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c-Jun N-TERMINAL KINASE BINDING DOMAIN–DEPENDENT PHOSPHORYLATION OF MITOGEN-ACTIVATED PROTEIN KINASE KINASE 4 AND MITOGEN-ACTIVATED PROTEIN KINASE KINASE 7 AND BALANCING CROSS-TALK BETWEEN c-Jun N-TERMINAL KINASE AND EXTRACELLULAR SIGNAL-REGULATED KINASE PATHWAYS IN CORTICAL NEURONS

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Abstract—The c-Jun N-terminal kinase (JNK) is a mitogen-activated protein kinase (MAPK) activated by stress-signals and involved in many different diseases. Previous results proved the powerful effect of the cell permeable peptide inhibitor D-JNKI1 (D-retro-inverso form of c-Jun N-terminal kinase-inhibitor) against neuronal death in CNS diseases, but the precise features of this neuroprotection remain unclear. We here performed cell-free and *in vitro* experiments for a deeper characterization of D-JNKI1 features in physiological conditions.

This peptide works by preventing JNK interaction with its c-Jun N-terminal kinase–binding domain (JBD) dependent targets. We here focused on the two JNK upstream MAPKs, mitogen-activated protein kinase kinase 4 (MKK4) and mitogen-activated protein kinase kinase 7 (MKK7), because they contain a JBD homology domain. We proved that D-JNKI1 prevents MKK4 and MKK7 activity in cell-free and *in vitro* experiments: these MAPKs could be considered not only activators but also substrates of JNK. This means that D-JNKI1 can interrupt downstream but also upstream events along the JNK cascade, highlighting a new remarkable feature of this peptide. We also showed the lack of any direct effect of the peptide on p38, MEK1, and extracellular signal-regulated kinase (ERK) in cell free, while in rat primary cortical neurons JNK inhibition activates the MEK1–ERK–Ets1/c-Fos cascade. JNK inhibition induces a compensatory effect and leads to ERK activation via MEK1, resulting in an activation of the survival pathway—(MEK1/ERK) as a consequence of the death pathway—(JNK)

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Abbreviations: D-JNKI1, D-retro-inverso form of c-Jun N-terminal kinase-inhibitor; ERK, extracellular signal-regulated kinase; JBD, c-Jun N-terminal kinase–binding domain; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MKK4, mitogen-activated protein kinase kinase 4; MKK7, mitogen-activated protein kinase kinase 7.

This study should hold as an important step to clarify the strong neuroprotective effect of D-JNKI1. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: JNK, MKK4, MKK7, ERK, MEK1, D-JNKI1.

Mitogen-activated protein kinases (MAPKs) are a family of enzymes involved in the transduction of signals from the extracellular environment into the cell. In particular, c-Jun N-terminal kinase (JNK) is an important stress-activated protein engaged in many aspects of cellular regulation including gene expression, cell proliferation, sprouting and programmed cell death.

As in the other MAPK systems, the immediate upstream activators of JNK are MKKs: JNK appears to be directly activated on Thr183 or Tyr185 residues (Weston and Davis, 2002) by mitogen-activated protein kinase kinase 4 (MKK4) or mitogen-activated protein kinase kinase 7 (MKK7) depending upon the cell type, developmental stage, and stressful stimulus. In consequence of its activation, JNK can phosphorylate nuclear substrates including c-Jun, ATF-2, Elk-1 as well as non-nuclear targets such as the mitochondrial proteins-Bcl2 family (Yao et al., 2005; Lei and Davis, 2003), MAP2 (Chang et al., 2003), Tau (Yoshida et al., 2004), MADD/DENN (Del Villar and Miller, 2004) and JIPs (Centeno et al., 2007). The way by which JNK activates its substrates is thought to require prior binding to the so-called c-Jun N-terminal kinase–binding domain (JBD), present in many JNK targets. JNK pathway activation has been observed in various diseases states and its inhibition represents an important research focus in order to protect cells against neurodegeneration, diabetes, tumors (Ennis et al., 2005) and other pathologies. In the last few years many studies have been directed to the screening and/or design of JNK-selective inhibitors with the aim to test their potential as drugs. Among the JNK inhibitors those which easily cross the blood–brain barrier and exert neuroprotective action with fewer side effects are the most important.

Recently it was reported that the biological action of JNK could be strongly inhibited *in vitro* and *in vivo* (Borsello et al., 2003a) by the cell-permeable JNK inhibitor peptide, D-retro-inverso form of c-Jun N-terminal kinase-inhibitor (D-JNKI1) (Borsello and Bonny, 2004). This peptide powerfully prevented neuronal death in permanent and tran-

sient brain ischemia (Borsello et al., 2003a; Repici et al., 2007), excitotoxicity of hippocampal organotypic cultures (Borsello et al., 2003b), hair-cell loss in animal models of sudden deafness and sound trauma-induced hearing cell loss (Wang et al., 2003, 2007), retinal ganglion cell death after optic nerve crush (Tezel et al., 2004), heart ischemia (Milano et al., 2007) and it also protected against viral infection (Beckham et al., 2007).

D-JNK11 was engineered by linking the 20-amino acid domain of the JIP-1/IB1 protein, which specifically binds JNK, to the 10-amino acid HIV-TAT cell-permeable sequence. It is a highly specific JNK substrate inhibitor which competes with the JBD₂₀-containing JNK targets (Centeno et al., 2007) blocking JNK action but not its activation (Borsello and Bonny, 2004). However, the properties of the D-JNK11 peptide are still only partially known.

Here, we report a detailed re-examination of D-JNK11 action on the JNK signaling pathway in cell-free and in basal conditions in primary cultures, using a high-purified D-JNK11 peptide. We characterized the effects of D-JNK11 (dose-response) on the two upstream JNK activators MKK4 and MKK7 and examined how specific JNK inhibition influenced p38 and extracellular signal-regulated kinase (ERK) pathways in primary cortical neurons.

EXPERIMENTAL PROCEDURES

Alignment of JBDs

Each factor was analyzed using the LALIGN program LAST P on the website EXPASY (Geneva, Switzerland). Factors were found by homology searches and were incorporated in the database. NCBI Protein Database accession numbers are marked. IB1/JIP1 protein accession numbers are AAD22543; IB2/JIP2 AAF32323; JIP3/JSAP1 BAA85874; c-Jun CAA35084; SEK1/MKK4 AAB81554; MKK7 AAD15821. The sequence alignments indicated that all sequences obeyed the previous 11 residue region described by Holland and Cooper (1999) including the motif $+X_{0-2}+X_{0-4}L/IVXL/IV$, where + is R or K, and X is any amino acid.

Cortical neuronal culture

All animal experiments were authorized by the Swiss veterinary authorities and were conducted according to legal guidelines. All experimental procedures on live animals were performed according to the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were authorized by Switzerland legal guideline. All efforts were made to minimize the number of animals used and their suffering. Small pieces of cortex were dissected from the brains of 2-day-old rat pups, incubated with 200 units of papain for 30 min at 34 °C, treated with trypsin inhibitor (10 µg) and mechanically dissociated. Neurons were then plated at densities of approximately 1×10^6 cells/plate on dishes pre-coated with 25 µg/ml poly-D-lysine. Experiments were done after 11–13 days in culture, by which time the neurons had elaborate axonal and dendritic arbors and had formed many synapses. D-JNK11 was added to the dishes at concentrations of 2–4 µM for 24 h before cell lysis. Total protein extracts were obtained by scraping cells in lysis buffer (Bonny et al., 2001).

Lactate dehydrogenase (LDH) cytotoxicity-assay

Neuroprotection was evaluated by LDH assay: 24 h after NMDA treatment, LDH released into the culture medium was measured using the Cytotox 96 non-radioactive cytotoxicity assay kit (Promega, WI).

Peptide sequences

JBD₂₀ sequence: RPXRPTTLNLFQVPRSQDT

JBD_{mut} sequence: RPXRPTYYNFQVPRSQDT

TAT sequence: GRKKRRQRRRPP

D-JNK11 sequence: TDQSRPVQPFNLTTPRXPRPPRRRQRRKKRG

For the in cell-free assay we used JBD₂₀ and JBD_{mut} sequences without the 10 aa of TAT to avoid precipitation due to high concentration of positive charged aa contained in this sequence. Instead in the *in vitro* experiments we used D-JNK11 peptide, which contains the TAT sequence and allows its permeation into neurons.

JNK kinase-assays

Before starting the kinase reactions, 2 µCi [γ -³³P]ATP and 40 µM of peptides (JBD₂₀ or JBD_{mut}) were added to the activated recombinant JNK2 or JNK3 (0.5 µg, Upstate Biotechnology, Lake Placid, NY, USA) for 15 min. Kinase reactions (Bonny et al., 2001) were done for 30 min at 30 °C using 1 µg of the GST-MKK4, GST-MKK7, GST-c-Jun, GST-ATF2 or GST-Elk1 fusion proteins. Samples were then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography.

Kinase-assays

Kinase assays to test MKK7 β , MKK4, JNK1 α 1, JNK2 α 2, MKK6, MEK1, ERK and p38 α activity were done at a concentration of 10 µM JBD₂₀ peptide as previously described, using the following fusion proteins: GST-JNK1 α 1 (2 µM) for MKK7 and MKK4 activity, GST-ATF2 (3 µM) for JNK1 α 1 and JNK2 α 2 activity, GST-p38 α (1 µM) for MKK6, GST-MAPK2 (1 µM) for MEK1 activity, GST-ATF2 (3 µM) for p38 activity and GST-ELK1 (3 µM) for ERK activity.

Western blot analysis

Proteins were separated on 10% SDS polyacrylamide gel and transferred to a PVDF membrane. Incubation with primary antibodies was overnight at 4 °C using: 1:10,000 anti-MKK7 (#M-86920, BD Transduction Laboratories, San Jose, CA, USA), 1:1000 anti-P-MKK7 (#4171 Cell Signaling Technology, Beverly, MA, USA), 1:50,000 anti MKK4 (#SC-964, Santa Cruz Biotechnology, CA, USA), 1:2000 anti-P-MKK4 (#9151 Cell Signaling Technology), 1:4000 anti-JNK (#9252, Cell Signaling Technology), 1:4000 anti-P-JNK (#4671 Cell Signaling Technology), 1:2000 anti-c-Jun (#9162 Cell Signaling Technology), 1:5000 anti-P-c-Jun (#06-659 Upstate Biotechnology), 1:5000 anti-ERK (#13-6200 Zymed, Carlsbad, CA, USA), 1:1000 anti-P-ERK (#SC-7383, Santa Cruz Biotechnology), 1:4000 anti-ATF-2 (#9226 Cell Signaling Technology), 1:2000 anti-P-ATF2 (#9221 Cell Signaling Technology), 1:1000 anti-p38 (#9212 Cell Signaling Technology), 1:1000 anti-P-p38 (#9211 Cell Signaling Technology), 1:1000 anti-Elk-1 (#SC 1355, Santa Cruz Biotechnology), 1:1000 anti-P-Elk-1 (#9181S Biolabs, Ipswich, MA, USA), 1:25,000 anti-MEK1-2 (#9126 Cell Signaling Technology), 1:25,000 anti-P-MEK1 (#9127 Cell Signaling Technology), 1:1000 anti-Ets1 (#SC-111, Santa Cruz Biotechnology), 1:1000 anti-P-Ets1 (#44-1104G Biosource, Camarillo, CA, USA) and 1:1000 anti-c-fos (#SC-253 Santa Cruz Biotechnology). P-antibodies are specific and recognize only the phosphorylated form of these proteins. Blots were developed using horseradish peroxidase-conjugated secondary antibodies and the ECL chemiluminescence system.

All blots were normalized against tubulin level (#SC-8035, Santa Cruz Biotechnology) and quantified by densitometry analysis (ImageQuantTLv2005, GE Healthcare).

Statistical analysis

All experiments were repeated at least five times using independent culture preparations. Data were calculated as mean \pm S.E.M. Differences between groups were compared using Student's *t*-test (single comparisons) or one-way ANOVA (multiple comparisons) with Bonferroni post-test. *P*-values <0.05 were considered significant.

RESULTS

The Δ -JNK1 peptide works by preventing JNK interaction with its JBD dependent targets. We here performed cell-free and *in vitro* experiments in primary cortical neurons for a deeper characterization of Δ -JNK1 features in physiological conditions.

Previous studies have led to the identification of factors that interact with JNK through a JBD, obeying the consensus MAPK binding domain (Pawson and Scott, 1997; Pawson and Nash, 2000). These include c-Jun (Dai et al., 1995), IB1/JIP1 (Bonny et al., 1998), IB2/JIP2 (Bogoyevitch and Arthur, 2007), MKK4 (Ho et al., 2003) and MKK7 (Ho et al., 2006). We ran a GenBank database search using the JBD sequence of IB1/JIP1 as bait in the LALIGN program: IB1/JIP1, IB2/JIP2, IB3/JIP3, c-Jun, MKK4 and MKK7 were aligned with the JBD of IB1/JIP1 (Fig. 1A). As a result in MKK4 and MKK7 we identified an 11 amino acid region showing evident similarities with the JBD of IB1/JIP1.

This confirms a putative JBD on the two upstream kinases, MKK7 and MKK4, and has important implications because Δ -JNK1 could exert an upstream action preventing MKK4 and MKK7 interactions with JNK. We therefore decided to further analyze Δ -JNK1 action on the JNK signaling pathway in cell-free and *in vitro* systems.

In cell-free conditions: MKK4 and MKK7 are JNK activators but also JNK targets

The ability of JNK to phosphorylate its activators MKK4 and MKK7 and also the JBD₂₀ effect on this phosphorylation was assessed by a JNK kinase assay in a cell-free system. GST-MKK4 and GST-MKK7 were incubated with equal amounts of JNK2 or JNK3, 2 μ Ci [γ -33P] ATP and 40 μ M with either JBD₂₀ or JBD_{mut} (see Experimental Procedures for the sequences) for 30 min at 30 °C. As shown in Fig. 1B, JNK2 and JNK3 specifically phosphorylated MKK4 and MKK7. No phosphorylation was observed in the presence of the JBD₂₀ sequence, whereas its mutated counterpart at three conserved residues, JBD_{mut} (Bonny et al., 2001), reestablished MKK4 and MKK7 phosphorylation by JNK2 and JNK3 (Fig. 1B). These results confirm a JNK action on MKK7 and MKK4 phosphorylation.

In cell-free conditions: JBD specifically interrupts upstream events along the JNK signaling cascade

Previously JBD domains were considered necessary for the activation of JNK downstream targets. However, the finding that both MKK4 and MKK7 contain a bona fide JBD (Ho et al., 2003, 2006) indicates that they might also be direct substrates as well as activators of JNK itself (Fig.

1A). Thus, JBDs might be necessary for signaling along the JNK cascade at more upstream levels than originally thought. If this holds true, JBD might regulate other kinases and signaling pathways. It is therefore important to determine the specificity of the JBD module in relation to the other kinases and signaling pathways.

To provide a more complete analysis of the JBD₂₀ peptide properties and to compare its inhibitory effect, we tested its activity by kinase assay on MKK7 β , MKK4, JNK1 α 1, JNK2 α 2, MKK6, MEK1, ERK and p38 (Fig. 1C). The substrates used for each kinase are indicated in the Experimental Procedures. Assays were done at a concentration of 10 μ M of JBD₂₀ peptide and 10 μ M of 2 μ Ci [γ -33P] ATP.

In the case of MKK4, MKK7 β , JNK1 α 1 and JNK2 α 2 the peptide specifically prevented kinase phosphorylation signaling by blocking their action on each substrate. Instead no effect was observed on MEK1, MKK6, ERK and p38, providing evidence that the JBD₂₀ peptide had no direct effect on these JNK-related kinases. Interestingly, the peptide was more efficient in inhibiting the MKK4 kinase (91% inhibition), than the two JNK isoforms' activity (80% inhibition) and MKK7 (57% inhibition) in cell-free conditions. Thus, the peptide at 10 μ M inhibits through a JBD mechanism both the activities of MKK4 and MKK7 on JNK and JNK action on its substrates like c-Jun.

In vitro: Δ -JNK1's action in primary cortical neurons in physiological conditions

Up to now Δ -JNK1 action has been studied in stress conditions (Borsello et al., 2003a; Borsello and Bonny, 2004; Centeno et al., 2007) due to its powerful neuroprotective effect. Here we used it in physiological conditions in cortical neurons for a more complete analysis of its properties. First of all we studied Δ -JNK1 action on MKK7, MKK4 and JNK activation.

Western blot analysis was performed to examine the ratios P-MKK7/MKK7, P-MKK4/MKK4 and P-JNK/JNK following Δ -JNK1 treatment (2 and 4 μ M, concentrations used in previous works) for 24 h (Fig. 2A–C). At 2 and 4 μ M of Δ -JNK1 the P-MKK7 was the same as controls; 2 μ M did not change the P-MKK4 level while 4 μ M caused a 52% reduction. We then looked at the P-JNK/JNK ratio: as expected 2 μ M Δ -JNK1 for 24 h had no effect on P-JNK (Borsello et al., 2003a) but 4 μ M decreased JNK activation by 40%.

These *in vitro* results are in line with the in cell-free assay: Δ -JNK1 has an upstream action along the JNK cascade, acting at 4 μ M on MKK4 activation, while MKK7 activation is not significantly influenced by Δ -JNK1 at the same concentration. Therefore as hypothesized in our previous work we proved that Δ -JNK1's effect is dose-dependent (Centeno et al., 2007): in fact at 2 μ M it does not interfere with the upstream events but at 4 μ M it acts both upstream and downstream JNK. We could not increase the peptide concentration up to 10 μ M (concentration used in cell-free) because of toxic effects.

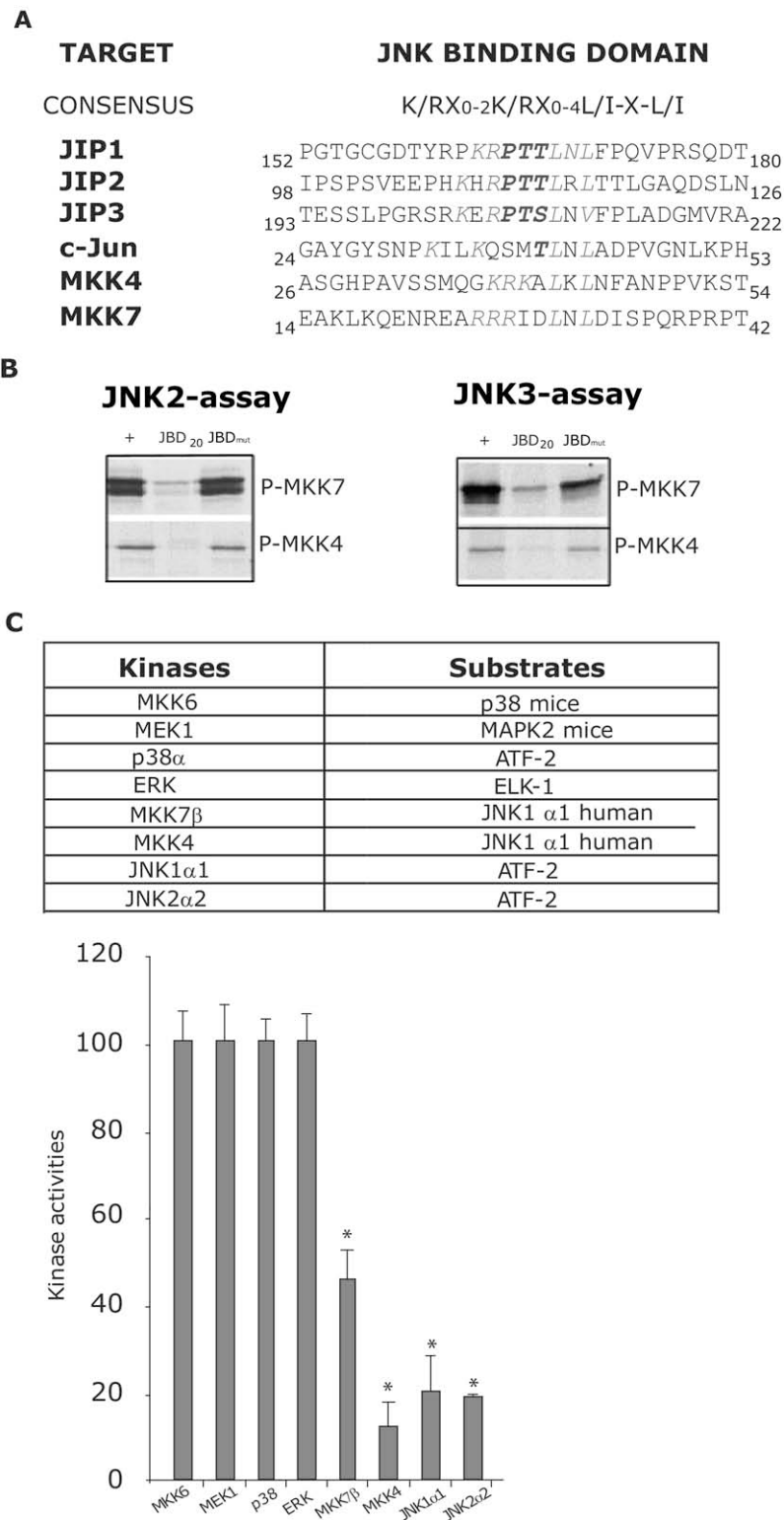


Fig. 1. JBD₂₀ peptide in cell-free. (A) JIP1, JIP2, JIP3, c-Jun, MKK4 and MKK7 sequences alignment using the LALIGN program. Sequences of and around the putative JBD are shown, consensus-matching residues are boldface (JBD preserved amino acids into the JIP family are in grey). (B) Kinase assays with recombinant activated JNK2 and 3, using GST-MKK4 and GST-MKK7 as substrates. The JBD₂₀ peptide inhibited MKK4 and MKK7 phosphorylation while the mutated peptide JBD_{mut} had no such effect. (C) Inhibitory specificity of JBD₂₀ on different kinases. Assays were run at a concentration of 10 μ M JBD₂₀ peptide (10 μ M ATP) to test MKK7, MKK4, JNK1 α 1, JNK2 α 2, MKK6, MEK1, ERK and p38 activities on their respective substrates (Fig. 1C, table). JBD₂₀ specifically prevented MKK4, MKK7, JNK1 α 1 and JNK2 α 2 activity while MKK6, MEK1, ERK and p38 activities were unchanged.

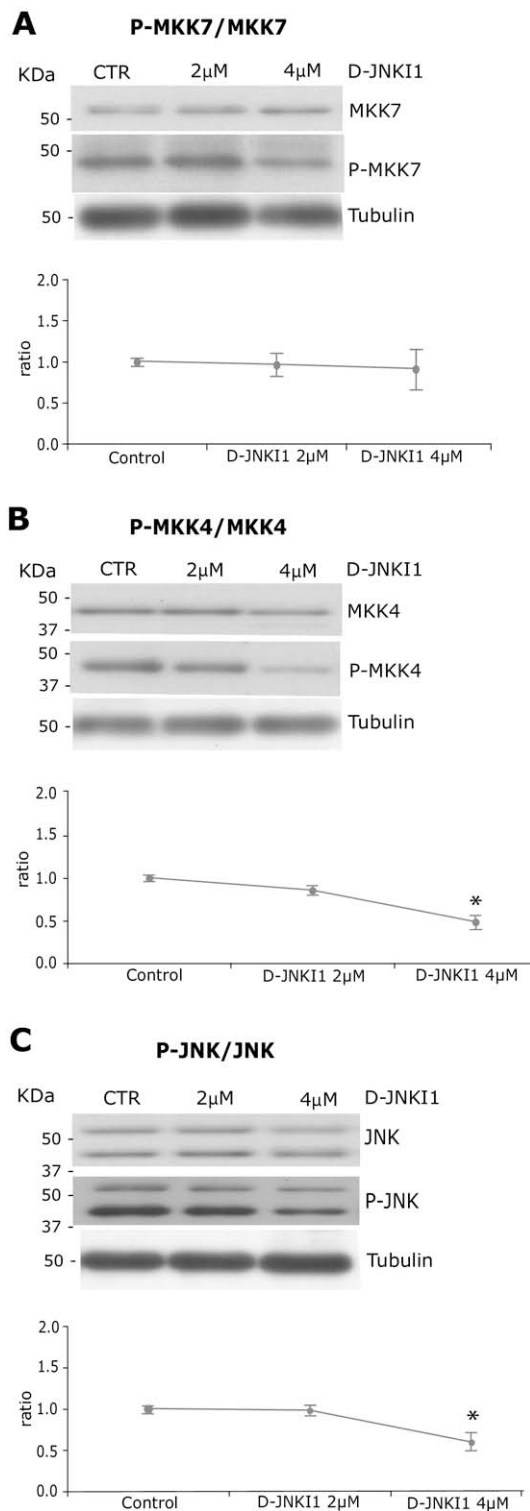


Fig. 2. D-JNKI1's effects on MKK4, MKK7 and JNK in cortical neurons. Cortical neurons were treated with 2 and 4 μ M D-JNKI1 for 24 h, and their lysates were analyzed by Western blot. The P-MKK7/MKK7 ratio was unaltered at both concentrations (A), while the ratio P-MKK4/MKK4 was reduced by 52% at 4 μ M (B). JNK activation also decreased of 40% with 4 μ M D-JNKI1 (C). Loading controls=tubulin. Data for WB quantification are mean \pm S.E.M. of 13 independent experiments. * $P < 0.05$ vs. control.

D-JNKI1's effects on c-Jun, ATF-2 and Elk-1 transcription factors

Although c-Jun is an elective JNK substrate, JNK can also phosphorylate and activate other transcription factors through a JBD interaction, such as ATF-2 (also target of p38) and Elk-1 (also target of p38 and ERK). We studied D-JNKI1 effects on these three transcription factors in cell-free and *in vitro* (Fig. 3).

In cell-free conditions in the presence of JBD₂₀ we found a strong reduction in the phosphorylation of c-Jun, ATF2 and Elk1 (Fig. 3A). Our data in cell-free conditions strongly correlate with our Western blot results on cortical neurons lysates.

In fact *in vitro* D-JNKI1 markedly reduced P-c-Jun/c-Jun levels, as expected (Fig. 3B) (Borsello et al., 2003a). At 2 μ M the reduction of P-c-Jun/c-Jun was 20% and at 4 μ M it reached 57%. The P-ATF2/ATF2 ratio was similar to that for c-Jun (reductions of 17% and 40% at 2 and 4 μ M D-JNKI1) (Fig. 3C). Finally the reduction in Elk-1 activation was approximately 18% at 2 μ M and 29% at 4 μ M D-JNKI1 (Fig. 3D). In conclusion D-JNKI1 prevented the phosphorylation of all three targets in control cortical neurons.

D-JNKI1's effects on the MAPK family: p38 and ERK

We already reported that 500 μ M of JBD₂₀ did not reduce p38 and ERK substrate phosphorylation in cell-free assays, meaning that the peptide cannot directly interfere with p38 and ERK activity (Borsello et al., 2003a). We confirmed here that the JBD₂₀ in a kinase assay had no direct effect on p38, ERK and MEK1 (Fig. 1C). However, *in vitro* the situation might be slightly different because of the cross-talk between MAPK signaling pathways (Davis, 2000).

Therefore we examined the effect of specific JNK inhibition on the associated MAP-kinases p38 and ERK (Figs. 4 and 5).

We first analyzed p38 activation by quantifying the P-p38/p38 ratio after 24 h of D-JNKI1 treatment (2 μ M and 4 μ M). With 2 μ M the ratio rose slightly to 1.24 and with 4 μ M it returned to basal levels (1.03) (Fig. 4A). D-JNKI1 did not interfere with p38 activation, even though 4 μ M partially inhibited MKK4 activation (Fig. 2B).

We then evaluated ERK activation: it increased 2.98-fold with 2 μ M and rose up to 9.36-fold with 4 μ M D-JNKI1 (Fig. 4B). This strong activation was due to an increase in the ERK phosphorylated/activated form, since the level of the unphosphorylated protein was almost unchanged. When we analyzed the peptide effect on Ets1 and c-Fos as ERK targets (Fig. 5B, C), we found a P-Ets1/Ets1 ratio increment of 1.66-fold at 2 μ M D-JNKI1 and 2.28-fold at 4 μ M. We also found activation of c-Fos of 1.64-fold at 2 μ M D-JNKI1 and 1.65-fold at 4 μ M. These data confirmed ERK pathway activation after JNK inhibition with D-JNKI1.

We checked more upstream in the ERK signaling pathway by assessing MEK1 activation after D-JNKI1 treatment, since MEK1 is an activator of both ERK1/2 and JNK2 pathways (Waetzig and Herdegen, 2005). From our cell-free study we know that the peptide had no direct

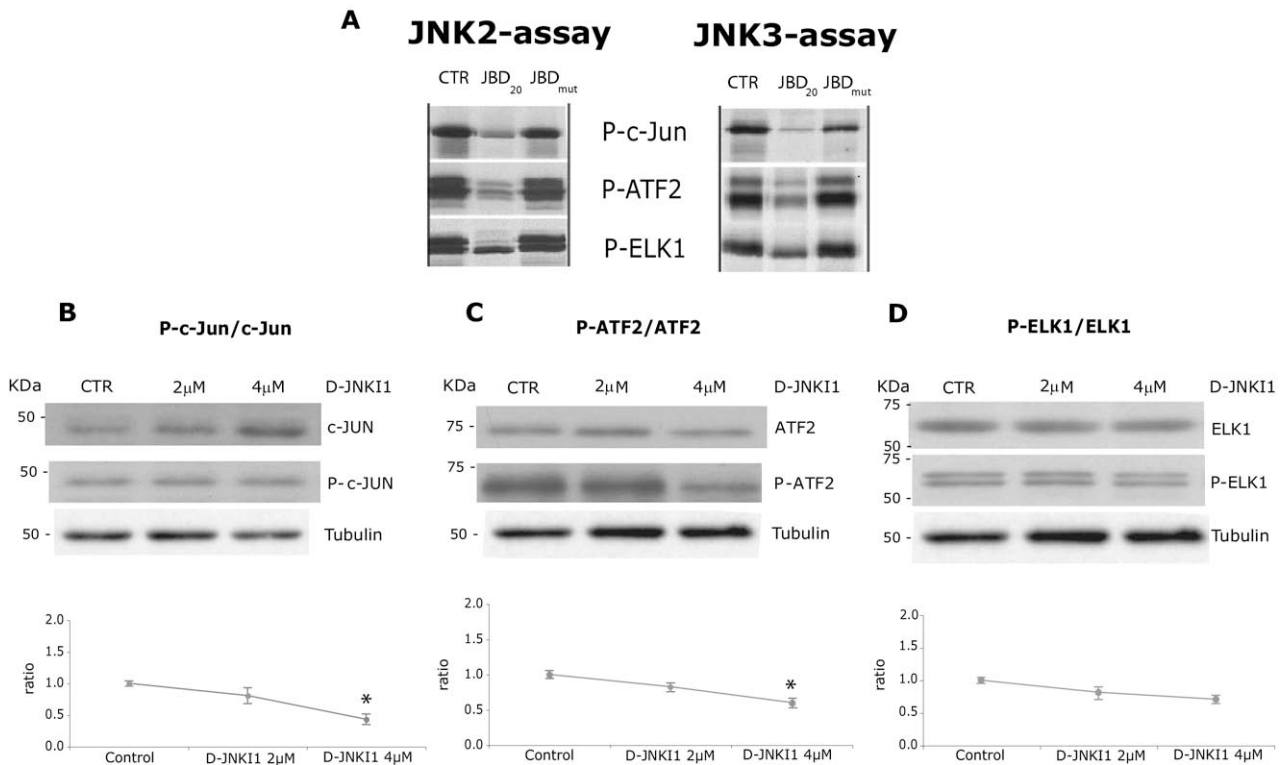


Fig. 3. D-JNK11's effects on c-Jun, ATF-2 and Elk-1 transcription factors. Kinase assays with recombinant activated JNK2 and -3, using GST-c-Jun, GST-ATF-2 and GST-Elk-1 as substrates (A). The JBD₂₀ peptide inhibited c-Jun, ATF-2 and Elk-1 phosphorylation while the mutated peptide JBD_{mut} had no effect. Cortical neurons were treated with 2 and 4 μ M D-JNK11 for 24 h and lysates were immunoblotted against c-Jun, P-c-Jun, ATF-2, P-ATF-2, Elk-1 and P-Elk-1 (B–D). There was a 20% reduction at 2 μ M and 57% at 4 μ M D-JNK11 for P-c-Jun/c-Jun (B), a 17% reduction at 2 μ M and 40% at 4 μ M D-JNK11 for P-ATF-2/ATF-2 (C), and 15% reduction at 2 μ M and 47% at 4 μ M D-JNK11 for P-Elk-1/Elk1 (D). Loading controls=tubulin. Data for WB quantification are mean \pm S.E.M. of 13 independent experiments. * $P < 0.05$ vs. control.

effect on MEK1 activity (Fig. 1C), but *in vitro* (cortical neurons) MEK1 was activated after 24 h of D-JNK11 treatment. The P-MEK1/MEK1-2 ratio increased 1.48-fold with 2 μ M compared with untreated neurons and rose further by 1.85-fold with 4 μ M D-JNK11 (Fig. 5A).

DISCUSSION

Many different studies have shown that JNK is strongly involved in neuronal death machinery. We have previously demonstrated that the specific JNK inhibitor peptide, D-JNK11, is capable of preventing neuronal death *in vitro* and *in vivo* (Borsello et al., 2003a; Centeno et al., 2007; Repici et al., 2007). This peptide (D-JNK11 or a shorter 1, TI-JIP (Barr et al., 2002)) prevents necrosis and apoptosis in different *in vivo* models (Wang et al., 2003, 2007; Tezel et al., 2004; Arthur et al., 2007; Eshraghi et al., 2007; Milano et al., 2007; Repici et al., 2007), implying an important neuroprotective quality because of the co-existence of these two death types in several pathologies.

Although D-JNK11 is one of the most powerful neuro-protectants and may hold promise for future clinical applications, its properties are still not fully identified.

A key finding of past studies was that at the concentration of 2 μ M D-JNK11 prevented JNK's action on the JBD-substrates without interfering with the activation of the

enzyme itself (Borsello et al., 2003a; Centeno et al., 2007; Repici et al., 2007), showing only a JNK downstream effect in the JNK signaling pathway.

However, in view of its powerfully neuroprotective feature we had always studied D-JNK11 action in stress conditions. Here we extend our earlier work by examining the peptide's properties in the cell-free and by characterizing its action mechanisms on cortical neurons in physiological conditions.

With a blast analysis we found that MKK7, as MKK4 (Ho et al., 2003), has a JBD homologue domain (Ho et al., 2006). Thus MKK4 and -7, currently considered the only two direct JNK activators, could also be JNK substrates. The significance of MKK4 and MKK7 phosphorylation by JNK is still not understood but the presence of a JBD homologue domain on MKK4 and MKK7 is an intriguing aspect of the JNK pathway, since it offers another regulation level for the JNK cascade. The JNK pathway might have a feedback loop pathway to quickly shut down the stimuli and/or to ensure the specificity, timing, and strength of its action.

We here showed in cell free that the peptide could inhibit the activity of MKK4 and MKK7. The effect of the JBD is stronger on MKK4 than on MKK7 in cell-free experiments, this induces us to speculate that in cortical neurons the ratio of P-MKK4/MKK4 will be more reduced compared

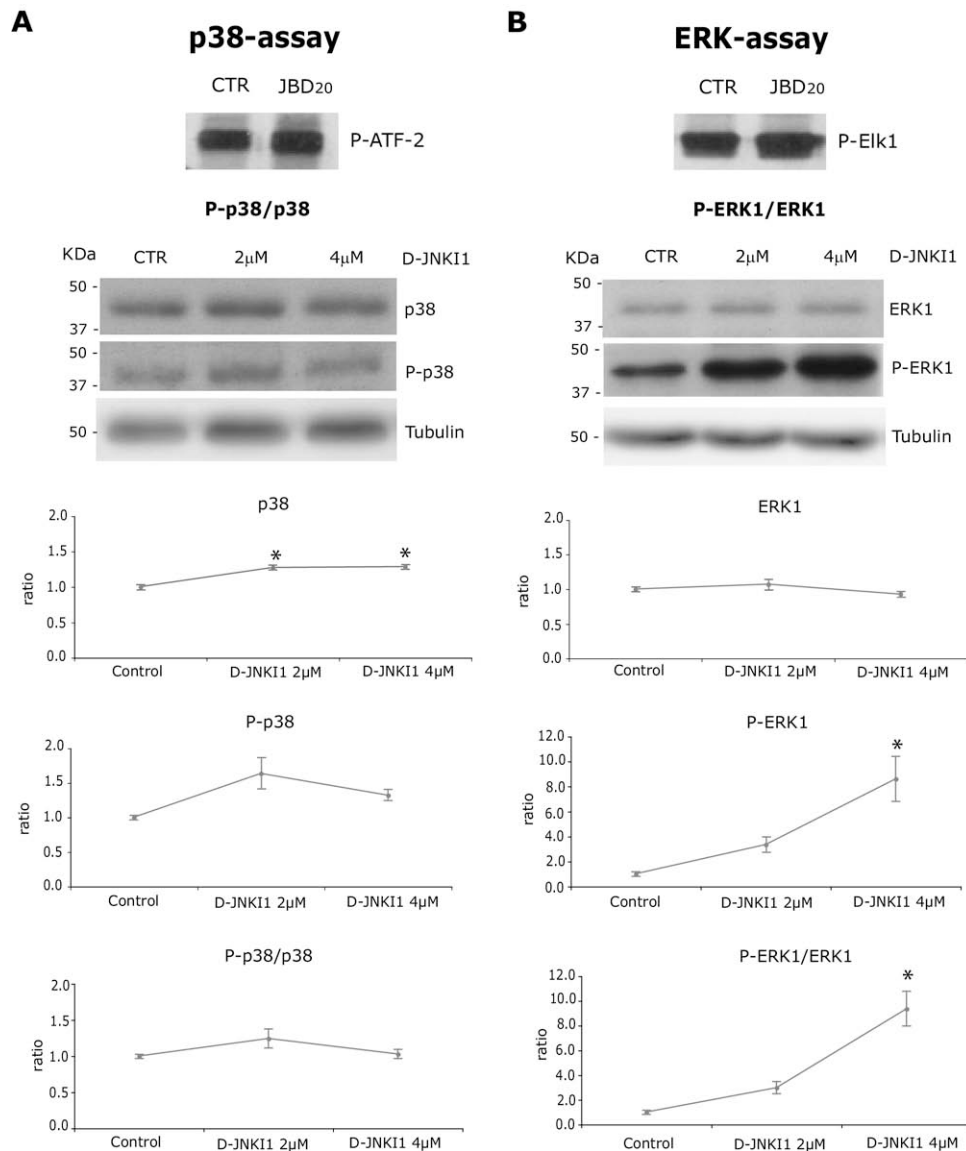


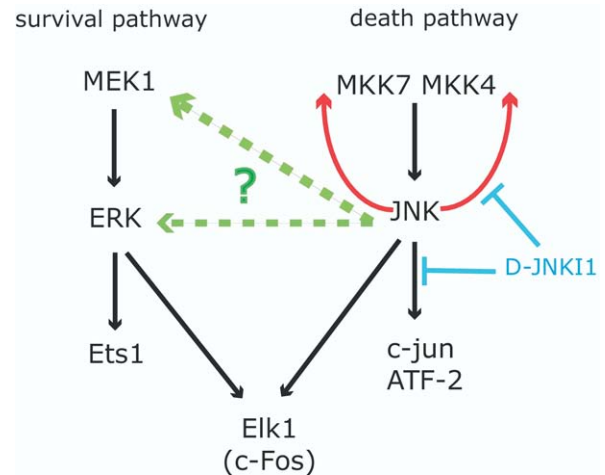
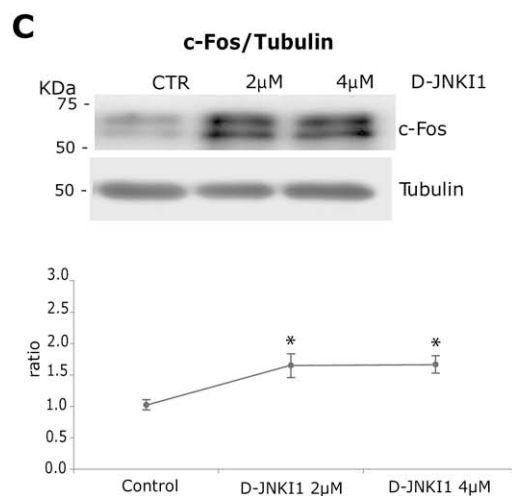
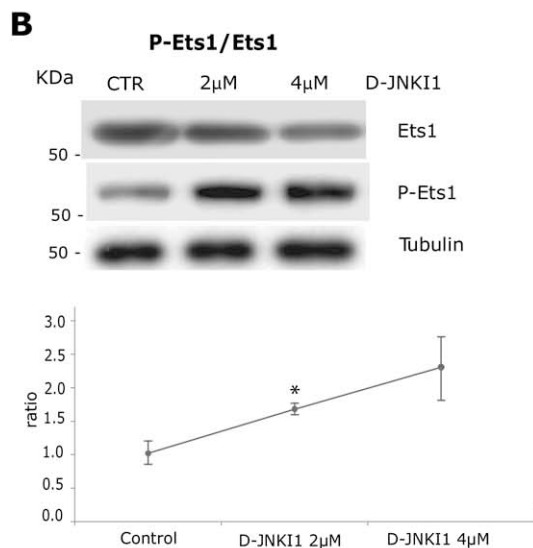
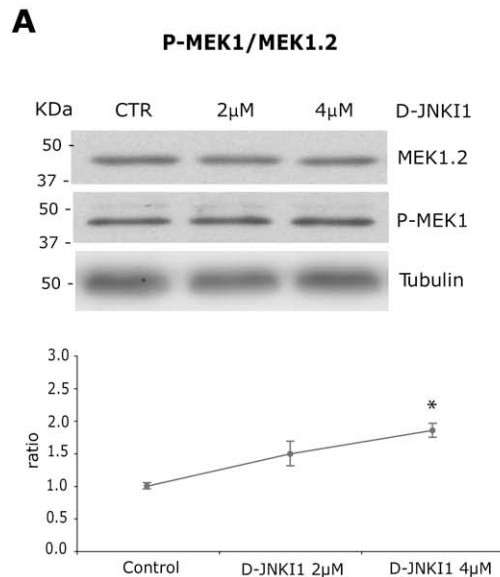
Fig. 4. D-JNKI1's effects on p38 and ERK MAPK. Kinase assays with recombinant activated p38 and ERK using GST-ATF2 (3 µM) for p38 and GST-Elk-1 (3 µM) for ERK as substrates. The JBD₂₀ peptide had no effect on p38 and ERK activities (A, B). Cortical neurons were treated with 2 and 4 µM D-JNKI1 for 24 h and the activation of p38 and ERK was evaluated as the phospho-protein/protein ratio. D-JNKI1 did not interfere with p38 activation (A), but strongly increased ERK activation (B) (2.98-fold and 9.36-fold with 2 and 4 µM). Loading control=tubulin. Data for WB quantification are mean±S.E.M. of 13 independent experiments. * $P < 0.05$ vs. control.

with the P-MKK7/MKK7 after D-JNKI1 treatment. Indeed this hypothesis is confirmed by our results: 4 µM D-JNKI1, the higher concentration used, produced an effect on MKK4 activation while it did not affect MKK7. We could not increase the peptide concentration up to 10 µM (concentration used in cell-free) in cortical neurons, because of toxic effects. As a consequence of the strong inhibition on MKK4, which is also a p38 activator, we expected a down-regulation of p38 pathway but neither in cell free nor in cortical neurons did we find any effects (Figs. 1, 4). Subsequently we can exclude an action of D-JNKI1 on the p38 signaling pathway.

We also examined the possible consequence of JNK inhibition on ERK MAPK since the ERK pathway has been

associated with pro-survival mechanisms and this would be consistent with the neuroprotective action of JNK inhibition by D-JNKI1. Our experiments indicate that by inhibiting JNK with 4 µM of D-JNKI1, ERK activation was increased, while there was no significant effect with 2 µM D-JNKI1. JNK inhibition in control conditions activates ERK pathway, implying an antagonist cross-talk between JNK and ERK pathways in physiological conditions in cortical neurons.

This represents a fascinating and controversial finding: the biochemical characterization of the peptide we did in cell-free assay did not completely bridge the gap from the *in vitro* state (Fig. 5). Earlier we have reported results proving that 500 µM of D-JNKI1 did not interfere with the



Scheme 1. Schematic representation of the ERK (survival) and JNK (death) pathways and their possible interactions. Downstream ERK and JNK targets (transcription factors) are indicated by black arrows. MKK4 and MKK7 are direct JNK activators but also JNK substrates (see the feedback loop). In the presence of D-JNKI1, JNK action is prevented on its JBD-dependent targets both downstream and upstream JNK (see the grey lines). JNK inhibition activates MEK/ERK survival pathway (the dotted lines indicate the hypothetical levels of activation in the ERK cascade).

ERK pathway in a cell-free assay (Borsello et al., 2003a). However, we here show that in cortical neurons D-JNKI1 4 μ M inhibits the JNK cascade and simultaneously induces powerful activation of the ERK pathway.

The hypothesis of a physiological balance of the MAPK family might explain the increased activation of ERK (Hall and Davis, 2002) after JNK inhibition: by preventing the stress-cascade (JNK) an activation of the survival pathway (ERK1, 2) occurs. We also know from cell-free experiments that D-JNKI1 has not direct effect on MEK1, the ERK upstream kinase, but in cortical neurons treated with D-JNKI1 MEK1 is activated. MEK1 is a kinase implicated in neuronal regeneration and sprouting (Matsuoka et al., 2004) and has an antagonist effect on the MKK7-JNK signalosome (Waetzig and Herdegen, 2005), confirming our *in vitro* results. This suggests that MEK1 might be the cross-point between JNK and ERK pathways: inhibition of JNK with D-JNKI1 in cortical neurons induces compensatory activation of the MEK1-ERK pathway. Finally, our results suggest that the powerful neuroprotective effect of D-JNKI1 may also be due to the activation of ERK/survival pathway and not only to the prevention of JNK activation/stress pathway.

Fig. 5. D-JNKI1's effects on ERK pathway. D-JNKI1 effects on MEK1, Ets1 and c-Fos. Cortical neurons were treated with 2 and 4 μ M D-JNKI1 for 24 h and the activation of MEK1, ETS1 was evaluated as the phospho-protein/protein ratio. The peptide effect on c-Fos was evaluated as the c-Fos protein expression level. D-JNKI1 increased MEK1 (A) (1.48-fold and 1.85-fold with 2 and 4 μ M) and ETS1 activation (B) (1.66 and 2.28-fold with 2 and 4 μ M), and c-Fos expression (C) (1.64-fold and with 2 and 4 μ M). Loading controls=tubulin. Data for WB quantification are mean \pm S.E.M. of five independent experiments. * $P < 0.05$ vs. control.

We here try to summarize possible interactions between JNK and ERK pathways in light of our own results (Scheme 1) hypothesizing the possible links. However, further studies are needed to discover other molecular links between these two pathways, considering also MAPK phosphatases, particularly MKP-7 (Katagiri et al., 2005), a JNK specific phosphatase, that may connect the JNK and ERK kinases. The cross-talk between JNK and ERK needs to be clarified in *in vivo* experiments (in progress) in order to better understand the physiological meaning of these compensatory cross-talks. This is of particular interest since understanding the control mechanisms of neuronal survival/death will allow the development of new tools for preventing degeneration.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at [10.1016/j.neuroscience.2008.11.049](https://doi.org/10.1016/j.neuroscience.2008.11.049).

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