Distinct Requirements for p38α and c-Jun N-terminal Kinase Stress-activated Protein Kinases in Different Forms of Apoptotic Neuronal Death

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The stress-activated protein kinases c-Jun-activated kinase (JNK) and p38 are implicated in neuronal apoptosis. Early studies in cell lines suggested a requirement for both in the apoptosis induced by withdrawal of nerve growth factor. However, studies in neuronal cells typically implicate JNK but not p38 in apoptosis. In some cases, p38 is implicated, but the role of JNK is undefined. It remains unclear whether p38 and JNK have differing roles dependent on cell type, apoptotic stimulus, or mechanism of cell death or whether they are redundant and each sufficient to induce identical forms of cell death. We investigate the relative roles of these protein kinases in different death mechanisms in a single system, cultured cerebellar granule neurons. Apoptosis induced by withdrawal of trophic support and glutamate are mechanistically different in terms of caspase activation, DNA fragmentation profile, chromatin morphology, and dependence on de novo gene expression. Caspase-independent apoptosis induced by glutamate is accompanied by strong activation of p38, and dominant negatives and inhibitors of the p38 pathway prevent this apoptosis. In contrast, withdrawal of trophic support induces caspase-dependent death accompanied by JNK-dependent phosphorylation of c-Jun, and inhibition of JNK is sufficient to prevent the death induced by withdrawal of trophic support. Inhibition of p38 does not block withdrawal of trophic support-induced death, nor does inhibition of JNK block glutamate-induced death. We propose that mechanistically different forms of apoptosis have differing requirements for p38 and JNK activities in neurons and demonstrate that only inhibition of the appropriate kinase will prevent neurons from undergoing apoptosis.

Stress-activated protein kinases (SAPKs)1 are believed to play an obligatory role in neuronal cell death; however, the relative roles of the JNK and p38 kinase groups have been unclear. Initial studies in the PC12 cell line suggested a role for both JNK and p38 in caspase-dependent cell death induced by withdrawal of nerve growth factor (1). Subsequent studies in primary cultured neurons failed to demonstrate a requirement for p38 in apoptosis induced by deprivation of trophic support, whereas a role for JNK in developmental, trophic withdrawal-induced, excitotoxic, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced death has been substantiated in a variety of neuronal systems (2–7). There are proposals that p38 contributes to axotomy-induced apoptosis of retinal ganglion cells, excitotoxicity-induced apoptosis of cerebellar granule neurons, and ceramide-induced death of cortical neurons (8–10). However, these reports rely on SB203580, the specificity of which has recently been called into question, since it inhibits other kinases more potently than it does p38 (11), and it discriminates poorly between p38 and certain JNK isoforms (12, 13). Furthermore, the nature of the cell death under investigation, whether apoptotic, necrotic, or intermediate, is often unclear. Whether JNK and p38 are required for different forms of death in the same cell type or whether cell type or development-specific properties are responsible for these observations have remained as unanswered questions. We set out to investigate the roles of p38 and JNK in cell death induced in the same neuronal cell type and at the same developmental stage but in different cell death paradigms, which we characterize in detail. Withdrawal of trophic support, proposed to model developmental and axotomy-induced death, leads to condensation of the nucleus to a single mass accompanied by many of the characteristics of classical caspase-dependent death, such as caspase-dependent internucleosomal DNA fragmentation and pyknosis, in a manner dependent on de novo protein expression. In contrast, glutamate treatment, a model for neuronal death in ischemia, leads to formation of lumpy chromatin and proceeds via a caspase-independent but nonnecrotic pathway associated with high molecular weight DNA fragmentation and rapid pyknosis independent of de novo protein synthesis. Glutamate treatment induces strong activation of p38, and the cell death is prevented by dominant negative inhibitors of the p38 pathway but not by the JNK inhibitor. In contrast, death induced by withdrawal of trophic support is prevented by JNK inhibitor but not by p38 inhibitor. Both forms of cell death are inhibited by Bcl-2 overexpression, suggesting a common or related intermediate despite the divergent upstream and downstream requirements of the cell death pathway. Our data indicates that

oromethylketone; GFP, green fluorescent protein; KD, kinase-dead; PBS, phosphate-buffered saline; GST, glutathione S-transferase.

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distinct members of the SAPK group are required for these neuronal cells to enter into different forms of apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Glutamate, and Withdrawal of Tropic Support Treatments**

Cerebellar granule neuron cultures were prepared as previously described (14), and neurons used were between 7 and 9 days in vitro. Glutamate treatment was carried out by rinsing cells in magnesium-free Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 1.3 mM CaCl₂, 5.6 mM d-glucose, 5 mM Hepes, pH 7.4) and placing them in the same buffer with glutamate (50 μM or as shown) for 30 min or as shown; 10 μM glycine was routinely included with glutamate, since it is an essential co-agonist for the NMDA receptor. Subsequently, cells were rinsed in Locke’s buffer with 1 mM MgCl₂, and conditioned medium was replaced for the time indicated. Withdrawal of tropic support was carried out as previously described (13). Pharmacological agents (2βAD-fmk (100 μM), MK-801 (2 μM), SB203580 (1 μM), and 1-β-antipyrarole “SP600125” (1 or 3 μM)) were added 60 min before withdrawal stress or glutamate treatment, and the agents were also present in all media in which the cells were subsequently incubated. Plasmids were introduced into neurons as indicated, 24 h before stress treatments, using transfection methods as previously described (13). When tested, co-transfection efficiency was near 100%, as previously reported (15).

**Immunoblotting**

Immunoblotting was carried out as previously described (13), by treating cells as indicated, rapid rinsing in ice-cold sodium phosphate buffer (phosphate-buffered saline; PBS), lysis in 1× Laemmli buffer, boiling, clearing, resolution by SDS-PAGE, and electrotransfer. Western blotting was carried out by standard protocols, and blots were developed using ECL reagents according to the manufacturer’s instructions.

**Antibodies and Plasmids**

Mouse anti-GFP (Clontech), rabbit anti-cleaved rat caspase-3 (Trevigen, Gaithersburg, MD), anti-phospho-p38 and anti-phospho-JNK (New England Biolabs, Beverly, MA), anti-pan-JNK from Upstate Biotechnology, Inc. (Lake Placid, NY), anti-phospho-p38 and c-Jun from Transduction Laboratories (Lexington, KY), and anti-actin (generous gift of Sander van den Heuvel (Massachusetts General Hospital). Empty vector pCMV was a generous gift of Marion MacFarlane, and Gerry Cohen (Center for Mechanisms of Human Toxicity, Leicester, UK), Bruce Mayer (Children’s Hospital, Boston, MA), Paul Whitten (University of Nottingham, Nottingham, UK), respectively. Empty vector pCMV was a generous gift of Sander van den Heuvel (Massachusetts General Hospital).

**Caspase Assays**

Caspase 3-like (DEVDase) activity in cell extracts was assayed using fluorogenic substrate DEVD-7-amino-methylcoumarin (Pharmingen) using amounts of extract and substrate according to the manufacturer’s protocol. Cell extract was prepared by the treatment of cells with ice-cold cell extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 130 mM NaCl, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100). DEVDase activity was measured after incubation of extract with 20 μM DEVD-7-amino-methylcoumarin for 1 h at 37°C.

**Electron Microscopy**

Cells were fixed for 30 min with 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4, washed three times with the same buffer, and then postfixed with 1% osmic acid in PBS, dehydrated, and embedded with LX-112 resin (Ladd Research Industries, Inc.). Sections (30–70 nm thick) were mounted on copper grids and then examined by transmission electron microscopy using a JEM-1200 EX microscope (JEOL, Tokyo, Japan).

**Analysis of DNA Integrity**

This was performed essentially as described earlier (18). Briefly, cells were embedded into low melting point agarose drops followed by incubation for 1 h at 37°C in lysis buffer (20 mM Tris-HCl, pH 7.5, 20 mM EDTA, 0.5% SDS) containing 100 μg/ml Proteinase K and 100 μg/ml RNase A. Agarose drops containing deproteinized DNA samples were loaded quantitatively to the wells of a 1% agarose gel and subjected to either conventional or field inversion gel electrophoresis. Conventional gel electrophoresis was carried out at 70 V for 3 h using 0.5× TBE buffer (0.89 M Tris, pH 8.3, 0.89 M boric acid, 0.002 M EDTA). Field inversion gel electrophoresis was performed at 85 V for 18 h in 0.5× TBE buffer under constant pulses of electric field (24 s forward and 8 s backward), allowing resolution of DNA molecules of size up to 500 kb (19).

**Reportor Assays**

Luciferase Reporter Assays—Luciferase reporter assays were carried out in primary cultured cerebellar granule neurons as previously described (4, 13). Briefly, cells were transfected with a firefly luciferase reporter plasmid driven by five GAL4 elements in tandem, pG3-5E4Δ38, a plasmid expressing a fusion protein of the p38α-specific substrate ME2A with the DNA binding domain of GAL4, and pRL-CMV expressing sea panay luciferase as an internal standard against which signals were normalized, and either pCDNA3-MKK6E expressing a constitutively active MKK6 and pEBGp38α expressing empty vector pCMV as indicated. For withdrawal-induced c-Jun reporter assays, GAL4-Mef2 was replaced with GAL4-Jun-6–89, and MKKGp38α wild-type

**Viability and Pyknosis Assays for Transfected and Untransfected Neurons**

7 days after plating, 12-well plates of cells were transfected as described previously (4). They were co-transfected with GFP marker plasmids and either empty vector (pCMV), pEBST-Bcl2, pcDNA3-p38α-AF, or pM73-MKK3a-kinase-dead “M KK3a-KD” as shown. 24 h after transfection, cells were treated with or without glutamate treatment as described above, and culture medium was replaced for 24 h. Immunoblotting-based Viability Assay—24 h after stimulation with or without glutamate, cells were rinsed in ice-cold PBS, lysed in 75 μl of Laemmli sample buffer, and processed for immunoblotting as described below.

**Cell Counting-based Viability Assay Method—**Cerebellar granule neurons were transfected as above. After a 24-h incubation, they were treated with or without glutamate as described above. Transfected cells in four evenly spaced fields per coverslip were counted by epifluorescence using 450–490-nm excitation light and a ×20 air objective. After a further 24 h of incubation, the transfected cells were counted again as above. Viability was calculated as the proportion of GFP-positive cells remaining after stimulation.

**Pyknosis Assay for Transfected and Untransfected Neurons—**3 h (as shown) after stimulation with or without glutamate or 24 h after withdrawal of tropic support, cells were fixed with 4% paraformaldehyde, rinsed with PBS and stained with Hoechst 33342. For transfected neurons, fluorescence image fields of GFP emission were taken to locate transfected cells, and the corresponding image of Hoechst fluorescence is examined to determine whether the transfected cell has a pyknotic nucleus. Four evenly spaced fields were counted per coverslip. Imaging of DNA dyes and transfected fluorescent proteins was carried out with a cooled Apogete IX85 CCD and IX70 Olympus microscope with appropriate filter cubes.
plasmids were not used. Dominant negative \( p38^{\alpha}\) and MKK3-KD plasmids were co-transfected where indicated. Empty vector pCMV was added to equalize transfections to 2 \( \mu \)g of total DNA/24-well plate. The following day, cells were lysed in passive lysis buffer (Promega). Firefly (reporter) and Renilla (internal standard) luciferase activities were assayed with the dual luciferase assay kit (Promega) according to the manufacturer’s instructions.

**Kinase Assays—**Kinase assays of \( p38^{\alpha}\)-transfected neurons were carried out as previously described for JNK1 (4). Briefly, 35-mm dishes of neurons at 7 days in vitro were co-transfected with the plasmids pEBG-p38\( ^{\alpha}\) together with either empty vector pCMV or pMT3-HA-MKK3KD as indicated. 24 h after transfection, cells were treated as described for 5 min or left untreated as indicated, rinsed once with ice-cold PBS, and lysed in 500 \( \mu \)l of lysis buffer (20 mM HEPES, pH 7.4; 2 mM EGTA; 50 mM \( \beta\)-glycerophosphate; 1 mM dithiothreitol; 1 mM Na<sub>3</sub>VO<sub>4</sub>; 1% Triton X-100; 10% glycerol; 50 mM NaF; 1 mM benzamidine; 1 \( \mu \)g/ml aprotinin, leupeptin, and pepstatin; and 100 \( \mu \)g/ml phenylmethylsulfonyl fluoride). Homogenized and precleared supernatants were incubated with 10 \( \mu \)l (bed volume) of prewashed 50-\( \times\)glutathione-agarose beads (Sigma) for 3 h at 4°C. Beads were spun out and washed three times in lysis buffer followed by boiling aspirated pellet in 40 \( \mu \)l of 1× Laemmli sample buffer and immunoblotting. GST-p38\( ^{\alpha}\) ran at about 65 kDa, and anti-phospho-p38 and anti-pan-p38 immunoreactive bands shown migrate at this molecular mass.

**Subcellular Fractionation**

8 days after plating in 6-cm dishes, cerebellar granule neuron cultures were treated with glutamate (50 \( \mu \)M), or trophic support was withdrawn as described above. 60 min after beginning the 30-min glutamate treatment or after 7 h of trophic support withdrawal, cells were rinsed with ice-cold PBS once and lysed in 700 \( \mu \)l of homogenization buffer (0.32 M sucrose; 1 mM EDTA; 2 mM MgCl<sub>2</sub>; 20 mM Tricine-NaOH, pH 7.8; 10 \( \mu \)g/ml aprotinin, leupeptin, and pepstatin; and 100 \( \mu \)g/ml phenylmethylsulfonyl fluoride). Homogenized lysates were cleared of nuclei and unbroken cells by centrifugation at 100 \( \times\)g for 5 min, followed by collection of crude mitochondrial pellet by centrifugation at 16,000 \( \times\)g for 20 min. The location of nuclei and mitochondria were confirmed by staining DNA with Hoechst 33342 and mitochondria with Mitotracker (Molecular Probes). Crude mitochondrial pellets were lysed in Laemmli sample buffer. Protein was precipitated from 250 \( \mu \)l of supernatants by adding 1250 \( \mu \)l of acetone, incubating at −20°C for 10 min, and centrifuging at 12,000 \( \times\)g for 5 min. The precipitated cytosolic protein was dried and lysed in Laemmli sample buffer. The location of cytochrome \( c\) was assessed by immunoblotting crude mitochondria and cytosol samples with monoclonal anti-cytochrome \( c\) (SA-226; Biomol). Equal loading of mitochondria and cytosol was assessed by immunoblotting with mouse anti-cytochrome oxidase subunit IV (A21348; Molecular Probes) and by Coomassie staining, respectively.

**RESULTS**

Glutamate is widely believed to underlie the neuronal death that occurs during stroke and a number of other neurodegenerative conditions. The SAPKs JNK and p38 are frequently implicated in neuronal death induced in these conditions. Therefore, we investigated the regulation of SAPKs in a manner corresponding to our previous analysis of the response of these cells to withdrawal of trophic support (13). The addition of glutamate to mature cultured cerebellar granule neurons induced a rapid and large increase of phospho-p38 signal without detectable increase of total phospho-JNK level (Fig. 1, A and B). As previously described, the stress-activated JNK pool in these cells is not detectable as a result of the high constitutive activity, but c-Jun phosphorylation serves as a sensitive indicator by alternative methods. Since the cerebellar neurons express p38\( ^{\alpha}\) and not p38\( ^{\beta}\) (Fig. 3A), we utilized expression plasmids for a dominant negative form of p38\( ^{\alpha}\) (nonphosphorylatable mutant “AF”) (16) and MKK3 (kinase-dead (KD)) (25). First, we examined the specificity of these dominant negatives, by testing their effects on actions of JNK activated by withdrawal of trophic support. For this purpose, we utilized a revealed that glutamate induces a large calcium spike that precedes the peak of p38 phosphorylation, followed by a sustained elevated level of neuronal cytoplasmic free calcium (Fig. 1C). We did not detect the previously described “necrotic delayed calcium deregulation” (21) under our conditions. The observed calcium response was dependent on calcium in the extracellular medium and NMDA receptors (data not shown). The p38 activation was entirely dependent on extracellular calcium (Fig. 1D). This contrasts with the reported calcium independence of glutamate-evoked JNK activation in striatal cultures (22). The p38 response could be considerably reduced by NMDA receptor antagonist MK-801 (Fig. 1E).

The activation of p38 and the stress-sensitive JNK pool by glutamate is reminiscent of the response induced by withdrawal of trophic support, although the latter response is slower and more sustained. The death induced by withdrawal of trophic support is believed to require JNK, but conditions that inhibit p38/\( \beta\) fail to prevent the death (13, 23). Therefore, we investigated the sensitivity of glutamate-induced death, inferred from the formation of pyknotic nuclei, to inhibitors of SAPKs. The small molecule 1,9-anthrapyrazolone (SP600125) inhibits JNK preferentially over other mitogen-activated protein kinases (24) (data not shown), and 1–3 \( \mu \)M inhibits the c-Jun phosphorylation shift induced by withdrawal of trophic support from cerebellar granule neurons (Fig. 2A, *upper panel*), whereas even 3 \( \mu \)M does not prevent p38\( ^{\alpha}\) from activating MeF2A (Fig. 2B). This compound at 3 \( \mu \)M (Fig. 2C) and 1 \( \mu \)M (data not shown) strongly inhibits pyknosis induced by withdrawal of trophic support but has no effect on pyknosis induced by glutamate treatment. In contrast, SB203580, which at 1 \( \mu \)M inhibits p38 action in neurons (Fig. 2B) without effect on either JNK (Fig. 2A, *lower panel*) or withdrawal of trophic support-induced death (13), strongly inhibits glutamate-induced pyknosis (Fig. 2D).

SB203580 inhibits p38\( ^{\alpha}\) and -\( \beta\) but not the higher molecular weight \( \gamma\) or the \( \delta\) isoform; however, it is unclear which of the \( \alpha/\beta\) isoforms are expressed by cerebellar granule neurons. Lysates of cerebellar granule neurons and 293 cells transfected with either FLAG-p38\( ^{\alpha}\) or FLAG-p38\( ^{\beta}\), containing similar amounts of total p38, were loaded on replicate gels and immunoblotted with FLAG, pan-p38, p38\( ^{\alpha}\), and p38\( ^{\beta}\) antibody (Fig. 3A). The FLAG and pan-p38 blots indicated equal amounts of FLAG-tagged and total p38 in each lysate, and the p38\( ^{\alpha}\) antiserum recognized endogenous protein in granule neurons (and 293 cells) and a higher signal in the p38\( ^{\alpha}\)-transfected 293 cells, whereas prolonged exposure to an immunoblot probed with p38\( ^{\beta}\) antibody revealed no band in cerebellar granule neurons (or 293 cells), although an amount of p38\( ^{\beta}\) equal to the amount of p38\( ^{\alpha}\) loaded was strongly detected. This suggests that the antibodies are isoform-specific, that these neurons do not express detectable p38\( ^{\beta}\), and therefore that the \( \alpha\) isoform is responsible for p38 responses that are SB203580-sensitive.

Sensitivity to SB203580 has been widely interpreted as evidence of a requirement for p38, although concentrations that also block JNK isoforms are sometimes used. Now, even data using low concentrations of the drug need to be re-evaluated, since it has been reported to inhibit some targets as effectively as and more effectively than it does p38 (11). Therefore, it is necessary to substantiate the results obtained with this inhibitor by alternative methods. Since the cerebellar neurons express p38\( ^{\alpha}\) and not p38\( ^{\beta}\) (Fig. 3A), we utilized expression plasmids for a dominant negative form of p38\( ^{\alpha}\) (nonphosphorylatable mutant “AF”) (16) and MKK3 (kinase-dead (KD)) (25). First, we examined the specificity of these dominant negatives, by testing their effects on actions of JNK activated by withdrawal of trophic support. For this purpose, we utilized a
reporter assay with GAL4-c-Jun-(6–89), since we had previously demonstrated that this reporter responds to TFD-induced JNK activation (13). Withdrawal of trophic support induces ~3-fold activation of this reporter in the presence of empty vector, and the presence of p38αAF or MKK3-KD in place of empty vector did not reduce this response (Fig. 3B). In contrast, GAL4-Mef2A activity in neurons, which is increased ~5-fold by the co-transfection of constitutively active MKK6 and p38α (Fig. 2B), was inhibited ~5-fold by the co-transfection with p38αAF (Fig. 2C), consistent with the previously reported effectiveness of this construct (16). Since this reporter system involves overexpression of MKK6, it is not a suitable assay for inhibition by MKK3-KD. Therefore, we developed a transfection-based assay of glutamate-evoked neuronal p38 activity, based on our previous transfection-based neuronal JNK assay (4). Cells were transfected with GST-tagged p38α and either empty vector or MKK3-KD and stimulated with glutamate. The tagged p38α was purified by affinity chromatography, and p38α activity was assessed by immunoblotting with phosphospecific antibody. This demonstrated that glutamate induces phosphorylation of the α isoform of p38 and that the presence of the MKK3-KD construct inhibited p38α activation (Fig. 3, D and E). Based on these results, we concluded that the p38αAF and MKK3-KD constructs specifically inhibited the p38 pathway. We then tested the hypothesis that glutamate-induced death did indeed require the p38 pathway as follows. When neurons are transfected with GFP expression plasmid, soluble GFP is produced. This protein is clearly visible in cell bodies of healthy neurons but is rapidly lost when membrane integrity is compromised, as occurs 24 h after glutamate exposure (Fig. 3F). Cells were co-transfected with GFP marker and either empty vector or the dominant negative p38αAF and MKK3-KD expression plasmids. Cells were untreated or treated with glutamate, and the number of cells was counted as described under “Experimental Procedures.” These experiments revealed that glutamate-induced loss of membrane integrity was reduced or prevented by dominant negatives of the p38 pathway (Fig. 3G).
These results suggest that despite the superficially similar mitogen-activated protein kinase response to glutamate and withdrawal of trophic support, there are distinct requirements for the SAPKs JNK and p38 in the ensuing cell death. Therefore, we compared the death pathways under the conditions exhibiting these distinct kinase requirements. Examination of cerebellar granule cultures 24 h after exposure to glutamate reveals degeneration of processes and shrinkage of cell soma accompanying the pyknosis described above. These events are unaffected by high levels of pancaspase inhibitor zVAD-fmk but are prevented by the presence of NMDA receptor channel blocker MK-801 (Fig. 4, A and B). This is in sharp contrast to the pyknosis induced by withdrawal of trophic support, which is strongly prevented by zVAD-fmk (Fig. 4B). Necrotic cell death is considered to be involved in cell lysis preceding nuclear changes (26). To investigate the sequence of events in the p38-dependent death induced by glutamate and whether caspases might play some regulatory role, neuronal cultures were exposed to glutamate in the presence or absence of zVAD-fmk, and pyknosis and lactate dehydrogenase release to the extracellular medium were measured at time points from 3 to 24 h after glutamate exposure. Pyknosis was complete by 3 h, whereas the lactate dehydrogenase release began after the 6-h time point, and the pancaspase inhibitor had no effect on these parameters (Fig. 4C). The lack of effect of zVAD-fmk on glutamate-induced death cannot be explained by lack of stability of the compound, because it did inhibit the pyknosis induced by withdrawal of trophic support, which takes place with considerable delay (>12 h; data not shown) compared with glutamate-evoked pyknosis.

We subsequently compared the activation of caspases during p38-dependent death induced by glutamate and JNK-dependent death induced by withdrawal of trophic support. Withdrawal of trophic support from neurons transfected with epitope-tagged caspase-7, a caspase-3 substrate, followed by immunoblotting of lysates revealed a time-dependent accumulation of cleaved caspase-7 peaking at about 8 h (Fig 5A, upper panel). Therefore, we used the 8-h withdrawal of trophic support time point as a positive control for caspase activation in subsequent experiments. Cotransfection of Bcl-2 prevented production of the cleaved caspase-7 form (Fig. 5A, lower panel). Withdrawal of trophic support strongly induced the formation of cleaved caspase 3 and DEVDase activity, a property of active caspases 3 and 7. In contrast, cleaved caspase 3 was undetectable, and DEVDase activity was not significantly different from control during glutamate-induced death (Fig. 5B, C and D). Furthermore, we previously observed no glutamate-evoked caspase activity with other caspase substrates or at other time points (28).

We further probed the possible contribution of caspases by co-transfecting neurons with GFP as transfection marker and cowpox virus cytokine response modifier A, CrmA. The wild type inhibits caspase-1 and -8, whereas mutation of the pseudosubstrate region in CrmA-DQMD has been shown to be an excellent caspase-3 inhibitor (29). We found that neither construct inhibited glutamate-induced pyknosis of transfected cells, whereas the caspase-3-inhibiting mutant significantly reduced pyknosis induced by withdrawal of trophic support, indicating the functionality of the constructs (Fig. 5D). The different stresses thus not only evoked pyknotic nuclei with contrasting sensitivities to caspase inhibitor but also differed in caspase activation.

Chromatin morphology at electron microscopic resolution is commonly used to discriminate different forms of neuronal apoptosis (26). This approach revealed that cerebellar neurons deprived of trophic support for 24 h exhibit compaction of chromatin to a single globular electron-dense mass, unlike the diffuse chromatin of the larger nucleus of a control cell (Fig. 6A, top and middle panels). The presence of multiple subnuclear fragments described in other systems during apoptosis was
FIG. 3. Requirements for p38 and JNK in neuronal death induced by glutamate and withdrawal of trophic support addressed with dominant negative constructs. A, the relative abundance of the SB203580-sensitive p38α and β isoforms was investigated in cerebellar granule neurons. First, 293 cell lysates expressing recombinant FLAG-tagged p38α/H9251 and p38β/H9252 were normalized by FLAG-immunoblotting (upper panel); equal amounts of FLAG-p38α, FLAG-p38β, and endogenous p38 from neuron lysates were loaded as assessed by pan-p38 immunoblotting (second panel). Parallel blots were probed with p38α-specific antibody (third panel) and p38β-specific antibody (bottom panel). Pan-p38 antibody detects endogenous p38 and recombinant p38α/H9252 as two distinct bands (second lane, second panel). The p38α antibody does not detect the recombinant p38β, because only a single band is visible in the second lane of the third panel, although equal amounts of p38α and β are present (upper panel). Correspondingly, the p38β antibody does not detect recombinant p38α (first lane, bottom panel). Although similar total p38 levels are loaded in all lanes, neuron lysates (and 293 cells) have undetectable endogenous p38β levels even after prolonged exposure of film (bottom panel), whereas endogenous p38α levels are similar in 293 cells (second lane, 293 s with expressed p38β and endogenous p38, third panel) and neurons (third lane, only endogenous protein present in untransfected neurons, third panel). Therefore, a dominant negative of the α isoform of p38 was used in subsequent experiments. B, the specificity of dominant negatives of the p38 pathway was assessed by examining the effects of co-transfected p38αAF and MKK3-KD on JNK activity induced by withdrawal of trophic support, assessed by the transcriptional activity of JNK-specific substrate c-Jun fused to GAL4. Cells were transfected with GAL4-driven luciferase plasmid pGL3-65E438, GAL4-c-Jun-6–89, and pRL-CMV internal control, together with empty vector pCMV or dominant negatives as indicated. c-Jun activity was assessed by a dual luciferase assay and luciferase ratios were normalized to control values. Means ± S.E. (n = 4) are shown, revealing a robust response to withdrawal of trophic support and no inhibition by p38 pathway dominant negatives. C, to demonstrate the ability of dominant negative p38αAF to block p38 responses, a reporter assay was used with the p38α-specific substrate Mef2A fused to GAL4, GAL4-driven luciferase reporter, and pRL-CMV internal control. Cells were co-transfected with MKK6E and p38α to activate Mef2A (Fig. 2B). The additional presence of p38αAF strongly reduced the MKK6E-p38α driven response. D, to demonstrate the ability of the dominant negative MKK3 construct to block p38α activation, neurons were co-transfected with pBE-p38α and either empty vector (pCMV) or dominant negative MKK3 (MKK3-KD) as shown, stimulated with glutamate (GLU), or left unstimulated (CTRL), and phospho-p38 and total GST-p38 bound to glutathione-beads were detected as in A. E, means ± S.E. (n = 3) of replicates as in C are shown. Activation of p38 by glutamate was significantly different in the presence of MKK3-KD than in its absence by paired t test (p < 0.01). F, cerebellar granule neurons were transfected with soluble GFP expression plasmid as a marker of secondary necrosis, to permit assessment of long term viability. Cells were treated for 30 min with or without glutamate (control), returned to conditioned medium, and 24 h later stained with Hoechst. Control cultures show healthy nuclei and GFP-expressing cells, whereas glutamate-treated cultures exhibit predominantly pyknotic nuclei and only small GFP-positive fragments of cells (indicated by the arrows). Fields shown are 450 μm across (upper panel). Neurons co-transfected with either empty vector (pCMV) or expression plasmid were treated 24 h later with or without glutamate (50 μM,
A significant difference from viability after glutamate of cells transfected with pCMV (shown are 225 different stresses are associated with individual patterns of chromatin morphology induced by the different stresses. Thus, the distinct chromatin morphologies induced by the different stresses are associated with individual patterns of DNA fragmentation.

Bcl-2 promotes the integrity of the mitochondrial membrane and inhibits release of proapoptotic factors (32), such as cytochrome c, which activates caspase-9, leading to activation of caspase-3, which acts on effector caspases such as caspase-7. However, the effect of Bcl-2 on the p38-dependent death evoked by glutamate could not be predicted, since it does not involve caspases. Therefore, we compared the pyknosis of glutamate-treated neurons transfected with empty vector or Bcl-2 expression vector (and GFP as a transfection marker) and control samples that had not been treated with glutamate. The co-expression of Bcl-2 resulted in a significant reduction in the proportion of cells with pyknotic nuclei (Fig. 7, A and B) without effect on the glutamate-evoked calcium response (not shown). To assess the ability of Bcl-2 to maintain long term viability subsequent to glutamate, the GFP-based viability assay described above (Fig. 3, F and G) was exploited either by counting cells as in Fig. 3G or by immunoblotting for GFP protein that could be isolated from the neuronal culture (Fig. 7C). Cells co-transfected with empty vector (pCMV) had lower levels of GFP subsequent to glutamate, whereas cells co-transfected with Bcl-2 retained almost as much GFP as mock-treated cultures (Fig. 7, C and D). Cell counting produced similar results (data not shown).

The withdrawal of trophic support paradigm results in reduced calcium levels, whereas activation of the glutamate receptor subtypes evokes large increases in cytoplasmic free calcium in these neurons (20, 35). Increased calcium plays a central role in ischemic and glutamate-evoked neuronal death, and there are reports that Bcl-2 can modulate cellular calcium levels (34, 35). However, co-transfection of Bcl-2 or empty vector with transfection marker EGFP-F (which did not interfere with fura-2 measurements) (data not shown) revealed that.

30 min), and a further 24 h later viability could be assessed by counting GFP-positive cells. G, neurons were co-transfected with GFP-marker plasmid and either empty vector (pCMV) or dominant negative proteins of the p38 pathway, p38aAF, or MKK3a KD as indicated and treated 24 h later with or without glutamate (50 μm, 30 min) as shown in F. The number of GFP-positive cells was counted again, and survival was assessed as the proportion of GFP-positive cells remaining after glutamate or control treatment in each case. Means ± S.E. (n = 3) are shown, †, a significant difference from control (p < 0.002) (i.e. glutamate reduces survival). *a significant difference from viability after glutamate of cells transfected with pCMV (p < 0.02) (i.e. the plasmids marked with an asterisk were protective).
Bcl-2 had no significant effect on either peak or sustained increase in cytoplasmic free calcium response (data not shown). A similar experiment with SB203580, which despite its limited specificity does inhibit p38α/β (11), also demonstrated no changes in the glutamate-evoked calcium response (data not shown). Therefore, the dramatic inhibition of glutamate-induced death by Bcl-2 cannot be ascribed to an influence on the cytoplasmic free calcium response, nor does inhibition of p38 prevent the calcium response.

The ability of Bcl-2 to prevent both forms of death is consistent with a role for release of mitochondrial proteins in both forms of cell death. Release of cytochrome c from mitochondria is expected to lead to activation of caspases, yet we did not detect caspase activation after glutamate treatment. Therefore, we investigated the release of cytochrome c into the cytosol after withdrawal of trophic support or after withdrawal with glutamate. Although caspase inhibitors are commonly added to such experiments to reduce loss of cytosolic cytochrome c via secondary necrosis, we did not add caspase inhibitors so that we could avoid selectively amplifying caspase-dependent responses. In contrast, the level of cytosolic cytochrome c detected after glutamate should not be an underestimate, since the time point used preceded the loss of membrane integrity (Fig. 4C). Nevertheless, cytosolic cytochrome c was easily detected after withdrawal of trophic support, whereas cytosolic cytochrome c, although present, was at a lower level after glutamate treatment (Fig. 7E).

DISCUSSION

Stress induces apoptosis, and the stress-activated protein kinases are believed to contribute to neuronal apoptosis. Early observations suggested a contribution from both p38 and JNK families in caspase-dependent apoptosis of PC12 cells (1). However, studies in primary cultured neurons typically implicate one or the other family, and no coherent explanation has emerged for any differential roles of these kinases in neuronal cell death. Thus, JNK is required for death induced by withdrawal of nerve growth factor from sympathetic neurons (2, 3), excitotoxicity- and β-amyloid-induced apoptosis of hippocampal neurons (5, 36), apoptosis during early brain development (8), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced apoptosis of dopaminergic neurons (7). The use of SB203580 has implicated a p38 contribution to axotomy-induced apoptosis of retinal ganglion cells (8), glutamate-induced apoptosis of cerebellar granule neurons (9) and ceramide-induced death of cortical neurons (10). However, it has recently been revealed that SB203580 exhibits higher specificity for kinases other than p38 (11), and at the concentrations typically used to target p38 it can even inhibit JNK-dependent death (13). Therefore, conclusions relying on SB203580 as the only strategy to inhibit p38 must now be reconsidered. This is particularly so, since there is even support for a role of the p38 kinase in survival pathways (27, 37). Furthermore, it is rare that the form of cell death prevented by the drugs is fully characterized. This has become a critical issue for interpretation of existing data, because some stimuli, such as glutamate, have been described by different authors to induce different forms of cell death even in the same cell type (e.g. necrosis in Refs. 21 and 38; apoptosis in Ref. 9). It is not clear whether or not the same form of death is discussed in these reports. In addition, no report has demonstrated different forms of death dependent on p38 and JNK, respectively, in the same neuronal type. This has left open the possibility that SAPKs may contribute differentially to cell death.
death pathways as a result of cell-specific and death pathway-specific requirements.

In order to shed some light on this, we compared in parallel experiments cerebellar granule neuron death induced by glutamate and by withdrawal of trophic support. The relative contributions of JNK and p38 stress-activated protein kinases to the latter form of death have been explored in detail in cerebellar granule neurons (e.g. see Refs. 13 and 39). Both p38 and a stress-activated JNK subpool are activated. The latter leads to phosphorylation of c-Jun, which is believed to mediate expression of death genes and neuronal death. Conditions that inhibit p38 activity but not JNK had no effect on the death induced by withdrawal of trophic support. Neuronal death induced by glutamate may be more clinically relevant, since it is considered a model of neuronal death during stroke and is implicated in other disorders. Whereas there is evidence that JNK inhibition reduces neuronal death after cerebral ischemia (40), protection is incomplete. Different forms of cell death are reported in ischemic brain (41); therefore, it is likely that multiple death pathways with distinct signaling requirements may be involved. Here we show that the p38, JNK, and c-Jun responses are superficially similar whether cerebellar granule neurons are exposed to glutamate concentrations in the range recorded in stroke models (30) or trophic support is withdrawn (13). One difference is the more transient nature of the glutamate response. This may be significant, since no consistent induction of c-Jun protein is detected, raising the possibility that glutamate does not induce a death gene program in this model. Supporting this, the glutamate-induced death is not blocked by inhibitors of transcription and translation, whereas withdrawal-induced death is (data not shown) (42).

The glutamate response requires extracellular calcium and NMDA receptor activity, suggesting that calcium influx through the NMDA receptor channel mediates the response. In contrast, withdrawal of trophic support involves removal of a depolarizing level of elevated KCl, and there is evidence suggesting that it is the calcium elevation that provides trophic support (43). Thus, either increase or decrease of intracellular calcium leads to activation of stress-activated protein kinases.

Despite the SAPK response profile superficially similar to that of glutamate and the withdrawal of trophic support, the SAPK dependence of the ensuing cell death, measured by pyknosis, is strikingly different. Glutamate-induced pyknosis is potently blocked by treatments that selectively inhibit p38 and have no effect on the death induced by withdrawal of trophic support (13). In contrast, the JNK inhibitor 1,9-pyrazoloanthrone (SP600125) eliminates death induced by withdrawal of trophic support without effect on glutamate-induced death. The glutamate-induced death is probably mediated by the α isoform of p38, since (i) sensitive isoform analysis was unable to detect any p38β in these cells and (ii) the MKK3 dominant negative isoform used does not interact with p38β (44).

These data clearly indicate differences in the mechanisms of cell death induced by the two stimuli in the conditions we use. The JNK-dependent death induced by withdrawal of trophic support induces delayed and transcription-dependent activation of caspases in a manner sensitive to Bcl-2, whereas p38-dependent death evoked by glutamate induces no detectable...
Fig. 7. Bcl-2 prevents glutamate-induced cell death. A, cerebellar granule neurons were transfected with soluble GFP expression plasmid to allow assessment of pyknosis in transfected cells. Cells were treated for 30 min with or without 50 μM glutamate ("control"), returned to conditioned medium, and fixed 3 h later, before loss of membrane integrity. Pyknosis of GFP-positive cells was assessed by Hoechst 33342 staining. Left panels, GFP fluorescence; middle panels, Hoechst 33342 staining; right panels, merged image. Glutamate-treated cultures transfected with empty plasmid show predominantly pyknotic nuclei, whereas those transfected with Bcl-2 plasmid exhibit healthy nuclei after glutamate treatment. B, corresponding replicates as shown in A were quantitated. Means ± S.E. are shown (n = 3 cultures). C, cerebellar granule neurons were transfected with soluble GFP expression plasmid as a marker of secondary necrosis to permit assessment of long term viability. Neurons co-transfected with either empty vector (pCMV) or Bcl-2 expression plasmid were treated 24 h later with or without glutamate (50 μM, 30 min), and a further 24 h later, viability was assessed by immunoblotting culture lysates with anti-GFP antibody. D, replicate immunoblots were quantitated, and viability was shown as percentage of control immunoreactivity (no glutamate) (lower panel). Means ± S.E. are shown (n = 3 cultures). In all cases, an asterisk indicates a significant difference between cells transfected with Bcl-2 compared with empty vector pCMV by paired t test (p < 0.02). E, cells were treated with glutamate (GLU), by withdrawing trophic support (W) or left unstimulated (CTL) as shown and fractionated. The presence of cytochrome c in crude mitochondria (mito) and cytosols (cyto) was assessed by immunoblotting. Equal loading of crude mitochondria and cytosols was assessed by immunoblotting with anti-cytochrome oxidase subunit IV (COX IV) and by Coomassie staining, respectively.

Fig. 8. Scheme depicting the selective contribution of p38 and JNK to different forms of neuronal cell death. See "Discussion" for details.
activation of caspases, and the rapid cell death is not prevented by transcription or translation inhibitors. Furthermore, inhibition of caspases with CrmA-based plasmids or with cell-permeable pancaspase inhibitor zVAD had no effect on the p38-dependent death. Since zVAD also inhibits caspase B (45), we conclude that this protease is also not required for the p38-dependent death. In contrast, JNK-dependent death is blocked by either zVAD or caspase-3-inhibiting CrmA, consistent with previous observations that peptide inhibitors of caspases inhibit death induced by withdrawal of trophic support (46).

Caspases typically confer specific chromatin cleavage patterns and chromatin morphologies to cell death (26). Indeed, JNK-dependent death exhibited internucleosomal DNA fragmentation and uniformly condensed chromatin that did not fragment to multiple bodies. In contrast, p38-dependent death led to accumulation of high molecular weight DNA fragments, and an atypical, "lumpy" chromatin morphology previously observed after caspase-independent death mechanisms and in ischemic brain (26, 41, 47, 48). Despite these substantial differences in JNK/death gene and p38-dependent death of cerebellar granule neuron death are very clear in the present data, although the differences between these two forms of cerebellar granule neuron death induced by the withdrawal of trophic support and gluta mate, respectively, both forms of cell death were prevented by Bcl-2 overexpression, lending credence to the possibility that Bcl-2 family-mediated regulation of death effectors from intracellular stores such as mitochondria may play a central role in both these forms of cell death. This is supported by our observation that we did observe a small but detectable release of cytochrome c into the cytoplasm after glutamate treatment, although it was not as clear as the response to withdrawal of trophic support. There are a number of possibilities that might explain why this may not result in activation of caspases. It is possible that an insufficient amount of cytochrome c is released or alternatively that other pathways prevent the activation of cytochrome c. For example, it has been reported that calpain cleaves Apaf-1 and can prevent caspase activation in response to excitotoxic stimulus (49, 50).

Although the differences between these two forms of cerebellar granule neuron death are very clear in the present data, evidence exists for a role of JNK and/or of caspases in excitotoxicity in other models (5, 40, 51). It should be anticipated that the cell death mechanisms invoked by glutamate depend on multiple factors (e.g. see Ref. 50), and there is evidence for heterogeneity of cell death mechanisms in ischemia. In light of the present data, different SAPKs may be required in different mechanisms. Thus, there is excellent correlation in some cases between the reported involvement for JNK and for caspases (6, 23). However, there has been virtually no literature on p38 and caspase-independent death to date.

In conclusion, we find that exposure of cerebellar granule neurons to different neurodegenerative stimuli (withdrawal of trophic support and glutamate) induces forms of death distinguishable on multiple levels and in a manner dependent on distinct limbs of the SAPK pathway (Fig. 8). Only inhibition of the effector SAPK involved is sufficient to prevent the corresponding neuronal death.

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