All JNKs Can Kill, but Nuclear Localization Is Critical for Neuronal Death

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JNKs are implicated in a range of brain pathologies and receive considerable attention as potential therapeutic targets. However, JNKs also regulate physiological and homeostatic processes. An attractive hypothesis from the drug development perspective is that distinct JNK isoforms mediate “physiological” and “pathological” responses. However, this lacks experimental evaluation. Here we investigate the isoforms, subcellular pools, and c-Jun/ATF2 targets of JNK in death of central nervous system neurons following withdrawal of trophic support. We use gene knockouts, gene silencing, subcellularly targeted dominant negative constructs, and pharmacological inhibitors. Combined small interfering RNA knockdown of all JNKs 1, 2, and 3, provides substantial neuroprotection. In contrast, knockdown or knock-out of individual JNKs or two JNKs together does not protect. This explains why the evidence for JNK in neuronal death has to date been largely pharmacological. Complete knockdown of c-Jun and ATF2 using small interfering RNA also fails to protect, casting doubt on c-Jun as a critical effector of JNK in neuronal death. Nonetheless, the death requires nuclear but not cytosolic JNK activity as nuclear dominant negative inhibitors of JNK protect, whereas cytosolic inhibitors only block physiological JNK function. Thus any one of the three JNKs is capable of mediating apoptosis and inhibition of nuclear JNK is protective.

Application of stressful stimuli to cells leads to the activation of the stress-activated protein kinase c-Jun N-terminal kinase (JNK) pathway. Initial studies using JNK knock-out mice indicated redundant roles for JNKs 1 and 2 in survival and death during early brain development (1, 2). JNK3 deletion on the other hand reduced neurodegeneration after excitotoxic-induced seizures in adult (3). Roles for JNK1 in physiological responses during neuronal development were revealed more recently (4 – 6). Overall, there has been an emphasis on JNK3 as a specific target for drug development (7, 8). Although some studies have shown a contribution from JNK3 in neuronal stress responses (9 – 11), the proposal that JNK3 plays a dominant role in neuronal death (reviewed in Refs. 7, 12–15, and 45) is not supported by the neuronal responsiveness of JNK3 in mice to stress (16), and studies suggest a possible contribution from JNK2 (16 – 18). Surprisingly, however, a comparative analysis of different JNK isoforms has not been reported. A major constraint in determining JNK isoform roles in cell death has been the absence of JNK1/2/3 triple knock-out mice and consequent lack of studies with neurons depleted of JNKs 1, 2, and 3. As a result, analysis of JNK isoform involvement in neuronal death and, in most cases, even addressing whether JNK is involved at all has depended on the use of the less specific pharmacological and dominant negative approaches. These fail to unequivocally show the contributions of JNK or individual isoforms, leaving this issue unresolved.

Similarly, conclusions regarding JNK substrate involvement in central nervous system neuron death responses have largely relied on correlation (19). c-Jun is a major target of the JNK pathway; it contributes to the response to cellular stress and is believed to mediate apoptotic neuronal cell death (20). Knock-in mice expressing c-Jun with mutations to alanine at the JNK phosphorylation sites Ser-63/73 (c-Jun-AA) exhibited the same deficiencies in the excitotoxic stress response as JNK3 knock-out mice (3, 20). This suggested that c-Jun and JNK3 were on the same phenotypic pathway and led to the widely held dogma that JNK3 \( \rightarrow \) c-Jun signaling mediates neuronal death (reviewed in Refs. 7, 12–15, and 45).

In this report, a combination of methods, knock-out mice, small-molecule inhibitors, siRNAs, and compartment-targeted dominant negative inhibitors establish that JNK is required for death of cerebellar granule neurons from which trophic support was withdrawn and is used to investigate the involvement of JNK isoforms and JNK substrates. We observed that nuclear activity of JNK was required for the death, but the JNK sub-localization sequence; JBD, JNK binding domain; DIV, days in vitro; WTS, withdrawal of trophic support.
strategies c-Jun and ATF2 were not required for the death in this system. JNK2 and JNK3 were together shown to be the main regulators of c-Jun following withdrawal of trophic support (WTS). Unexpectedly, however, they were not required for neuronal death. Only simultaneous knockdown of JNKs 1, 2, and 3 was sufficient to confer neuroprotection. This indicates that the presence of any one of the JNKs is sufficient to produce a dominant apoptotic signal.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Mouse anti-JNK1 (clone G151-333) and mouse anti-JNK1/2 (clone G151-666) were from Pharmingen. Rabbit anti-JNK3 (clone C05T), rabbit anti-JNK2/3 ("SAPK1ba," catalogue number 06-748, characterized in Ref. 16), and rabbit anti-JNK1/2 ("SAPK1b/SAPKβ," catalogue number 06-749) were from Upstate Biotechnology (Lake Placid, NY). Mouse anti-actin was a gift from Brigitte Jockusch (Technical University of Braunschweig). Rabbit anti-ATF2 (catalogue number 9222), anti-phospho-ATF2 (Thr-69/71; catalogue number 9225), and anti-phospho-JNK (Thr-183/Tyr-185; catalogue number 9255) were from Cell Signaling Technology (Beverly, MA). Mouse anti-c-Jun (clone J31920) was from BD Transduction Laboratories, and mouse anti-phospho-c-Jun (Ser-63; clone KM-1) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit Alexa-546 and antimouse Alexa-555 were from Molecular Probes ( Eugene, OR). siRNA pools for silencing of ATF2 and JNK3 (set I) were obtained from Dharmacon (Chicago, IL). siRNA oligonucleotides for c-Jun (5'-AACAGAAAGUCAUGACCG-3') and JNK1/2 (set I) (5'-GAAGUGCUCUACCUUCUA-3') were synthesized with a deoxynucleoside deoxynucleoside leader sequence by MWG-Biotech AG (Ebersberg, Germany) as described previously (21). siRNA oligonucleotides set II for JNKs were as follows: JNK1 pool (5'-GAAAGAACUGAUAACAA-3' and 5'-GAAGACAC-GUGAACA-3'), JNK2 pool (5'-CCGTGAACCTCGTCTC-3' and 5'-GTGATGACCTGGAAGAA-3'), and JNK3 pool (5'-GAAAGACAUUAUCUCA-3' and 5'-CCA-GUAACAUUGUACUGUA-3'). Kinase inhibitors SB203580 and SP600125 were from Calbiochem and Sigma-Aldrich, respectively. GFP-NLS-SEK1kd and GFP-NES-SEK1kd were prepared, as were GFP-NLS-JBD and GFP-NES-JBD, as described previously (6). δMEKK1-(1174–1493) was previously described (16).

**Cell Culture**—Cerebellar granule neurons were prepared as described previously (16) from postnatal day seven JNK2−/−, JNK3−/−, and JNK2−/−/JNK3−/− mice where indicated. Genotyping was as described previously (1). Otherwise, neurons were prepared from FVB/N mice for JNK siRNA experiments or from Sprague-Dawley rats for ATF2/c-Jun siRNA experiments. Briefly, cells were cultured in minimal essential medium supplemented with 10% (v/v) fetal bovine serum (HyClone, Logan, UT), 33 mM glucose, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 20 μM supplementary KCl (final 25.4 mM KCl). Cells were plated at 250,000/cm² onto culture surfaces coated with poly-1-lysine (50 μg/ml). Culture medium was replaced after 24 h with the inclusion of 10 μM cytosine arabinofuranoside (Sigma) to reduce non-neuronal proliferation. Cells were cultured in a humidified 5% CO2 atmosphere at 37 °C.

**RESULTS**

A number of studies suggest that JNK-mediated phosphorylation of c-Jun contributes to neuronal cell death (reviewed in Ref. 25), particularly in response to WTS. The majority of the
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The total level of phospho-Ser-63 c-Jun is a somewhat composite parameter representing the specific phosphorylation of c-Jun at this site multiplied by the total level of c-Jun (16). Therefore we measured constitutive JNK1 activity (16, 22). Using 10 μM SB203580, which inhibits (at least) JNK2/3 isoforms in these cells, we found that the JNK2/3 pool contributes to c-Jun phosphorylation in neurons upon withdrawal of trophic support (16). However, this conflicts with the expectation that JNK3 has a specific role in neuronal c-Jun stress responses alone, largely supported by the finding that both JNK3−/− and c-Jun-AA knock-in mice possess similar resistance to kainate-induced seizure activity and the subsequent death of hippocampal neurons (3, 15, 20). We therefore investigated which JNKs contributed to the trophic withdrawal responses using cultured cerebellar granule neurons from single and compound knock-out mice.

The c-Jun Stress Response Is Strongly Suppressed in JNK2−/−JNK3−/− Neurons—Neurons were prepared from wild-type, JNK2−/−, JNK3−/−, and JNK2−/−JNK3−/− mice and treated according to the WTS paradigm. We also included in these experiments the use of small molecule JNK inhibitors that had previously been shown to influence these responses. As reported previously, phosphorylation of the JNK substrate c-Jun increased in response to WTS, and this response was blocked by 10 μM SB203580 (which inhibits JNK2/3 isoforms (16) and 1 μM SP600125 (sufficient to inhibit JNK3 (16, 29)) (Fig. 1A). Importantly, 1 μM SB203580, which is sufficient to inhibit p38α/β but not JNKs (16, 22, 29), did not influence c-Jun phosphorylation (Fig. 1A). When the experiments were repeated with cells from JNK2−/−, JNK3−/−, or JNK2−/−JNK3−/− mice, the size of the response was somewhat diminished in JNK3−/− cultures but most strongly reduced in JNK2−/−JNK3−/− cultures, indicating that JNK2 in addition to constitutive JNK1 activity (16, 22). Using 10 μM SB203580, which inhibits (at least) JNK2/3 isoforms in these cells, we found that the JNK2/3 pool contributes to c-Jun phosphorylation in neurons upon withdrawal of trophic support (16). However, this conflicts with the expectation that JNK3 has a specific role in neuronal c-Jun stress responses alone, largely supported by the finding that both JNK3−/− and c-Jun-AA knock-in mice possess similar resistance to kainate-induced seizure activity and the subsequent death of hippocampal neurons (3, 15, 20). We therefore investigated which JNKs contributed to the trophic withdrawal responses using cultured cerebellar granule neurons from single and compound knock-out mice.

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FIGURE 1. c-Jun mobility shift in response to withdrawal of trophic support is inhibited in JNK2−/−JNK3−/− neurons. Cerebellar neuron cultures were prepared from wild-type (wt), JNK2−/−, JNK3−/−, and JNK2−/−JNK3−/− mice. Trophic support was withdrawn (WTS) from these cultures for 0, 30, or 240 min as indicated in the presence of 1 μM SB203580 (SB) to block p38α/β, 10 μM SB203580, which also blocks neuronal JNK2/3, and 1 μM SP600125 (SP) to block JNK as indicated. Cell lysates were prepared from equal numbers of cells, and levels of c-Jun phosphorylated on serine 63 were detected by immunoblotting. A, phospho-Ser-63 c-Jun bands (c-Jun P63) are indicated by arrows; * indicates a nonspecific band. WB, Western blot. B, the same lysates were immunoblotted for c-Jun. Mobility shifted c-Jun bands are indicated with arrows. C, immunoreactive density profiles were quantitated from c-Jun blots and normalized to the amplitude of the most intense band, and intensity migration profiles were superimposed. This analysis demonstrates similar effects on relative mobility profiles upon loss of JNKs 2 or 3 and a greater effect in the double knock-out cultures. The inset shows a magnified image of the blots from which c-Jun mobility profiles were calculated. arb. units, arbitrary units. D–G, replicate immunoblots from B were quantified and normalized to signals from wild-type neurons from which trophic support was withdrawn. Data from four separate experiments are shown as mean ± S.E. Statistical analysis was performed using one-way analysis of variance. Significance levels, * p < 0.05, and *** p < 0.001, indicate significant differences with respect to WTS in the same graph, #, p < 0.002 indicates significant difference when compared with corresponding sample in wild type.

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The total level of phospho-Ser-63 c-Jun is a somewhat complex parameter representing the specific phosphorylation of this site multiplied by the total level of c-Jun (16). Therefore we immunoblotted the lysates with c-Jun antibody in parallel (Fig. 1B). This indicates that the total amount of c-Jun, as well as evidence comes from observations that dominant negative c-Jun constructs prevent neuronal cell death upon removal of trophic support (23–28). We have reported that withdrawal of trophic support from rat cerebellar granule neurons leads to selective activation of JNK2/3 isoforms in the presence of con-
relative phosphorylation status, visualized by the retarded mobility, is the result of phosphorylation at serines 63 and 73 and threonines 91 and 93 (30). To more directly compare the c-Jun mobility profiles, we plotted the film density profile against migration distance in the gel, normalizing to the peak intensity for each trace. This analysis clearly showed that following WTS, c-Jun migrates as three distinct peaks. In cells from JNK2−/−JNK3−/− mice, there was a marked reduction in retarded mobility bands when compared with JNK3−/− neurons (Fig. 1C). The c-Jun induction in the JNK2−/−JNK3−/− cultures, reduced when compared with wild type, was blocked by the pan-inhibitor of JNK, SP600125 (Fig. 1G). This indicates that the residual JNK1 is sufficient to elicit some c-Jun stress response. In summary, we have shown using knock-out cells that JNks 2 and 3 are both required to elicit c-Jun phosphorylation in neurons, consistent with earlier results using inhibitors (16).

**JNK1 Expression Is Up-regulated in the Absence of JNKs 2 and 3**—We investigated the levels of JNK and P-JNK under the conditions shown in Fig. 1. Withdrawal of trophic support with or without inhibitors caused no substantial change in total JNK levels, detected with a pan JNK antibody (16). Similarly, there was no clear change in phospho-JNK level that could not be attributed to variations in total JNK (Fig. 2A). As we noted, up-regulation of c-Jun expression in JNK2−/−JNK3−/− cells (Fig. 1, B and G), we examined whether this gene deletion may have led to an increase in JNK1. Immunoblotting with an antibody that was specific for JNK1 (16) revealed a significant increase in JNK1 p46 and p54 isoform expression in JNK2−/−JNK3−/− cells when compared with wild type (Fig. 2, B and C).

**Neurons from Mice Lacking JNks 2 and 3 Are Not Protected from WTS**—An underlying assumption in the neuronal cell death field has been that c-Jun regulation by JNK can lead to cell death (reviewed in Ref. 25). This led to the subsequent expectation that inhibition of JNK-regulated c-Jun would be neuroprotective. Withdrawal of trophic support in the JNK2−/−JNK3−/− background led to a marked inhibition of c-Jun mobility relative to the wild-type background (Fig. 1). We therefore investigated the extent to which this regulation of c-Jun correlated with pyknosis induced by WTS. Once again, we included inhibitors of JNK and p38 in experiments carried out on cultures from both wild-type and knock-out backgrounds. Treatment of wild-type cells with 10 μM SB203580 provided significant protection from WTS as described previously (Fig. 3) (Ref. 16). SP203580 at 1 μM also provided significant protection. However, the extent of death following WTS did not differ significantly between wild-type cells and JNK2−/−, JNK3−/−, or even JNK2−/−JNK3−/− cells (Fig. 3). This was especially surprising given that the c-Jun phosphorylation shift was severely reduced in JNK2−/−JNK3−/− neurons (Fig. 1). Moreover, 10 μM SB203580, which effectively inhibits JNK2/3 activities (16) provided a similar degree of protection in wild-type and in JNK2−/−JNK3−/− neurons. One possible explanation is that JNK1 isoforms can also contribute to neuronal death and that SB203580 also inhibits a subset of JNK1 isoforms (as reported in Ref. 31) that are up-regulated in JNK2−/−JNK3−/− neurons (Fig. 2). Indeed the JNK inhibitor SP600125 (1 μM) is also protective in neurons from JNK2−/−JNK3−/− mice.

**Knockdown of JNK2 and JNK3 Does Not Protect Neurons, All Three JNks Must Be Depleted to Confer Significant Neuroprotection**—The use of JNK knock-out mice did not demonstrate that JNK is required for WTS-evoked neuronal death, nor did it implicate specific isoforms, as no protection was observed either in JNK2−/−JNK3−/− (Fig. 3), neurons or in JNK1−/− neurons (data not shown). Surprisingly, SB203580 (10 μM), which inhibits JNks 2 and 3, was neuroprotective (16) (Fig. 3) even in JNK2−/−JNK3−/− neurons. One explanation would be that a JNK1 isoform is sufficient to induce death and that this
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FIGURE 3. JNK2−/−,JNK3−/− neurons are not protected from withdrawal of trophic support-evoked death. Cerebellar granule neurons were isolated from wild-type (wt), JNK2−/−, JNK3−/−, or JNK2−/−,JNK3−/− mice, and trophic support was withdrawn (WTS) for 24 h in the presence or absence of inhibitors as described in the legend for Fig. 1. Cells were fixed and DNA-stained with Hoechst 33342. The percentage of surviving (non-pyknotic) cells was calculated from 4346 (wild type), 4418 (JNK2−/−), 3917 (JNK3−/−), and 4226 (JNK2−/−,JNK3−/−) cells, respectively. Data from four sets are shown as mean ± S.E. Neuroprotection was conferred in all cultures by treatment with 10 μM SB203580 (SB), which inhibits p38α/β and JNK2 and 3, or with SP600125 (SP), which is a pan-JNK inhibitor (**, p < 0.01; ***, p < 0.001).

isoform is sensitive to SB203580 (31). Consistent with this, SP600125 also protected JNK2−/−,JNK3−/− neurons (Fig. 3). Indeed, no genetic evidence for JNK requirement in WTS-induced death of central nervous system neurons has been reported, only pharmacological evidence. Therefore we addressed these issues with gene silencing. First we established the silencing efficiency of JNK siRNA. We tested antibodies for JNK isoform specificity using wild-type and knock-out cells and tissues. Using this approach, we showed that JNK1/2 and JNK3 antibodies were specific (Fig. 4A). They recognized only bands corresponding to molecular weights of JNKs on SDS-PAGE and, most importantly, all immunoreactivity disappeared in knock-out tissue or cell homogenates that lacked the respective JNKs. We have previously demonstrated JNK isoform specificity for the JNK1 and JNK2/3 antibodies (16, 15). We then tested two separate sets of siRNAs targeting mouse JNKs 1/2 and JNK3. Silencing efficiency was determined by immunostaining of endogenous remaining JNKs using these antibodies (Set I siRNAs, Fig. 4, B and C; set II siRNAs, supplemental Fig. 1, A–C). All siRNA sets used induced a robust silencing of the targeted JNKs. Viability in response to WTS was then measured (Fig. 4, D and E). Neurons in which JNK3 expression alone was knocked down were not protected from death evoked by WTS. This is in contrast to the prevailing dogma of JNK3 involvement in neuronal death. Similarly, suppression of JNKs 1 and 2 was not protective (Fig. 4, D and E). Only combined suppression of all three JNKs provided significant and long-lasting protection from WTS-evoked death (Fig. 4, D–F). These data indicate that in wild-type neurons, at least one splice variant from each JNK gene can mediate apoptotic death and that products from any gene alone are sufficient.

Neither c-Jun nor ATF2 Is a Critical Player in WTS-induced Neuronal Death—Having established genetically that JNKs are indeed required for WTS-induced death, we tested whether the c-Jun stress response is critical. ATF2 is a JNK substrate that, like c-Jun, binds to AP1 sites by dimerizing with Jun family members (32, 33). It can therefore contribute to the c-Jun stress response. We detected a clear induction of phospho-Thr-69/71 ATF2 immunoreactivity in cerebellar granule neurons following stress, and this was prevented by treatment with the JNK inhibitor SP600125 (1 μM; Fig. 5A). To determine whether either of these factors was a critical mediator of JNK-regulated neuronal death, we used gene silencing. Neurons were transfected with siRNA as indicated, together with GFP-F to highlight transfected cells. The efficiency of silencing was demonstrated by immunostaining for endogenous c-Jun and ATF2 protein. There was no detectable staining for c-Jun or ATF2 visible following 48 h with respective siRNAs followed by 4 h of WTS, whereas a control non-targeting siRNA had no effect on endogenous expression of these transcription factors (Fig. 5B). Cells in which c-Jun and ATF2 were silenced were then subjected to 24 h of WTS (Fig. 5C). Loss of expression of c-Jun or ATF2 did not protect from stress-induced death, whereas treatment with SB203580 (10 μM) still protected. We then reasoned that either c-Jun or ATF2 expression alone may be sufficient to elicit JNK-mediated neuronal death, so the experiments were repeated in cells where both c-Jun and ATF2 were silenced. Dual silencing of c-Jun and ATF2 provided no protection from WTS-induced death at either early or late time points (Fig. 5D). Consistent with this, cerebellar granule neurons isolated from c-Jun-AA mice (JNK sites Ser-63/73 replaced with alanine), were not protected from WTS-evoked death (Fig. 5E). These data indicate that although c-Jun and ATF2 phosphorylation and expression (in the case of c-Jun) are regulated by JNK following removal of trophic support, neither one nor the other is necessary for neuronal death to proceed. This is consistent with the finding that JNK2−/−,JNK3−/− neurons are susceptible to death, although c-Jun phosphorylation is blocked (Figs. 1 and 3).

Nuclear JNK Is Critical for WTS-induced Neuronal Death, Cytosolic JNK Is Not—A growing number of JNK substrates has emerged in recent years (8), among them nuclear-localized regulators of transcription and cytosolic proteins. Having excluded the obvious candidates, c-Jun and ATF2, as critical death effec-
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FIGURE 4. Simultaneous knockdown of JNK1, JNK2, and JNK3 is required to protect cerebellar granule neurons from withdrawal of trophic support-evoked death. A, the specificity of JNK1/2 and JNK3 antibodies were tested using wild-type (wt) and JNK1/2−/− JNK2−/− mouse embryonic fibroblasts (MEF) and forebrain homogenates from wild-type and JNK3−/− mice. Both antibodies showed the expected isoform specificity and did not recognize additional bands. MW, molecular weight; WB, Western blots. B and C, cerebellar granule neurons at 5 DIV were transfected with pEGFP-F as a transfection marker together with Set I siRNAs targeting JNKs 1 and 2 (JNK1/2), JNK3, or non-targeting (NT) siRNA, as indicated. At 90 h following transfection, neurons were fixed and immunostained with antibodies recognizing JNK1, JNK1/2, JNK3, and JNKs 2 and 3 (JNK2/3). Representative widefield (B) or confocal (C) images are shown. siRNAs targeting JNK1/2 and JNK3 efficiently silenced expression at the protein level under the conditions used. D, neurons were transfected with non-targeting siRNA or set I siRNAs targeting JNKs 1/2 and JNK3 as described in B and once more with set I siRNAs targeting JNKs 1, 2, and 3. Cell survival is the percentage of transfected cells that survived 24 h of WTS. E, neurons were transfected with Set II siRNAs targeting JNKs 1/2 and JNK3 as in D, and survival was measured after 24 h of WTS. The only surviving neurons were those expressing siRNAs targeting all three JNKs. F, cerebellar granule neurons were transfected with non-targeting siRNA or with siRNAs targeting JNKs 1, 2, and 3 combined and subjected to WTS for 10, 24, or 42 h as shown. Cell survival is the percentage of transfected cells with non-pyknotic nuclei. The number of transfected cells counted is shown above the histogram bar. Data from four separate experiments are shown as mean ± S.E.
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FIGURE 5. Knockdown of c-Jun or ATF2 does not protect from withdrawal of trophic support-evoked death; c-Jun-AA neurons are not protected. A, cerebellar granule neurons were untreated (control) or subjected to WTS in the presence or absence of 1 μM SP600125 (SP), and lysates were immunoblotted with an antibody detecting phospho-ATF2. A representative blot is shown. WB, Western blot. B, cerebellar granule neurons were transfected with siRNA oligonucleotides targeting c-Jun, ATF2, or non-targeting (NT) siRNA together with pEGFP-F marker (green) at 5 DIV. 48 h later, cells were fixed and immunostained with antibodies recognizing c-Jun or ATF2 (red). Nuclei were stained with Hoechst 33342 (white). Immunoreactivity in the absence of 1° antibody is shown (–). c-Jun and ATF2 siRNAs effectively silenced transcription factor expression. C, neurons treated as described in B were transfected with c-Jun or ATF2 siRNAs or with non-targeting siRNA as indicated, and trophic support was withdrawn for 24 h in the presence or absence of 10 μM SB203580 (SB). Cell survival was assessed from the transfected cell population only. Suppression of c-Jun or ATF2 expression did not protect from WTS-evoked death. D, cerebellar granule neurons were treated as in C except that both c-Jun and ATF2 siRNAs were added simultaneously and cell survival was assessed from transfected cells at 10, 24, or 48 h following WTS. Suppression of both c-Jun and ATF2 expression did not protect. E, 7 DIV cerebellar granule neurons isolated from wild type (wt) or c-Jun-AA mice were subjected to WTS for 24 h. Neurons from c-Jun-AA mice were not protected when compared with wild-type cells. The number of transfected cells counted is shown above the histogram bar. Data from four separate experiments are shown as mean ± S.E. (***, p < 0.001).

DISCUSSION

In this study, we investigate the significance of JNKs 1, 2, and 3 and the JNK substrates c-Jun and ATF2 in apoptotic neuronal death. JNK is activated upon withdrawal of trophic support from neurons (16, 25, 35). This is accompanied by a well-documented increase in c-Jun Ser-63/73 phosphorylation leading to increased activity of the c-Jun transactivation domain (16). As a consequence, AP-1 complexes act at proximal jun1 and distal jun2 AP-1 motifs on the c-Jun promoter (19). c-Jun:ATF2 are the AP-1 complexes that bind most efficiently to the sequences of these motifs (32, 36). Here we show that the c-Jun stress response, c-Jun phosphorylation and subsequent c-Jun protein induction, are depleted upon knock-out of JNK2 and JNK3. Nonetheless, these neurons are not protected from apoptotic death. Moreover, knockdown in rat neurons of the AP-1 components c-Jun and ATF2 confers no protection from death induced by withdrawal of trophic support. Conversely, pharmacological and gene silencing approaches give consistent results that JNK is essential for neuronal death. These results imply that apoptotic death of cerebellar granule neurons induced by withdrawal of trophic support has an absolute requirement for JNK signaling, but c-Jun and ATF2 transcription factors are not critical JNK effectors in this response.

Having excluded the classic JNK substrate c-Jun as a potential mediator of JNK-induced death in neurons, we considered...
other proapoptotic proteins that are characterized JNK substrates. Among these are Bim and Bad (reviewed in Ref. 8), proapoptotic members of the Bcl-2 family that, unlike c-Jun and ATF2, function in the cytoplasm. In addition to these and other known JNK targets, there are likely to be a large number of substrates that are not yet identified. We decided to make use of compartmental targeted inhibitors of JNK, NES, and NLS-JBD that we had previously used to dissect the physiological functions of JNK (5, 6). Confinement of JNK inhibition to the cytoplasm failed to protect neurons from WTS, whereas a nuclear-targeted inhibitor provided robust protection. This suggested that proapoptotic JNK function was in the nucleus. In support of this, a structurally distinct inhibitor of JNK, SEK1kd, which prevents activation of JNK by a dominant negative mechanism (37), also provided complete protection when targeted to the nucleus and failed to protect if located in the cytoplasm. Moreover, the JNK activator MEKK1 was extremely toxic to neurons, and NLS-JBD provided complete protection, indicating that JNK phosphorylation of Bim and Bad in the cytosol is not critical in this death paradigm.

The neuroprotective attributes of the nuclear JNK inhibitors are in stark contrast to the lack of effect of nuclear inhibition on physiological JNK functions. The constitutively high activity that is characteristic of neuronal JNK is largely cytoplasmic (Ref. 22 and reviewed in Ref. 34). This activity regulates axodendritic architecture via mechanisms involving microtubule regulator protein targets of JNK (5, 6). Importantly, JNK regulation of this physiological function in neurons is blocked by NES-JBD but not by NLS-JBD. These data suggest that although physiological JNK function in neurons resides in the cytoplasm, proapoptotic function, at least in this death paradigm, is strictly nuclear. Targeting of JNK inhibitors to the nucleus in neurons provides a selective inhibition of pathological JNK function without interfering with physiological responses. This valuable information could be exploited when considering therapeutic targeting of JNKs for treatment of chronic disorders.

What then is the function of c-Jun activation following withdrawal of trophic support? Rapid elevation of c-Jun expression and transcriptional activation is a characteristic early event that occurs prior to apoptosis in central nervous system and peripheral nervous system neurons alike (16, 35, 38). The withdrawal of trophic support paradigm mimics the absence of growth factors or electrical stimulus among cells competing for trophic support in developing brain. This paradigm may also simulate the conditions that arise upon nerve fiber transection where there is loss of innervation. Interestingly, c-Jun up-regulation is also associated with regenerative responses following axotomy, although the relative contribution of c-Jun to regenerative or proapoptotic events in axotomized neurons remains controversial (reviewed in Refs. 39–41). Our data do not resolve the controversy, but we show clearly that suppression of c-Jun expression provides no protection from death induced by withdrawal of trophic support in cerebellar granule neurons, the

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**FIGURE 6. Nuclear JNK is required for WTS-induced neuronal death.**

**A**. The subcellular localization of GFP-tagged NES-JBD, NLS-JBD, NES-SEK1kd, and NLS-SEK1kd was tested in cerebellar granule neurons by confocal microscopy. Sections through nuclei are shown. Nuclei are stained with Hoechst 33342 (blue).

**B**. Neurons were transfected with GFP-F or GFP-tagged NES-JBD, NLS-JBD, NES-SEK1kd, or NLS-SEK1kd as shown and then either untreated (control) or subjected to WTS. The proportion of surviving cells was measured 24 h later. Dominant inhibitors of nuclear JNK signaling conferred significant protection.

**C**. Neurons were transfected with constitutively active MEKK1 in the presence or absence of NLS- or NES-JBD. MEKK1 strongly induced neuronal death. Expression of NLS-JBD completely blocked MEKK1-induced death, whereas NES-JBD was less protective.
most abundant central nervous system neuronal type. It is possible that the rapid phosphorylation of c-Jun and ATF2 on trans-activating sites reflects a regenerative signal that is overcome by robust apoptotic signaling.

We observe an increase in JNK1 expression accompanied by increased basal c-Jun levels in neurons from JNK2−/−JNK3−/− mice. Similar increases in JNK1 and c-Jun expression are reported upon chemical genetic block of JNK2 activity in mouse embryonic fibroblasts (42). The requirement for JNK2/3 to maintain basal c-Jun levels may reflect the powerful influence of mitogen-activated protein kinases (MAPKs) such as JNKs on transcriptional regulation. Development without the complete complement of JNK isoforms can be expected to impact on the levels of a number of genes. For example, knock-out of the JNK scaffold JIP3 results in substantially altered expression of diverse genes (43). The impact on gene regulation may also be indirect, i.e. the affected genes may themselves affect other target genes. Thus up-regulation of c-Jun in JNK2−/− mouse embryonic fibroblasts has been attributed to increased JNK1 activity (42). In addition, scaffolds and other sites selectively bound by JNK2/3 isoforms may become available for JNK1 in the JNK2−/−JNK3−/− background. This may also be true for JNK-dependent neuronal death as only deletion of all JNKs 1, 2, and 3 confers protection. Whether a single splice isoform of JNK is sufficient to allow apoptosis remains to be resolved. However, we show that the products of any single jnk gene are sufficient for the death.

It is somewhat surprising that JNK1 can also trigger neuronal apoptosis. JNK1 activity is elevated in brain and in neurons in the absence of stress (6, 22, 44) and is associated with maintenance of axonal and dendritic integrity (4–6, 34). Nonetheless, JNK2−/−JNK3−/− neurons are fully sensitive to withdrawal of trophic support-induced apoptosis and are protected by 1 μM SP600125. That JNK1, JNK2, or JNK3 alone permit neuronal death to proceed has important implications for therapeutic targeting of this pathway. Our data suggest that drugs selectively targeting individual jnk gene products (e.g. JNK3) will not be neuroprotective. At the same time, pan-JNK inhibitors are potentially hazardous as JNK1 has important physiological roles. Instead, we propose that it could be beneficial to target the nuclear pool of JNK, where death-promoting JNK action takes place. The JNK substrates eliciting neuronal death are still not known, and our data indicate that the AP-1 component c-Jun does not play the dominant role in neuronal death with which it has long been associated. Although our results indicate that the identity of the JNK effectors mediating neuronal death are not known, they underscore the importance of JNK in death of neurons deprived of trophic support.

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