; Eurobio), at a density of 7.10⁵ cells/cm². After 2 days in culture the media was replaced with N5 medium (Kawamoto and Barrett, 1986) with 180mg/l glucose and changed daily; FCS content was reduced to 1%; Ara-C (cytosine arabinoside, 3 μM, Sigma, Saint Louis, MO, USA) was added to prevent astrocyte proliferation (our cultures were at least 95% neuronal after this treatment), and MK-801 (1 μM, Research Biochemicals International, Natick, MA, USA) to prevent excitotoxicity (Knusel et al., 1990; Brugg et al., 1996). All experiments conformed to French and international guidelines on the ethical use of animals. We minimized the number of animals used and their suffering.

Serum deprivation

Cells were deprived in serum after 5 days of *in vitro* culture. Culture medium N5 5% HS and 1% FCS, MK-801, Ara-C was replaced by N5 without serum but with MK-801, after washing cells three times in this last medium.

Lipid extraction and ceramide quantization

Cells were washed with phosphate-buffered saline (PBS) and scraped. After spinning 5 min at 4 °C, cell pellets were resuspended in water and homogenized by brief sonication. Aliquots were saved for protein quantification. Lipids were extracted and

ceramide was quantitated using *Escherichia coli* diacylglycerol kinase and 32P-γ-ATP (NEN, Boston, MA, USA) according to previously published procedures (Van Veldhoven et al., 1992). Lipids were then resolved by thin-layer chromatography; radioactivity associated with ceramides was scraped and quantitated by liquid-scintillation spectrometry.

Ceramide treatment

Embryonic cortical neurons cultivated in 5% HS and 1% FCS, optimal conditions for their maturation and differentiation, were insensitive to c₂-ceramide treatment. That is why we reduced serum content to 1% HS, 24 h before any ceramide treatment (Brugg et al., 1996). Thus, the effects seen were not due to partial serum deprivation but rather to specific treatment, since control cells were in the same serum conditions during the same time as the treated cells. The culture conditions (concentration and time course) under which c₂-ceramide provokes cell death were determined by a cell-viability test.

Cell viability

Cell viability was determined by counting viable cells after a 10-min treatment with bisbenzimidazole (Hoechst; 0.8 μg/ml), Triton X-100 (0.005%) or with the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (the conversion of the yellow tetrazolium salt, MTT, to the purple formazan dye depends upon the mitochondrial activity; Slater et al., 1963; Altman, 1976). The MTT assay was performed on neurons in N5 medium 1% HS, with MTT (0.5 mg/ml) for 2 h in a humidified atmosphere at 37 °C, 5% CO₂. The purple formazan salt was solubilized in 100 μl of a solution containing 0.1 M HCl in isopropanol. The spectrophotometric absorbency of the samples was determined at a 560 nm wavelength.

Transfections

Transient transfection of primary cortical cultures was performed with LipofectAMINE 2000 (Invitrogen, Groningen, The Netherlands) as recommended by the manufacturer's protocol. Cells were transfected with 1 μg of enhanced green fluorescent protein (pEGFP-N3; Clontech, Cambridge, UK) alone or in the presence of 5 μg of the dominant negative c-Jun (DNC-Jun; Ham et al., 1995). After 6 h, the cultures were rinsed with fresh medium. Transfection efficacy was 3–5% of the cells. After treatments, the cells were fixed as described below. Immunocytochemical analysis of DNC-Jun expression was performed using a mouse monoclonal anti-flag M2 antibody (Sigma), and an anti-mouse Cy3 conjugated antibody (1:1000, Amersham, UK) with the protocol described below for P-JNK and P-c-Jun immunodetection.

Immunocytochemistry

After c₂-ceramide treatment, cells were fixed in the super cells cultured dishes with PBS containing 4% paraformaldehyde for 20 min and then incubated with methanol/acetone solution (50/50) for 10 min at 4 °C. Then, after washing three times with PBS, plates were treated with blocking buffer containing FCS 10%, bovine serum albumin (BSA) 1% in PBS for 2 h. Polyclonal antibodies raised against dually phosphorylated SAPK/JNK (Thr¹⁸³-Tyr¹⁸⁵-JNK) (P-JNK, 1:500, New England Biolabs, Ozyme, France) or Phospho-c-Jun (Ser⁷³-c-Jun) (P-c-Jun, 1:500, New England Biolabs) were incubated overnight at 4 °C in PBS containing 1% BSA, 0.05% Tween 20. Plates were rinsed in PBS and incubated with an anti-rabbit fluorescein isothiocyanate-conjugated antibody (1:750, Amersham) for 2 h. After counterstaining with propidium iodide, cells were mounted under coverslips using vectashield (Vector Abcys, France).

Subcellular fractionation

Neurons were cultured in six-well plates, placed on ice, and lysed in buffer containing Tris-EDTA 1 mM, NaCl 50 mM, NaPPi 330 mM, Triton X-100 10%, NaF 1 M and a mix of protease and phosphatase inhibitors (100 μ M Na₃VO₄, 1 mM dithiothreitol, 100 nM okadaic acid, 2.5 μ g/ml aprotinin, 2.5 μ g/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamide, 0.5 μ g/ml leupeptin). Cells were homogenized with a dounce A and then centrifugated (for 10 min at 1000 \times g at 4 °C). The supernatant corresponding to the cytosolic fraction was removed. Then pellets corresponding to the nuclear fraction were washed twice with lysis buffer. Protein extracts (10 μ g from each fraction) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before electrotransfer. Blots were blocked with 5% nonfat milk and incubated with rabbit polyclonal antisera raised against JNK (1:1000, New England Biolabs) or Egr-1 (1:500; Santa Cruz, Santa Cruz, CA, USA) overnight at 4 °C. After rinsing, the blots were incubated with goat horseradish peroxidase-conjugated antibody (1:2500) for 2 h at room temperature before exposure to the ECL kit (Amersham).

Western blot analysis

Cultured neurons were lysed in solubilization buffer (10 mM Tris-HCl, 50 mM NaCl, 1% Triton X-100, 30 mM Na₄P₂O₇, 50 mM NaF, 5 μ M ZnCl₂, 100 μ M Na₃VO₄, 1 mM dithiothreitol, 5 nM okadaic acid, 2.5 μ g/ml aprotinin, 3.6 μ M pepstatin, 0.5 μ M phenylmethylsulfonyl fluoride, 0.5 mM benzamide, 5.3 μ M leupeptin) at 4 °C. Insoluble material was removed by centrifugation (10,000 \times g for 30 min at 4 °C) and samples were stored at –80 °C. The proteins were dosed with the DC protein assay kit (Bio-Rad, Hercules, CA, USA). Cell extracts containing equivalent amounts of protein were boiled for 5 min in sample loading buffer. After a 10% SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (ICN Biochemicals, Costa Mesa, CA, USA). Non-specific sites were blocked with 5% skimmed dried milk for 2 h. Blots were incubated overnight at 4 °C with primary polyclonal antibodies raised against dually phosphorylated SAPK/JNK (Thr¹⁸³-Tyr¹⁸⁵-JNK, P-JNK; 1:1000, New England Biolabs) or P-c-Jun (Ser⁷³-c-Jun; 1:1000, New England Biolabs) in 5% BSA.

They were then incubated with horseradish peroxidase-conjugated secondary rabbit polyclonal antibody (1:2500) in 5% skimmed dried milk for 1 h. The revelation was processed with enhanced chemiluminescence substrate (Amersham). The blots were stripped with 0.1 M glycine-HCl (pH 2.8) twice for 30 min at 60 °C and SDS 2% for 10 min, followed by saturation in 5% skimmed dried milk overnight at 4 °C. They were then incubated with the primary antibodies (New England Biolabs) raised against synthetic peptides specific for JNK diluted 1:1000 detecting both phosphorylated and non-phosphorylated forms of JNK, in 5% skimmed dried milk. They were incubated with a horseradish peroxidase-conjugated secondary antibody (1:2500) in 5% skimmed dried milk for 1 h. The revelation was processed as described above. Quantification was processed using the Densylab software (Microvision Instruments, Evry, France).

RESULTS

Serum withdrawal-induced neuronal apoptosis increases ceramide levels in cortical neurons

Cortical neurons were deprived of serum after a 5-day maturation period *in vitro* and assayed for cell viability at various time points after treatment using the MTT metabolism assay (Fig. 1A). No significant loss of viability was

detected during the first 6 h of treatment; however, after this lag phase the neurons degenerated progressively. After 24 and 42 h, 52% and 37% of the cells survived in the culture respectively (Fig. 1A).

We measured ceramide levels, a stress and apoptotic lipid second messenger, during serum withdrawal in cultured cortical neurons. There was a biphasic increase in ceramide levels: a rapid two-fold induction after 0.5 h that then decreased and another 2.5-fold induction after a 16-h serum withdrawal, compared with the control value (Fig. 1B). Thus, serum withdrawal-induced apoptosis in cortical neurons induces ceramide levels increase in two distinct waves.

Cortical neurons were treated after a 5-day maturation period *in vitro* with c₂-ceramide and assayed for cell viability at various times after treatment using the MTT metabolism assay (Fig. 1C). After a 24-h treatment, survival was inversely correlated with c₂-ceramide concentration in the range between 10 and 75 μ M (Willaime et al., 2001). At the concentration we used (40 μ M) no significant loss of viability was detectable during the first 12 h of treatment; after that the neurons degenerated progressively. After 24 and 48 h, 50% and 10% of the cells survived in the culture respectively (Fig. 1C).

Serum withdrawal and ceramide use a common apoptotic pathway including JNK phosphorylation

We have previously described JNK activation in ceramide-induced apoptosis (Willaime et al., 2001) and here we have analyzed JNK activation during serum withdrawal in cortical neurons by Western blot. Three different genes (*JNK1*, *JNK2*, *JNK3*) encode for the JNK proteins, each in giving rise to alternatively spliced isoforms (Gupta et al., 1996) whose *in vitro* translation produces two major proteins of 54 and 46 kD. JNKs are activated by dual phosphorylation on residues Thr¹⁸³ and Tyr¹⁸⁵ (P-p54 and P-p46). Using an antibody specifically directed against active JNKs, we found that serum withdrawal gradually increased P-p54 and P-p46 levels throughout the 24 h of treatment (Fig. 2A, upper panel). Using the same antibody, we found that c₂-ceramide (40 μ M) increased P-p54 and P-p46 levels (Fig. 2B, upper panel), as previously described (Willaime et al., 2001). These increases resulted from phosphorylation, as the total JNK levels did not increase significantly during treatment (Fig. 2A and 2B, lower panels). P-p46 level increased more slowly with serum withdrawal than ceramide treatment, and reached 250% of the control values after a 24-h treatment under both conditions (Fig. 2C). Thus serum withdrawal and ceramide treatment induced a strong activation of JNKs.

C₂-ceramide induces P-JNK nuclear translocation and subsequent c-Jun phosphorylation during apoptosis of cortical neurons

As JNKs have a wide variety of potential phosphorylation substrates it is important to analyze the localization of P-JNK within the cell to understand its possible effects upon nuclear transcription factors. Immunocyto-

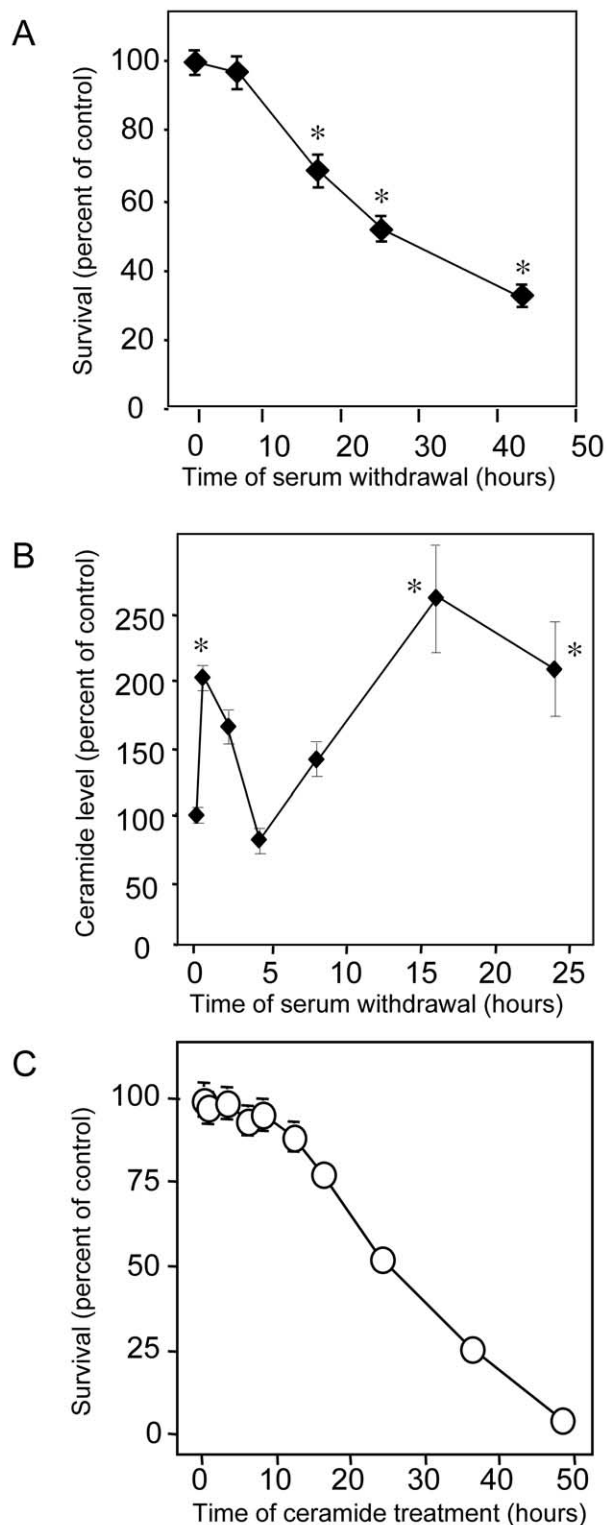


Fig. 1. (A) Serum withdrawal treatment induces neuronal death in cultured cortical neurons. The effect is time-dependent. Survival was assessed using the MTT assay. Data represent mean \pm S.E.M., n =eight independent experiments. * P <0.05 compared with the control value, according to the one-way ANOVA followed by Dunnett's post hoc test. (B) Ceramide level increases during serum withdrawal-induced neuronal death. Data represent mean \pm S.E.M., n =three inde-

pendent experiments. * P <0.05 compared with the control value, according to the one-way ANOVA followed by Dunnett's post hoc test. (C) C_2 -ceramide treatment induces neuronal death in cultured cortical neurons. The effect is time-dependent. Survival was assessed using the MTT assay. Data represent mean \pm S.E.M., n =four independent experiments.

chemical detection of the phosphorylated and thereby active form of JNKs (P-JNKs), was used and kinetics of JNK activation were analyzed in this model system by immunofluorescence (Fig. 3A). A low basal level of P-JNK immunoreactivity was found in control cells that corresponded, nevertheless, to an exclusive neuritic localization. After 8 h of c_2 -ceramide (40 μ M) treatment and thereafter, P-JNK immunoreactivity showed a distinct localization in a vast majority of neurons, confirmed by confocal microscopy analysis (Fig. 3A, insert). To confirm this biochemically, we fractionated cortical extracts into the nucleus and the cytoplasm. As a control of our fractionation procedure, Egr-1 immunoblotting was performed and showed an immunoreactive band at 57 kD, the expected molecular weight for Egr-1, in the nuclear extract almost exclusively. From these fractions, we then analyzed the distribution of JNK by immunoblotting. This shows a strong increase in nuclear JNK p46 isoform after ceramide treatment (Fig. 3B), corresponding to the nuclear labeling of P-JNK found by immunocytochemistry (Fig. 3A), with no translocation of JNK p54 isoform. This result together with immunocytochemical analyses of P-JNKs strongly support that p46 is the major JNK isoform (see Fig. 2B) phosphorylated and translocated upon ceramide treatment. Together these data show that JNKs are activated and translocated into the nucleus during ceramide treatment.

Within the nucleus, activated JNKs are known to control the phosphorylation state of c-Jun as well as its transcriptional control (Angel et al., 1988). By Western blot using an antibody specific for the phosphorylated form of c-Jun (P-c-Jun), we found that c_2 -ceramide increased phosphorylation of c-Jun at 10 and 16 h of ceramide treatment (Fig. 4A), compared with loading control with the total JNKs that did not change during ceramide treatment.

Next, we examined c-Jun phosphorylation by immunocytochemistry with the same antibody (Fig. 4B). In contrast to control neurons (t0), where we failed to detect any P-c-Jun-immunoreactivity (green), P-c-Jun-immunoreactivity appeared after 8 h of c_2 -ceramide (40 μ M) treatment and strong levels persisted well after 16 h of treatment. Thus, c-Jun phosphorylation appears with JNK activation and translocation.

Overexpression of dominant negative c-Jun blocks ceramide-induced apoptosis in primary cortical neurons

To address the role of c-Jun activation in ceramide-induced neuronal apoptosis, we overexpressed a dominant negative form of c-Jun (DNc-Jun) in primary cortical neurons. We used a construct corresponding to a deletion of

pendent experiments. * P <0.05 compared with the control value, according to the one-way ANOVA followed by Dunnett's post hoc test. (C) C_2 -ceramide treatment induces neuronal death in cultured cortical neurons. The effect is time-dependent. Survival was assessed using the MTT assay. Data represent mean \pm S.E.M., n =four independent experiments.

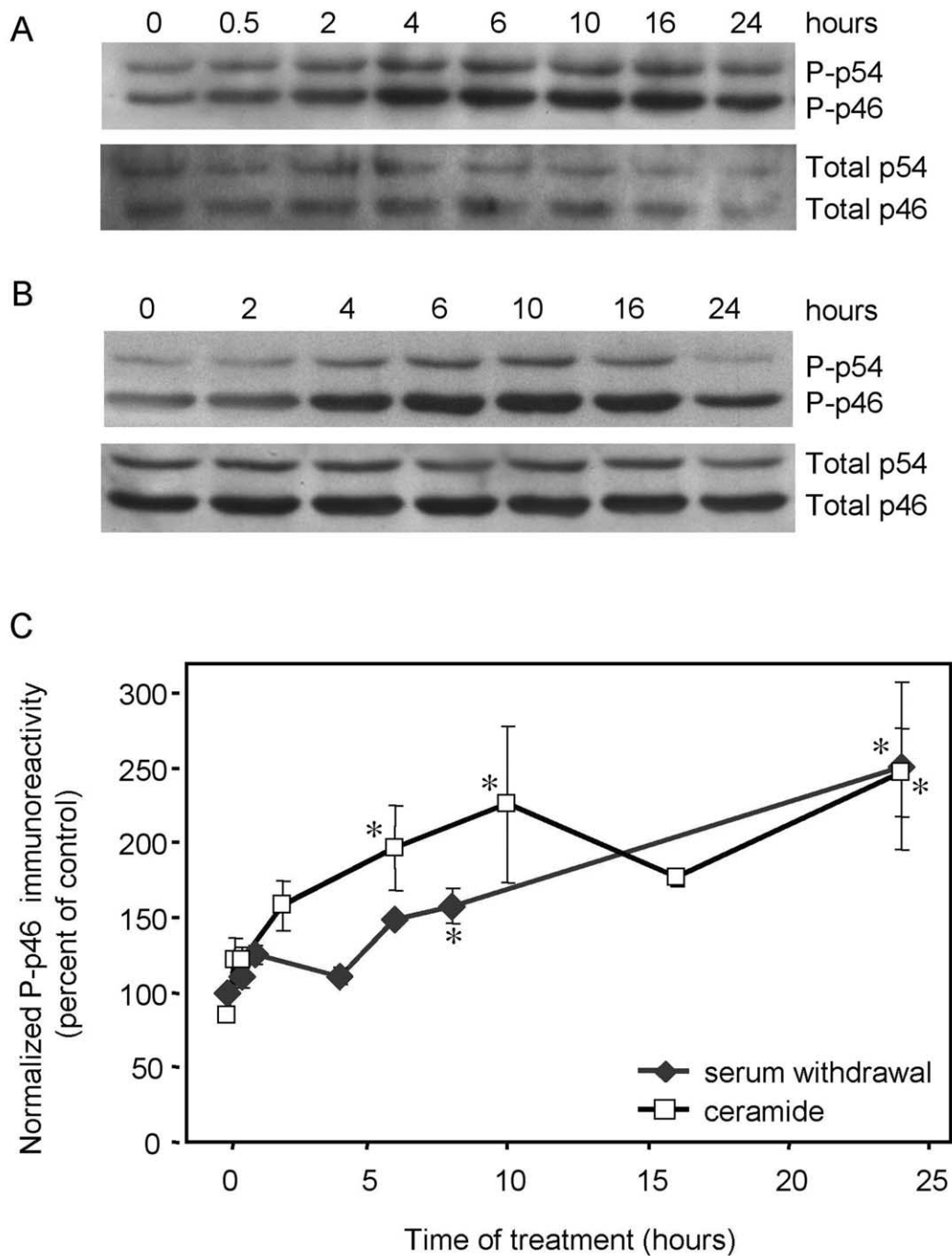


Fig. 2. Serum withdrawal (A) and c_2 -ceramide (B) induce an increase of JNK phosphorylation in cultured cortical neurons. Immunoblot analysis of P-JNKs (P-p54 and P-p46, upper panels) and total JNKs (total p54 and total p46, lower panels) in extracts of treated cortical neurons. (C) Quantification of P-p46 immunoreactivity in Western blot analyses during serum withdrawal or ceramide treatment. P-p46 signals were normalized to the corresponding total p46 signal. Data represent mean \pm S.E.M., $n \geq 4$ independent experiments. * $P < 0.05$ compared with the control value, according to the one-way ANOVA followed by Dunnett's post hoc test.

the first 169 amino acids, the region containing the trans-activation domain, which contains the phosphorylation target sites of activated JNK (Ham et al., 1995). Neurons were transfected with GFP alone or in combination with DNc-Jun. GFP expression allowed for visualization of the cytoarchitecture, including neuritic extension of transfected

neurons and cell body (analyzed after 24 h of c_2 -ceramide treatment) in transfected neurons. Immunocytochemical detection of DNc-Jun showed its nuclear localization, as well as coexpression with GFP in double-transfected neurons (Fig. 5H). In control conditions, neurons transfected with GFP alone (Fig. 5A, B) or in combination with DNc-

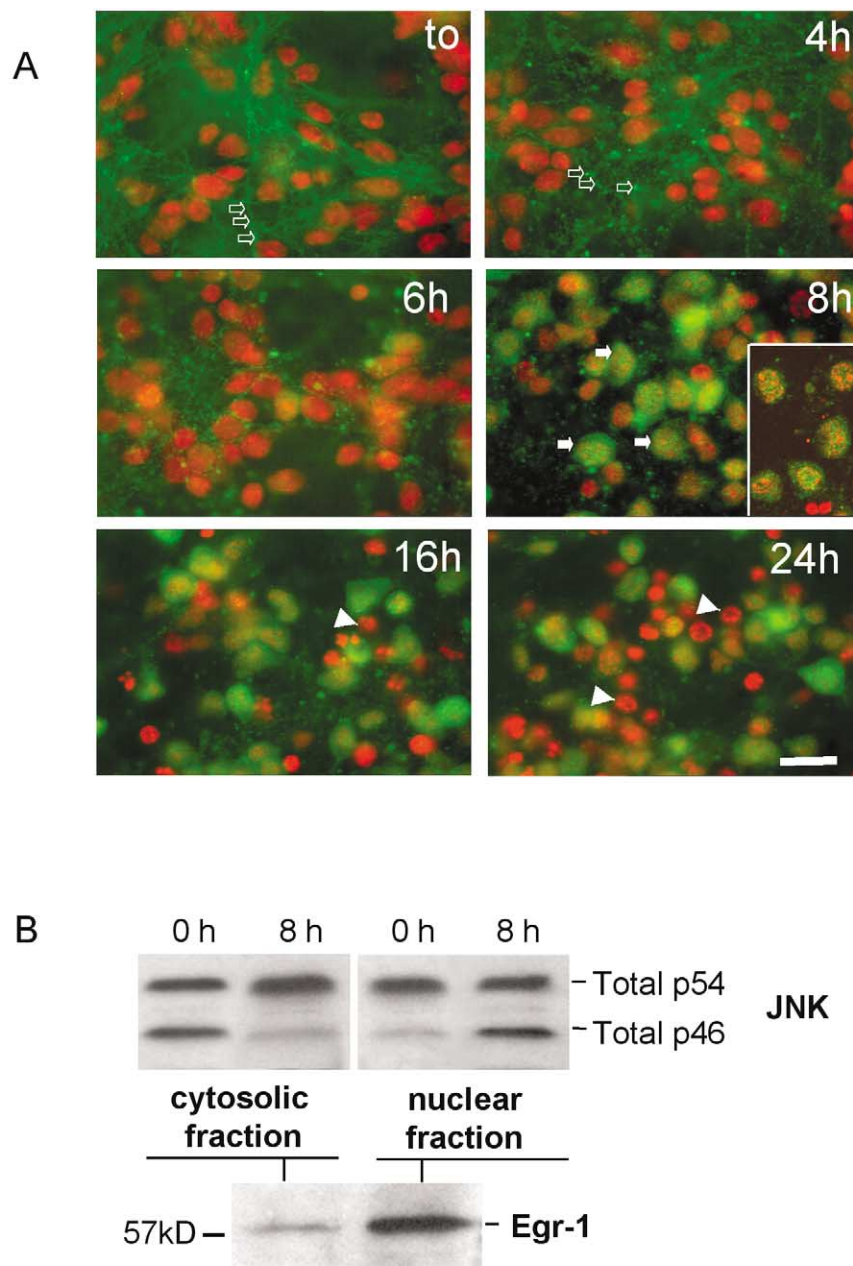


Fig. 3. (A) C_2 -ceramide induces a nuclear translocation of P-JNK in cultured cortical neurons. Neurons were untreated (0h) or C_2 -ceramide ($40 \mu\text{M}$) treated during indicated times. Neurons are immunostained for P-JNK (green) and nuclear labeled by propidium iodide (red) in cortical cultures. Nuclear localization of P-JNK shows yellow labeling in these merge images (white arrows) and is confirmed by confocal microscopy insert at 8 h. Empty arrows: P-JNK labeling in the dendrites; arrowheads: condensed and fragmented nuclei. Scale bar= $20 \mu\text{M}$. (B) Western blot analysis of JNK from subcellular fractionation of cortical neurons. Specific labeling corresponding to the expected molecular weights for JNK was detected in cytosolic and nuclear compartment during ceramide treatment. As a control of fractionation, Egr-1 immunoblotting was performed showing a specific nuclear immunostaining.

Jun (Fig. 5G–I) showed very long neuritic extensions and normal nuclei. After 24 h of C_2 -ceramide ($40 \mu\text{M}$) treatment, 48.7% of neurons transfected with GFP alone are alive whereas the others exhibited a strong retraction and fragmentation of neurites (Fig. 5C) and nuclear fragmentation (Fig. 5D), quantified in Table 1. In contrast, 77.3% of neurons coexpressing GFP and DNc-Jun and treated for 24 h with ceramide were alive (Fig. 5E, F, Table 1), thus

demonstrating that activation of c-Jun plays an important role in ceramide-induced neuronal apoptosis.

Treatment of DNc-Jun-expressing neurons with p38 inhibitor completely protects cortical neurons against ceramide-induced apoptosis

We previously described that SB203580, a specific inhibitor of p38 pathway, also partially protects against ceram-

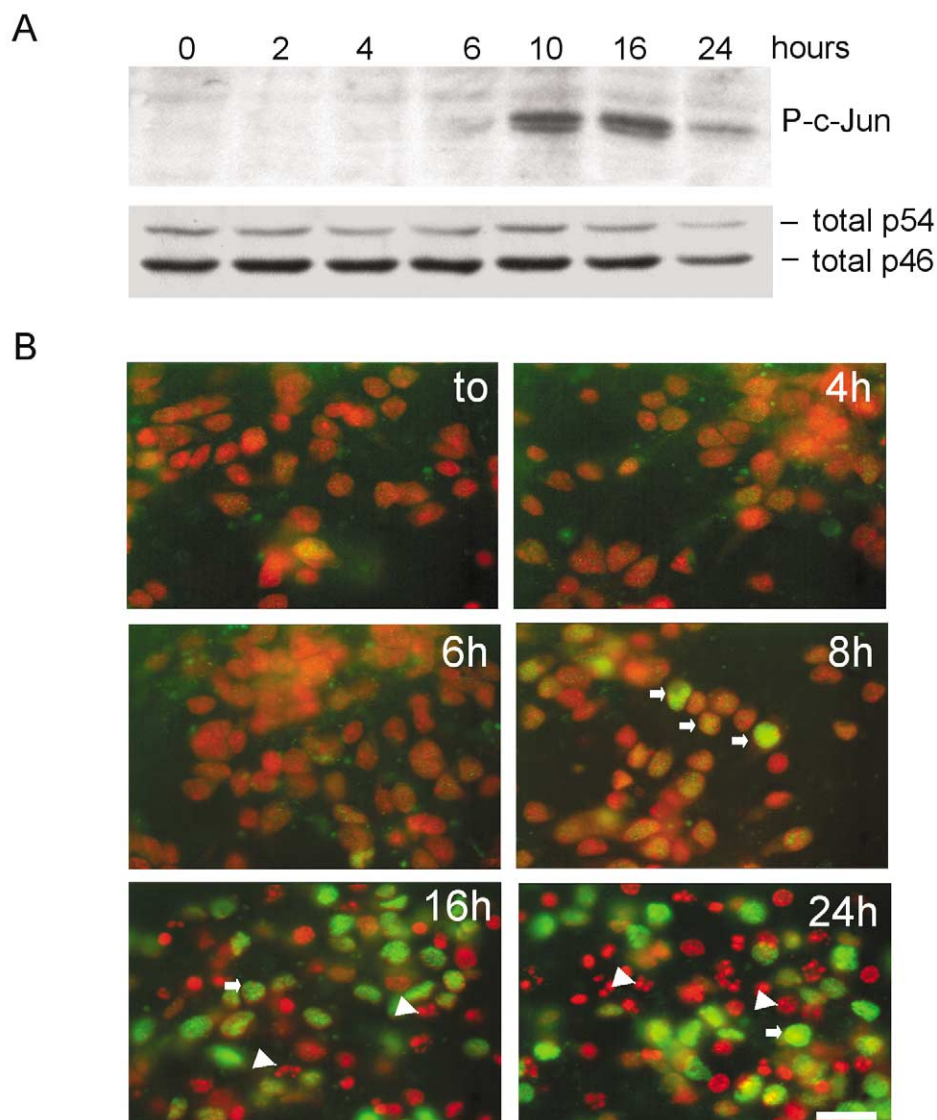


Fig. 4. (A) Immunoblot analysis of P-c-Jun (upper panel) in extracts of ceramide-treated cortical neurons and loading control with total JNKs (total p54 and total p46, lower panel). (B) C_2 -ceramide induces an increase of P-c-Jun in the nuclei of cultured cortical neurons. Neurons immunostained for P-c-Jun (green) and nuclear labeled by propidium iodide in cortical cultures. Neurons control (t0) or treated with C_2 -ceramide (40 μ M) during indicated times. White arrows: normal nuclei; arrowheads: condensed and fragmented nuclei. Scale bar=20 μ M.

ide-induced apoptosis (Willaime et al., 2001) in exactly the same conditions as in this new study (Table 1). Here we show that treatment of Dnc-Jun-expressing neurons with SB203580 completely protects cortical neurons against ceramide (40 μ M)-induced apoptosis: neuronal survival is 96.9% of the control with double treatment, versus 77.3% and 76.0% with Dnc-Jun and SB203580 respectively (Table 1).

DISCUSSION

Ceramide generation has been reported as a mechanism for the induction of apoptosis in response to different stress stimuli in a variety of non-neuronal cells types. The present study demonstrates that the second-messenger ceramide increases upon survival-factor withdrawal in primary cortical

neurons. Serum deprivation increased endogenous ceramide levels in a two-peak manner: the first peak occurred very rapidly within the first hour and the second peak at 16 h after the apoptotic process had already been engaged. These data, together with the previous findings which demonstrate that a 6-h pulse of C_2 -ceramide treatment is sufficient to commit neurons to die (Hunot et al., 1997), suggest that the first ceramide peak could be responsible for apoptosis induction. This rapid and transient increase of ceramide may reflect an activation of sphingomyelinases, as seen after stimulation of non-neuronal cells with TNF- α , IL-1 β and Fas-ligand (for review see Kronke, 1997). The mechanism of receptor mediated sphingomyelinase activation after survival-factor withdrawal, however, remains unidentified.

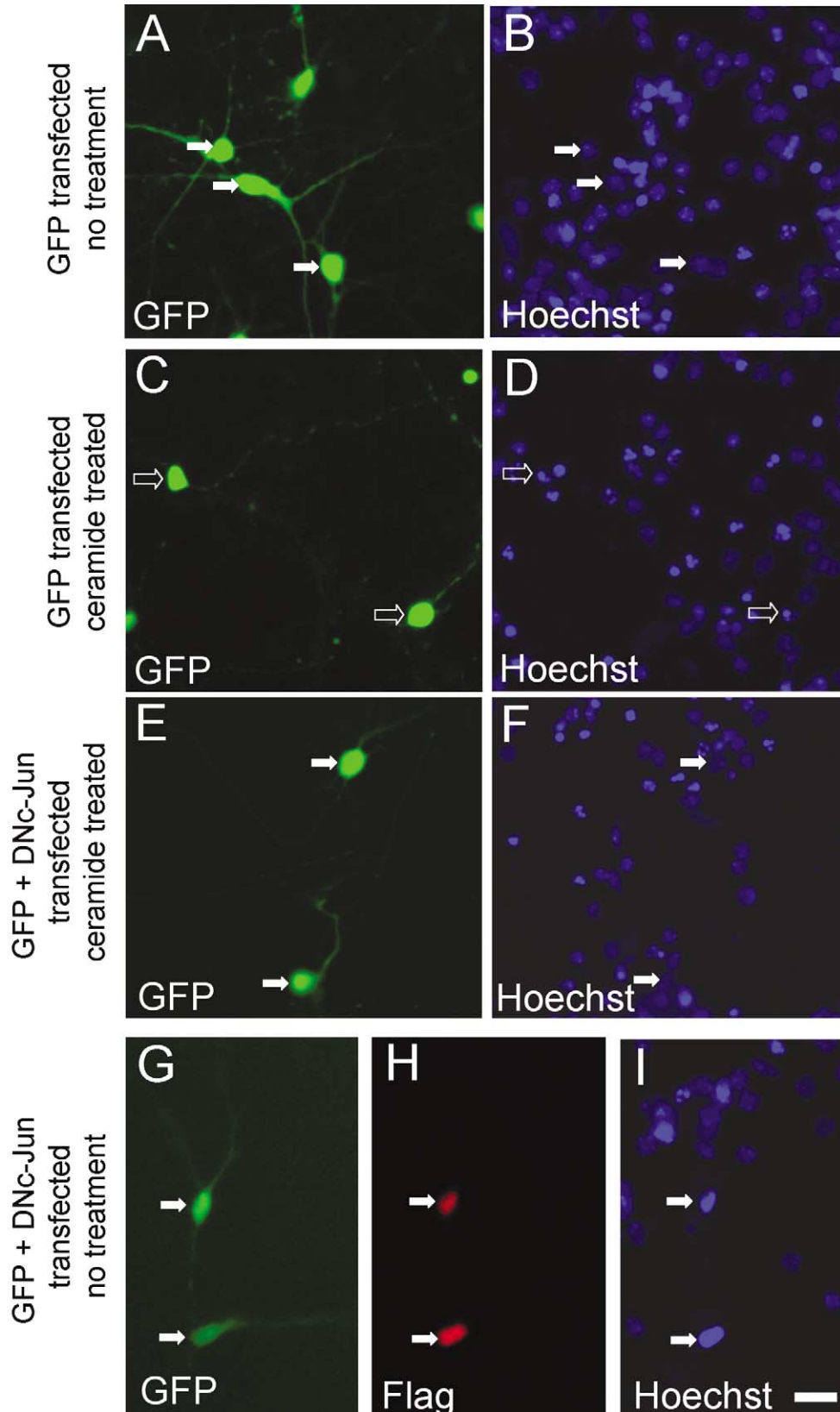


Fig. 5.

Table 1. c-Jun and p38 cooperate in ceramide-induced neuronal apoptosis

Transfection	Treatment	Survival of transfected neurons (percent of control)
GFP	–	100.00±6.1
GFP	cer	48.7±4.3
GFP	cer+SB	76.0±5.0*
GFP+Dnc-Jun	–	100.0±7.7
GFP+Dnc-Jun	cer	77.3±4.3*
GFP+Dnc-Jun	cer+SB	96.9±11.4**£

A dominant negative form of c-Jun (Dnc-Jun) protects neurons from apoptosis induced by c_2 -ceramide and treatment of Dnc-Jun-expressing neurons with SB203580 completely protects cortical neurons against ceramide-induced apoptosis. Primary cortical neurons were transfected with green fluorescent protein (GFP) alone or in combination with Dnc-Jun, treated with ceramide and/or SB203580, and transfected neurons having normal nuclei were quantified. Data represent mean±S.E.M. $n=15$ wells from three independent experiments, $P<0.05$ compared with GFP+cer value (*), with GFP+cer+SB value (#), or with GFP+Dnc-Jun+cer value (£), according to the one-way ANOVA followed by Student Newman-Keuls post hoc test; cer= c_2 -ceramide (40 μ M); SB=SB203580 (10 μ M).

The second ceramide peak could reflect an increase in ceramide synthase activity, since it has been reported that fumonisin B1, a ceramide synthase inhibitor, can decrease this second peak (Jaffrezou et al., 1998). This might reveal a cellular degeneration process since this late ceramide production is also observed under necrotic conditions (Bose et al., 1995; Jaffrezou et al., 1998). The implication of the rapid ceramide peak in apoptosis induction is furthermore supported by the finding that serum withdrawal or c_2 -ceramide treatment induces an almost identical time course of JNK phosphorylation (Fig. 2). Ceramide generation and JNK activation could also be correlated *in vivo* in animal models of ischemia, as data show that ceramide levels increase (Nakane et al., 2000) and JNK1 has been shown to activate and translocate to the nuclei (Mizukami et al., 1997).

To further evaluate JNK activation in neuronal apoptosis involving ceramide we have investigated JNK activation by immunocytochemistry. To our knowledge this is the first documentation showing that ceramide may induce nuclear translocation of JNK in neurons. This event coincides with the increase in JNK phosphorylation (Fig. 2) after 8 h of ceramide treatment. JNK activation and nuclear translocation in neurons have been reported *in vivo* under different pathological conditions. Nuclear localization of JNK has been detected in degenerating neurons of Alzheimer patients (Zhu et al., 2001) and in cerebellar granule neurons (Coffey et al., 2000, 2002). JNK1 and JNK3 translocation from the cytosol to the nucleus in neurons can also be observed in experimental models of hypoxia (Zhang et al., 1998) and ischemia (Mizukami et al., 1997) respectively.

The mechanism by which ceramide activates the JNK pathway in neurons, however, has yet to be identified. Several intermediate kinases described in other cellular systems may be involved in this process in neurons, including the small G-protein Rac1 (Brenner et al., 1997), the MAP kinase TGF β -activating kinase 1 (Schirakabe et al., 1997); the MAP 3 kinase 1 (Takekawa et al., 1997) or the

apoptosis signal-regulated kinase 1 (Chen et al., 1999). Further investigations will help to determine how ceramide and serum withdrawal induce the JNK pathway in neurons.

JNK activation, and its nuclear translocation, has been associated to transcriptional events that occur early in the apoptotic process (Ham et al., 2000). Consistent with this, we found an increase of the transcription factor c-Jun's phosphorylation in the neuronal nuclei after ceramide treatment.

To further investigate the specific role of the JNK/c-Jun pathway in ceramide-induced cortical neuron apoptosis, we overexpressed a Dnc-Jun in primary cortical neurons. Our results demonstrate that Dnc-Jun partially protects cortical neurons against ceramide-induced apoptosis. This is consistent with data obtained in others models, such as PC12 cells (Xia et al., 1995; Lambeng et al., 2003) and sympathetic neurons (Ham et al., 1995) deprived of NGF.

We previously described that SB203580, a specific inhibitor of the p38 pathway, also partially protects against ceramide-induced apoptosis (Willaime et al., 2001) in exactly the same conditions as in the present study. The cooperative role of p38 and JNK/c-Jun pathway remained to be addressed. Although the result was previously suggested in two articles, in each the specificity of pharmacological agents is contestable: Le Niculescu et al. used the p38 inhibitor SB202190 at high unspecific doses to also inhibit JNKs (Manthey et al., 1998; Le-Niculescu et al., 1999); Namgung and Xia used CEP1347 as a JNK inhibitor in cooperation with a p38 inhibitor (Namgung and Xia, 2000), but CEP1347 has since been shown to affect mixed lineage kinase family rather than directly JNKs (Maroney et al., 2001). In this study we have employed an alternative approach using p38 kinase inhibitor SB203580 and Dnc-Jun. We obtained a complete protection in our model. In others models, where Dnc-Jun alone almost completely protects cells against apoptosis, p38 kinase is not activated during apoptosis (Watson et al., 1998) or not described. Furthermore, Xia et al. showed that either Dnc-

Fig. 5. A Dnc-Jun protects neurons from apoptosis induced by c_2 -ceramide. Primary cortical neurons were transfected with GFP alone (A–D) or in combination with a Dnc-Jun (lacking the first 169 amino acids, E–I). Transfected neurons are visualized by GFP (A, C, E, G) and Hoechst (B, D, F, I). Control (A, B, G–I) or ceramide treated (C–F) neurons. In cotransfected neurons, detection of Dnc-Jun expression was performed using an antibody that recognizes the FLAG epitope (H). White arrows: alive cells; empty arrows: dead cells. Scale bar=20 μ M.

Jun or dominant negative form of MKK3, upstream kinase of p38s, protects completely PC12 cells from NGF withdrawal-induced apoptosis. They did not explore the cooperative role of p38 and JNK/c-Jun pathway. Thus, our data further demonstrate that p38 kinase and JNK/c-Jun act in parallel to induce neuronal apoptosis by ceramide.

The pro-apoptotic action of p38 in ceramide-induced apoptosis may be mediated by apoptotic effectors: Bax translocation from the cytosol to the mitochondria is dependent upon p38 (Ghatan et al., 2000); Bid is cleaved upon p38 activation and leads to mitochondrial dysfunction (Zhuang et al., 2000); Fas-ligand expression is regulated by p38 and is pro-apoptotic (Zhang et al., 2000); GADD153/CHOP activation is controlled by p38 and is involved in ceramide-induced apoptosis (Brenner et al., 1997). The pro-apoptotic action of JNK/c-Jun in ceramide-induced apoptosis may be mediated by apoptotic effectors such as Fas-ligand which expression is also regulated by JNK (Herdegen et al., 1998; Morishima et al., 2001), since the Fas-ligand promoter contains an AP-1 site that enables the promoter to be activated by the JNK pathway. Bim, a pro-apoptotic member of Bcl-2 family, which is strongly induced in sympathetic neurons on NGF withdrawal, has also been shown to be under the transcriptional control of the JNK pathway (Putchá et al., 2001; Whitfield et al., 2001).

Our results show that p38 kinase and JNK/c-Jun act in parallel to induce neuronal apoptosis by ceramide. With this double inhibition, we obtained a complete protection against neuronal apoptosis. These results suggest that multitherapy with specific inhibitors of these signaling pathways could have important synergistic effects for inhibiting neuronal apoptosis.

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REFERENCES

- Altman FP (1976) Tetrazolium salts and formazans. *Prog Histochem Cytochem* 9:1–56.
- Angel P, Hattori K, Smeal T, Karin M (1988) The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. *Cell* 55:875–885.
- Behrens A, Sibilía M, Wagner EF (1999) Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat Genet* 21:326–329.
- Bielawska A, Crane HM, Liotta D, Obeid LM, Hannun YA (1993) Selectivity of ceramide-mediated biology: lack of activity of erythrodiacylceramide. *J Biol Chem* 268:26226–26232.
- Bose R, Verheij M, Haimovitz-Friedman A, Scotto K, Fuks Z, Kolesnick R (1995) Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. *Cell* 82:405–414.
- Brenner B, Koppenhoefer U, Weinstock C, Linderkamp O, Lang F, Gulbins E (1997) Fas- or ceramide-induced apoptosis is mediated by a Rac1-regulated activation of Jun N-terminal kinase/p38 kinases and GADD153. *J Biol Chem* 272:22173–22181.
- Brugg B, Michel PP, Agid Y, Ruberg M (1996) Ceramide induces apoptosis in cultured mesencephalic neurons. *J Neurochem* 66:733–739.
- Chen Z, Seimiya H, Naito M, Mashima T, Kizaki A, Dan S, Imaizumi M, Ichijo H, Miyazono K, Tsuruo T (1999) ASK1 mediates apoptotic cell death induced by genotoxic stress. *Oncogene* 18:173–180.
- Coffey ET, Hongisto V, Dickens M, Davis RJ, Courtney MJ (2000) Dual roles for c-Jun N-terminal kinase in developmental and stress responses in cerebellar granule neurons. *J Neurosci* 20:7602–7613.
- Coffey ET, Smiciene G, Hongisto V, Cao J, Brecht S, Herdegen T, Courtney MJ (2002) c-Jun N-terminal protein kinase (JNK) 2/3 is specifically activated by stress, mediating c-Jun activation, in the presence of constitutive JNK1 activity in cerebellar neurons. *J Neurosci* 22:4335–4345.
- Crocker SJ, Lamba WR, Smith PD, Callaghan SM, Slack RS, Anisman H, Park DS (2001) c-Jun mediates axotomy-induced dopamine neuron death in vivo. *Proc Natl Acad Sci USA* 98:13385–13390.
- Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. *Cell* 103:239–252.
- Estus S, Zaks WJ, Freeman RS, Gruda M, Bravo R, Johnson EM Jr (1994) Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. *J Cell Biol* 127:1717–1727.
- France-Lanord V, Brugg B, Michel PP, Agid Y, Ruberg M (1997) Mitochondrial free radical signal in ceramide-dependent apoptosis: a putative mechanism for neuronal death in Parkinson's disease. *J Neurochem* 69:1612–1621.
- Ghatan S, Larner S, Kinoshita Y, Hetman M, Patel L, Xia Z, Youle RJ, Morrison RS (2000) p38 MAP kinase mediates bax translocation in nitric oxide-induced apoptosis in neurons. *J Cell Biol* 150:335–347.
- Gupta S, Barrett T, Whitmarsh AJ, Cavanagh J, Sluss HK, Derijard B, Davis RJ (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J* 15:2760–2770.
- Ham J, Babij C, Whitfield J, Pfarr CM, Lallemand D, Yaniv M, Rubin LL (1995) A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron* 14:927–939.
- Ham J, Eilers A, Whitfield J, Neame SJ, Shah B (2000) c-Jun and the transcriptional control of neuronal apoptosis. *Biochem Pharmacol* 60:1015–1021.
- Hartfield PJ, Mayne GC, Murray AW (1997) Ceramide induces apoptosis in PC12 cells. *FEBS Lett* 401:148–152.
- Herdegen T, Claret FX, Kallunki T, Martin-Villalba A, Winter C, Hunter T, Karin M (1998) Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinases after neuronal injury. *J Neurosci* 18:5124–5135.
- Herdegen T, Skene P, Bahr M (1997) The c-Jun transcription factor: bipotential mediator of neuronal death, survival and regeneration. *Trends Neurosci* 20:227–231.
- Hunot S, Brugg B, Ricard D, Michel PP, Muriel MP, Ruberg M, Faucheux BA, Agid Y, Hirsch EC (1997) Nuclear translocation of NF-kappaB is increased in dopaminergic neurons of patients with parkinson disease. *Proc Natl Acad Sci USA* 94:7531–7536.
- Jaffrezou JP, Maestre N, de Mas-Mansat V, Bezombes C, Levade T, Laurent G (1998) Positive feedback control of neutral sphingomyelinase activity by ceramide. *FASEB J* 12:999–1006.
- Kawamoto JC, Barrett JN (1986) Cryopreservation of primary neurons for tissue culture. *Brain Res* 384:84–93.
- Knusel B, Michel PP, Schwaber JS, Hefti F (1990) Selective and nonselective stimulation of central cholinergic and dopaminergic development in vitro by nerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. *J Neurosci* 10:558–570.
- Kronke M (1997) The mode of ceramide action: the alkyl chain protrusion model. *Cytokine Growth Factor Rev* 8:103–107.
- Lambeng N, Michel PP, Brugg B, Agid Y, Ruberg M (1999) Mecha-

- nisms of apoptosis in PC12 cells irreversibly differentiated with nerve growth factor and cyclic AMP. *Brain Res* 821:60–68.
- Lambeng N, Willaime-Morawek S, Mariani J, Ruberg M, Brugg B (2003) Activation of mitogen-activated protein kinase pathways during the death of PC12 cells is dependent on the state of differentiation. *Mol Brain Res* 111:52–60.
- Le-Niculescu H, Bonfoco E, Kasuya Y, Claret FX, Green DR, Karin M (1999) Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. *Mol Cell Biol* 19:751–763.
- Lozano J, Menendez S, Morales A, Ehleiter D, Liao WC, Wagman R, Haimovitz-Friedman A, Fuks Z, Kolesnick R (2001) Cell autonomous apoptosis defects in acid sphingomyelinase knockout fibroblasts. *J Biol Chem* 276:442–448.
- Manthey CL, Wang SW, Kinney SD, Yao Z (1998) SB202190, a selective inhibitor of p38 mitogen-activated protein kinase, is a powerful regulator of LPS-induced mRNAs in monocytes. *J Leukoc Biol* 64:409–417.
- Maroney AC, Finn JP, Connors TJ, Durkin JT, Angeles T, Gessner G, Xu Z, Meyer SL, Savage MJ, Greene LA, Scott RW, Vaught JL (2001) Cep-1347 (KT7515), a semisynthetic inhibitor of the mixed lineage kinase family. *J Biol Chem* 276:25302–25308.
- Mathias S, Pena LA, Kolesnick RN (1998) Signal transduction of stress via ceramide. *Biochem J* 335:465–480.
- Mielke K, Herdegen T (2000) JNK and p38 stress kinases: degenerative effectors of signal-transduction-cascades in the nervous system. *Prog Neurobiol* 61:45–60.
- Mitoma J, Ito M, Furuya S, Hirabayashi Y (1998) Bipotential roles of ceramide in the growth of hippocampal neurons: promotion of cell survival and dendritic outgrowth in dose- and developmental stage-dependent manners. *J Neurosci Res* 51:712–722.
- Mizukami Y, Yoshioka K, Morimoto S, Yoshida K (1997) A novel mechanism of JNK1 activation: nuclear translocation and activation of JNK1 during ischemia and reperfusion. *J Biol Chem* 272:16657–16662.
- Morishima Y, Gotoh Y, Zieg J, Barrett T, Takano H, Flavell R, Davis RJ, Shirasaki Y, Greenberg ME (2001) Beta-amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand. *J Neurosci* 21:7551–7560.
- Nakane M, Kubota M, Nakagomi T, Tamura A, Hisaki H, Shimasaki H, Ueta N (2000) Lethal forebrain ischemia stimulates sphingomyelin hydrolysis and ceramide generation in the gerbil hippocampus. *Neurosci Lett* 296:89–92.
- Namgung U, Xia Z (2000) Arsenite-induced apoptosis in cortical neurons is mediated by c-Jun N-terminal protein kinase 3 and p38 mitogen-activated protein kinase. *J Neurosci* 20:6442–6451.
- Obeid LM, Linardic CM, Karolak LA, Hannun YA (1993) Programmed cell death induced by ceramide. *Science* 259:1769–1771.
- Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JA, Strasser A, Johnson EM (2001) Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. *Neuron* 29:615–628.
- Santana P, Pena LA, Haimovitz-Friedman A, Martin S, Green D, McLoughlin M, Cordon-Cardo C, Schuchman EH, Fuks Z, Kolesnick R (1996) Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. *Cell* 86:189–199.
- Schirakabe K, Yamaguchi K, Shibuya H, Irie K, Matsuda S, Moriguchi T, Gotoh Y, Matsumoto K, Nishida E (1997) TAK1 mediates the ceramide signaling to stress activated protein kinase/c-Jun N-terminal kinase. *J Biol Chem* 272:8141–8144.
- Slater TF, Sawyer B, Sträuli U (1963) Studies on succinate-tetrazolium reductase systems. *Biochim Biophys Acta* 77:383–393.
- Spence MW, Burgess JK (1978) Acid and neutral sphingomyelinases of rat brain: activity in developing brain and regional distribution in adult brain. *J Neurochem* 30:917–919.
- Takekawa M, Posas F, Saito H (1997) A human homolog of the yeast Ssk2/Ssk22 MAP kinase kinase kinases, MTK1, mediates stress-induced activation of the p38 and JNK pathways. *EMBO J* 16:4973–4982.
- Van Veldhoven PP, Matthews TJ, Bolognesi DP, Bell RM (1992) Changes in bioactive lipids, alkylacylglycerol and ceramide, occur in HIV-infected cells. *Biochem Biophys Res Commun* 187:209–216.
- Watson A, Eilers A, Lallemand D, Kyriakis J, Rubin LL, Ham J (1998) Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons. *J Neurosci* 18:751–762.
- Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J (2001) Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. *Neuron* 29:629–643.
- Willaime S, Vanhoutte P, Caboche J, Lemaigre-Dubreuil Y, Mariani J, Brugg B (2001) Ceramide-induced apoptosis in cortical neurons is mediated by an increase in p38 phosphorylation and not by the decrease in ERK phosphorylation. *Eur J Neurosci* 13:2037–2046.
- Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 270:1326–1331.
- Zhang J, Gao JX, Salojin K, Shao Q, Grattan M, Meagher C, Laird DW, Delovitch TL (2000) Regulation of fas ligand expression during activation-induced cell death in T cells by p38 mitogen-activated protein kinase and c-Jun NH2-terminal kinase. *J Exp Med* 191:1017–1030.
- Zhang Y, Zhou L, Miller CA (1998) A splicing variant of a death domain protein that is regulated by a mitogen-activated kinase is a substrate for c-Jun N-terminal kinase in the human central nervous system. *Proc Natl Acad Sci USA* 95:2586–2591.
- Zhu X, Raina AK, Rottkamp CA, Aliev G, Perry G, Boux H, Smith MA (2001) Activation and redistribution of c-Jun N-terminal kinase/stress activated protein kinase in degenerating neurons in Alzheimer's disease. *J Neurochem* 76:435–441.
- Zhuang S, Demirs JT, Kochevar IE (2000) p38 Mitogen-activated protein kinase mediates Bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen, but not by hydrogen peroxide. *J Biol Chem* 275:25939–25948.