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Caspase-2 is essential for c-Jun transcriptional activation and Bim induction in neuron death

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SYNOPSIS

Neuronal apoptotic death generally requires de novo transcription, and activation of the transcription factor c-Jun has been shown to be necessary in multiple neuronal death paradigms. Caspase-2 has been implicated in death of neuronal and non-neuronal cells, but its relationship to transcriptional activation has not been clearly elucidated. Here, using two different neuronal apoptotic paradigms, β-amyloid treatment and NGF withdrawal, we examined the hierarchical role of caspase-2 activation in the transcriptional control of neuron death. Both paradigms induce rapid activation of caspase-2 as well as activation of the transcription factor c-Jun and subsequent induction of the pro-apoptotic BH-3 only protein Bim. Caspase-2 activation is dependent on the adaptor protein RAIDD, and both caspase-2 and RAIDD are required for c-Jun activation and Bim induction. Our work, thus, shows that rapid caspase-2 activation is essential for c-Jun activation and Bim induction in neurons subjected to apoptotic stimuli. This places caspase-2 at an apical position in the apoptotic cascade and demonstrates for the first time that caspase-2 can regulate transcription.

Keywords

Caspase-2; Bim; neurons; beta-amyloid; NGF; RAIDD

INTRODUCTION

Widespread neuron death occurs during normal development, after trauma, and in neurodegenerative diseases. Understanding the mechanisms of such death may provide the

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AUTHOR CONTRIBUTION:
Ying Jean, Elena Ribe, Lloyd Greene and Carol Troy conceived the study and designed the experiments. Ying Jean, Elena Ribe, Maria Elena Pero, Marina Moskalenko, Zarah Iqbal and Lianna Marks performed the experiments. Ying Jean, Elena Ribe, Lloyd Greene and Carol Troy analysed the data and wrote the paper.

basis for useful therapies. Caspases, cysteine aspartate proteases, are key mediators of neuronal apoptotic death [1]. In vertebrates, caspase-2 is the most highly evolutionarily conserved member of the caspase family and the closest in sequence to *C. elegans* ced-3 [2]. Yet, there is much uncertainty about the extent to which caspase-2 participates in apoptotic death, the mechanism by which it does so and its hierarchical position in apoptotic cascades [3, 4]. The present study addresses these issues in two different paradigms of neuron death: β-amyloid ($A\beta_{1\text{-}42}$) treatment and NGF (nerve growth factor) withdrawal.

Although caspase-2 has been less studied than most other caspase family members, it has been implicated as a required constituent in a variety of apoptotic cell death paradigms by a substantial body of evidence from silencing and knockout experiments in non-neuronal cells [5–8]. Moreover, silencing of caspase-2 expression rescues neurons from apoptotic death triggered by NGF withdrawal and β-amyloid ($A\beta_{1\text{-}42}$) treatment [9–12]. Additional studies indicate that caspase-2 siRNA protects retinal ganglion cells from death evoked by optic nerve transection [13] while in gray and white matter, injury in response to hypoxia-ischemia or excitotoxic stress is reduced in caspase-2 deficient neonatal mice [14]. Dopaminergic neuron loss in an MPTP model of Parkinson's disease is also significantly decreased in caspase-2 null mice [15]. Such findings support a role for caspase-2 in apoptotic death of neurons and other cell types.

The JNK/c-Jun pathway has been identified as a central element in apoptotic death mechanisms and has been shown to lead to induction of pro-apoptotic molecules including the BH3-only protein, Bim [16]. In this context, transcriptional upregulation of Bim is required for apoptotic neuron death in response to NGF withdrawal and exposure to β-amyloid [16–19]. Placing caspase-2 within the death pathway has been problematic. Findings that caspase-2 is activated by dimerization induced by interaction with signaling platforms that include the caspase-2 binding adaptor protein RAIDD [20, 21], have indicated that it is an initiator caspase. However, other findings identify caspase-2 as an effector that is downstream of other caspases [22, 23]. These issues have not been systematically addressed in neurons.

Our past work has shown that caspase-2 is required for apoptotic neuronal death associated with NGF withdrawal and $A\beta_{1\text{-}42}$ exposure. Here, we have examined the function and hierarchical role of caspase-2 in the death signaling pathways triggered by these two apoptotic stimuli. We show that caspase-2 is rapidly activated in response to apoptotic stimuli and, surprisingly, promotes induction of Bim mRNA and protein. Moreover, we find that this action is mediated by caspase-2-dependent activation of the transcription factor c-Jun. These findings causally associate caspase-2, c-Jun and Bim in the same apoptotic pathway and provide a novel mechanism by which activated caspase-2 triggers neuron death.

EXPERIMENTAL

For all animal experimentation, Institutional and National guidelines for the care and use of laboratory animals was followed.

Primary hippocampal neuron cultures

Neurons were cultured as previously described [24]. Briefly, embryonic day 18 rat fetuses were removed from CO_2-sacrificed pregnant Sprague Dawley rats (Charles River). The hippocampus was dissected from surrounding tissue and the meninges removed. Pooled hippocampi were mechanically dissociated in a serum-free defined medium. Medium consisted of a 1:1 mixture of Eagle's MEM and Ham's F12 (Invitrogen) supplemented with glucose (6 mg/ml), insulin (25 μg/ml), selenium (30 nM), progesterone (20 nM), transferrin

(100 μg/ml), putrescine (60 μg/ml), penicillin (0.5 U/ml), and streptomycin (0.5 μg/ml). Dissociated cells were grown on poly-D-lysine coated plates or 8-well chamber slides. Neurons were cultured for 7 days prior to experimental treatments.

Primary sympathetic neuron cultures

Neurons were cultured as previously described [11]. Briefly, sympathetic neurons were dissected from 1-day-old wild-type and caspase-2 null [5] mouse pups. Cultures were maintained in RPMI 1640 medium supplemented with 10% horse serum and mouse NGF (100 ng/ml) on collagen-coated 24-well dishes. For cells that were subjected to microscopic imaging, Matrigel-coated 8-well chamber slides were used. One day after plating, uridine (10 μM) and 5-fluorodeoxyuridine (10 μM) were added for 3 days to eliminate non-neuronal cells. Experiments were conducted after 6 days of culture.

Neuronal viability

Hippocampal or sympathetic neuron survival was scored as previously reported [12]. For hippocampal neurons, culture medium was removed by aspiration and 100 μl of detergent-containing lysis solution was added to the well. This solution dissolves cell membranes providing a suspension of intact nuclei. Intact nuclei were quantified using a hemacytometer. Triplicate wells were scored and values reported as mean ± SEM. Significance was calculated by Student's t-test. For sympathethic neurons, each culture was scored as numbers of living, phase-bright neurons counted in the same field at various times. Three replicate cultures were assessed for each condition, and data are normalized to numbers of neurons present in each culture at the time of $A\beta_{1\text{-}42}$ addition or NGF deprivation and reported as mean ± SEM.

β-amyloid Preparation

Lyophilized and HPLC-purified β-amyloid$_{1\text{-}42}$ ($A\beta_{1\text{-}42}$) was purchased from Dr. David Teplow (UCLA). Peptides were prepared according to Fa et al. [25] except that monomerized $A\beta_{1\text{-}42}$ was reconstituted in DMSO to 1 mM. To form $A\beta_{1\text{-}42}$ aggregates stocks of 1 mM peptide were resuspended in PBS to a concentration of 100 μM and incubated at 37°C for 24 hrs. 3 μM $A\beta_{1\text{-}42}$ was used in all experiments.

siRNA Conjugation and Use

siRNAs against caspase-2 and RAIDD were generated (Dharmacon). The sequence for caspase-2 is: GCCAUGCACUCCUGAGUUU. The sequence for RAIDD is: CCACAUUCAAGAAAUCAAA. The siRNAs were customized with a thiol group attached to the 5′ ends of the sense strands and carrying 3′ poly dT overhangs. Prior to use each siRNA sequence was conjugated to Penetratin1 (Pen1) [26]. Pen1-linked siRNA allows efficient delivery of siRNA into cells with minimal toxicity. For experiments all Pen1-siRNA were used at 80 nM.

Western Blot

Hippocampal neurons or sympathetic neurons were lysed in CHAPS lysis buffer (150 nM KCl, 50 mM HEPES, 0.1% CHAPS, protease inhibitor tablet, pH 7.4). Protein concentration was determined using BioRad protein assay reagent (Bio-Rad). Equal amounts of protein were loaded onto 10% or 12% polyacrylamide gels. The proteins were transferred onto nitrocellulose transfer membranes (Millipore). Subsequently, the membranes were blocked in 5% milk for 1 hr. Primary antibodies used for Western immunoblots include caspase-2 (Affinity purified, 1:250), phospho-cJun (Ser63) (Cell Signaling, 1:750), cJun (Cell Signaling, 1:750), ERK1 (Santa Cruz, 1:10,000), αTubulin (Abcam, 1:10,000), or Bim (Cell Signaling, 1:1,000). Proteins were detected using either enhanced chemiluminescence

(Thermo Scientific) or fluorescence using the Odyssey infrared imaging system (LI-COR Biosciences). The relative densities of immunopositive bands were analyzed using ImageJ.

Real Time PCR (RT-PCR)

Hippocampal or sympathetic neuron cultures grown in 24-well plates were harvested using iced cold 100% Trizol reagent (Invitrogen). cDNA was transcribed from RNA using Superscript RT II (Invitrogen). Equal amounts of cDNA were used for each PCR reaction for Bim, β tubulin. The sequence for the rat specific Bim primer was published previously [18]. cDNA was added to 25 μl of reaction mixture containing OmniMix HS master mix (Cepheid) and SYBR Green I (Invitrogen) together with appropriate primers. Quantitative PCR was performed using a Cepheid SmartCycler according to the manufacturer's directions.

Caspase-2 Activity Assay

This unbiased caspase-2 activity measurement was adapted from [21]. 50 μM of bVAD-fmk, a biotinylated pan-caspase inhibitor that traps active caspases, was added to neuronal cultures 2 hrs prior to $A\beta_{1\text{-}42}$ or NGF deprivation. Cells were lysed in CHAPS buffer. Active caspase-bVAD-fmk complex was pulled out with streptavidin-coated beads (Invitrogen). Active caspase-2 was detected by western blotting using affinity purified polyclonal caspase-2 antibody [12].

Immunocytochemistry

E18 rat hippocampal neurons and P1 mouse sympathetic neurons were cultured on 8-well chamber slides (Nunc) for 1 week. Cultures were then fixed in a solution of 3.7% formaldehyde and 5% sucrose at 37°C for 20 min. The cells were rinsed in TRIS buffer saline (TBS). Cultures were blocked in TBS supplemented with 3% normal goat serum (NGS) for at least 1 hr at RT. Primary antibodies used include anti-phosho-cJun (Ser63) (1:100, Cell Signaling) and anti-βIII-Tubulin (1:1000, Abcam).

Immunohistochemistry

Frozen mouse and human brain tissue sections were washed in PBS for 15 mins and then blocked in PBS with 1% bovine serum albumin (BSA), 10% NGS, and 0.5% Triton X-100 for 1 hr. Primary antibodies used include anti-Bim (1:100, Cell Signaling) and anti-caspase-2 (1:100, affinity purified). To prevent autofluorescence 1% Sudan black (prepared in 70% EtOH) was used to treat the sections for 5 min prior to cover-slip mount.

Fluoro-Jade B labeling

Frozen mouse brain sections were air dried at 45°C for 20 mins. Slides were incubated in 1% NaOH + 80% EtOH for 5 min, followed by a 2 min rinse in 70% EtOH and a 2 min rinse in ddH_2O. Sections were then incubated in 0.06% potassium permanganate for 10 min, and subsequently incubated with 0.0004% Fluoro-Jade B (made in 0.1% acetic acid) for 30 min. Finally, ddH_2O rinses for 1 min, followed by air drying for 5 min at 50°C, and then mounted with permount.

Mouse hippocampal $A\beta_{1\text{-}42}$ infusion

Adult wild-type or caspase-2 null male mice were used. Mice were anesthetized with xylazine (7 mg/kg) and ketamine (95 mg/kg) and then place onto a stereotaxic frame. The coordinates from Bregma 2.45 mm AP, 1.5 mm ML, and 1.7 mm DV were used to drill a hole into the skull and then a Hamilton syringe was inserted into the right CA1 region of the hippocampus. 4 μl of $A\beta_{1\text{-}42}$ (100 μM) was infused via convection enhanced delivery (CED)

at a rate of 0.2 μl per minute. Following injection, the head wound was closed using Vetbond (3M) and the animals were maintained for 2 weeks.

Mouse brain processing and sectioning

After the 2 week survival period animals were anaesthetized with xylazine (7 mg/kg) and ketamine (95 mg/kg) and transcardiacally perfused with 4% paraformaldehyde. Brains were removed and post-fixed with 4% paraformaldehyde for 24 hrs at 4°C, followed by 30% sucrose infiltration. The brains were embedded in Optimal Cutting Temperature embedding medium (Tissue-Tek) and stored at −80°C. For immunohistochemistry, brains were sectioned on a cryostat at 15 μm thickness and mounted onto SuperfrostPlus slides.

Alzheimer's disease and control brain samples

AD and control brains were obtained at autopsy by the New York Brain Bank at Columbia University (Dr. J.-P. Vonsattel, Director) as described in their website (http://nybb.hs.columbia.edu/index.htm). Detailed descriptions of the brains used are in [18].

Microscopy

Both *in vitro* and *in vivo* immunostainings were visualized at room temperature using PerkinElmer Ultraview spinning disc confocal system using Hamamatsu ORCA-ER digital camera, Nikon inverted TE-200 microscope, 20X/0.75, 40X/1.30 or 60XA/1.40 objectives. Images acquired using Volocity 6.0.1. Images were prepared in Adobe Photoshop CS3.

RESULTS

Bim and caspase-2 proteins are elevated and co-expressed in neurons from Alzheimer's disease patients

We have previously shown in cellular models of AD that $A\beta_{1\text{-}42}$ induces Bim transcripts and that Aβ-induced neuronal death requires Bim as well as caspase-2 [10, 18]. Additionally, we observed that Bim expression is elevated in more than 80% of entorhinal cortical neurons of AD patients [18], a brain region that shows early degeneration in AD. To determine whether caspase-2 might also be dysregulated along with Bim in AD, we co-immunostained brain sections from 6 AD patients and 6 age-matched non-demented controls for both proteins. Representative images are shown in Figure 1. We confirmed the increased expression of Bim in entorhinal cortical neurons, and consistently found an increase of caspase-2 (Figure 1), with AD brains containing more than 200 caspase-2 positive cells/field, controls containing less than 20. Moreover, Bim and caspase-2 co-localized almost completely within the same entorhinal cortical neurons in AD brains (Figure 1). In contrast, there was no increased expression of either protein in cerebellum, an area spared of AD pathology (data not shown).

Caspase-2 is rapidly activated by exposure to $A\beta_{1\text{-}42}$ and by NGF deprivation

The required roles of Bim and caspase-2 for neuron death in cellular models of AD and their increased expression and co-localization in AD neurons raised the possibility that they may function in the same apoptotic pathway. To assess a potential functional interaction between Bim and caspase-2 in neuron death, we utilized primary cultures of rat hippocampal neurons. Hippocampal neurons undergo apoptotic death in response to treatment with $A\beta_{1\text{-}42}$, which requires both Bim [18] and caspase-2 [10]. We also used a second, well-studied model of neuron death: cultured sympathetic neurons deprived of NGF. In this system also, NGF deprivation induces elevation of Bim transcripts and protein and the subsequent neuron death requires both Bim [17] and caspase-2 expression [11, 12]. We first aimed to detect caspase-2 activation in these death models and to determine the time at

which it occurs. To achieve this, we used an unbiased caspase activity probe that we previously adapted for use in neurons [24, 27]. This approach employs a biotinylated pan-caspase inhibitor, bVAD-fmk, that irreversibly binds and inhibits active caspases within cells and permits their subsequent isolation and identification by western immunoblotting [21]. When cells are pre-treated with bVAD-fmk and then exposed to a death stimulus, bVAD binds to proximal caspases (usually initiator caspases) and inhibits their activation, usually that of initiator caspases. All subsequent events dependent on activity of the proximal caspases are blocked. The specificity of the polyclonal caspase-2 antiserum used for these studies was confirmed by using brain lysates from wild-type and caspase-2 null animals (Figure 2A).

The levels of activated caspase-2 in cultured hippocampal neurons significantly increased within 30 min of treatment with 3 μM $A\beta_{1\text{-}42}$ (Figure 2B). Moreover, NGF withdrawal from sympathetic neurons elicited an increase in activated caspase-2 within 2 hours (Figure 2C). We have reported that sympathetic neurons undergo apoptotic death in response to $A\beta_{1\text{-}42}$ and do so by a caspase-2 dependent mechanism [10]. Consistent with this, $A\beta_{1\text{-}42}$ also activated caspase-2 in sympathetic neurons within 2 hours (Figure 2C). Total caspase-2 levels remained unchanged in all models at these times (Figure 2B and 2C). Taken together, these data show that caspase-2 is rapidly activated in neurons in response to death stimuli, consistent with our previous findings that neuronal apoptosis caused by $A\beta_{1\text{-}42}$ or NGF deprivation requires caspase-2.

Bim induction by $A\beta_{1\text{-}42}$ occurs after caspase-2 activation

If Bim and caspase-2 have the potential to function in the same apoptotic pathway, then it is important to determine the temporal relationship of Bim induction and caspase-2 activation. In other models Bim elevation precedes activation of caspases such as caspases-9 and -3 [28, 29]. Our previous studies with hippocampal neurons indicated that $A\beta_{1\text{-}42}$ induces Bim mRNA within 1 hr, with a maximal effect at 3–6 hrs and that Bim protein is elevated within 4 hrs of treatment [18]. We therefore examined Bim protein expression in hippocampal neuron cultures at relatively early times after $A\beta_{1\text{-}42}$ exposure, starting at 30 min (Figure 2D). This revealed no change in Bim expression at 30 and 60 min but a significant increase by 2 hrs. These data (Figure 2B and 2C) indicate that caspase-2 activation occurs prior to Bim protein elevation and suggest that caspase-2 activation is independent of Bim induction. They also raised the possibility that activated caspase-2 may be upstream of Bim regulation.

Caspase-2 activation by $A\beta_{1\text{-}42}$ does not require Bim induction

While our temporal data suggest that caspase-2 activation could be upstream of Bim induction, the canonical pathway places Bim upstream of caspase-2. To determine if Bim induction is required for caspase-2 activation, we utilized a Bim specific siRNA conjugated to the cell penetrating peptide Penetratin1 (Pen1) for highly efficient, low toxicity delivery into neurons [26]. We previously used this sequence with an shRNA [17] to suppress Bim expression and to provide protection from apoptotic stimuli and, as shown in Figure 3A, found that 4 hrs treatment of cultured hippocampal neurons with Pen1-siBim yielded substantial knockdown of Bim expression (Figure 3A). Cultures of hippocampal neurons were preincubated with Pen1-siBim for 3 hrs and then bVAD-fmk was added for 2 hrs followed by addition of $A\beta_{1\text{-}42}$. After 4 hrs of $A\beta_{1\text{-}42}$ exposure, the neurons were harvested and activated caspase-2 was detected by western immunoblotting. This revealed that knockdown of Bim did not alter the activation of caspase-2 by $A\beta_{1\text{-}42}$ (Figure 3B), thus indicating that Bim is not upstream of caspase-2 activation in this model.

Bim induction by $A\beta_{1-42}$ is blocked by a pan-caspase inhibitor

Since Bim does not appear to act upstream of caspase-2 activation, we next considered that it may act downstream. As a first step to determine whether caspase activity is required for Bim induction, we utilized the pan-caspase inhibitor bVAD-fmk, which we have shown above captures active caspase-2, and should inhibit any subsequent action of caspase-2 and any other captured caspases. Cultured hippocampal neurons were pretreated with bVAD-fmk for 2 hrs followed by addition of $A\beta_{1-42}$ for another 4 hrs and were then assessed for Bim mRNA and protein levels. Caspase inhibition by bVAD-fmk blocked induction of both Bim transcripts and protein (Figure 3C and 3D). Western immunoblotting confirmed the capture of activated caspase-2 under these conditions (Figure 3E). In contrast, when bVAD-fmk was added two hours after $A\beta_{1-42}$, a time at which caspase-2 has already been activated (Figure 2B), induction of Bim mRNA and protein still took place (Figure 3C and 3D). These findings thus support the idea that caspase activation occurs upstream of Bim induction.

Induction of Bim mRNA and protein by apoptotic stimuli requires caspase-2 expression

We next examined whether caspase-2 is specifically required for Bim induction by apoptotic stimuli. We used a caspase-2 specific siRNA conjugated to Pen1. In cultured hippocampal neurons knockdown of caspase-2 with this reagent was evident within 2 hrs and appeared to be maximal by 4 hrs (Figure 4A). Knockdown of caspase-2 in these neurons completely blocked the induction of Bim mRNA that occurs after 4 hrs of $A\beta_{1-42}$ treatment (Figure 4B). Similarly, Pen1-siCaspase-2 fully inhibited the capacity of $A\beta_{1-42}$ to induce Bim transcripts in cultured sympathetic neurons (Figure 4C). Finally, caspase-2 knockdown repressed Bim mRNA induction caused by NGF withdrawal from sympathetic neurons (Figure 4C). Taken together, these data suggest that caspase-2 expression is required for transcriptional regulation of Bim in two different apoptotic models and two different neuronal types. To further support this conclusion, we utilized sympathetic neurons cultured from caspase-2 null mice. Bim mRNA was measured following 4 hrs of $A\beta_{1-42}$ treatment or NGF deprivation. In both paradigms, in contrast with sympathetic neurons from wild-type mice (Figure 4C), Bim transcripts were unchanged in the caspase-2 null neurons (Figure 4D).

Parallel experiments were carried out to examine the role of caspase-2 in the elevation of Bim protein levels by $A\beta_{1-42}$ and NGF deprivation. Pen1-siCaspase-2 fully inhibited the capacity of $A\beta_{1-42}$ to increase Bim protein expression in cultured hippocampal neurons (Figure 4E) after 8 hrs of treatment. As a control, we employed a Pen1-siRNA targeting the firefly luciferase gene. In contrast with Pen1-siCaspase-2, this construct had no effect on the increase in Bim protein levels caused by $A\beta_{1-42}$ treatment (Figure 4F). We also compared the effects of apoptotic stimuli on Bim protein expression in wild-type and caspase-2 null sympathetic neurons. To achieve this, cultures (with and without $A\beta_{1-42}$ treatment or NGF deprivation for 5 hrs) were immunostained for Bim expression and scored in a blinded manner as previously described [17] for proportion of neurons with high Bim staining. In cultures from wild-type animals, there was a substantial increase in the proportion of neurons that showed high Bim staining and this response was completely blocked by pre-treatment with Pen1-siCaspase-2 (Figure 4G). In contrast, apoptotic stimuli caused no significant change in Bim expression in caspase-2 null neurons (Figure 4G). Collectively, these findings indicate that caspase-2 is required for induction of Bim mRNA and protein in neurons after $A\beta_{1-42}$ exposure and NGF deprivation.

Bim induction by $A\beta_{1-42}$ requires RAIDD expression/caspase-2 activation

The above studies indicate that caspase activity and caspase-2 expression are necessary for Bim induction by $A\beta_{1-42}$ exposure or NGF deprivation. We next specifically addressed whether such induction requires caspase-2 activation. To do so, we took advantage of prior findings that caspase-2 activation requires the death adapter RAIDD [30–32]. RAIDD

expression is also necessary for neuron death caused by NGF deprivation [31, 32] and $A\beta_{1-42}$ treatment [31]. We used a Pen1-linked RAIDD siRNA (Pen1-siRAIDD) that effectively knocks down RAIDD mRNA and protein levels in cultured hippocampal neurons (Figure 5A) and that protects hippocampal neurons from death induced by $A\beta_{1-42}$ [31]. Simultaneous treatment with Pen1-siRAIDD suppressed the induction of Bim protein elicited by 8 hrs of exposure to $A\beta_{1-42}$ (Figure 5B). Thus, it appears that RAIDD expression and therefore caspase-2 activation are required for Bim induction by $A\beta_{1-42}$.

Caspase-2 acts upstream of Bim induction by enabling activation of c-Jun

Bim induction by apoptotic stimuli requires transcriptional activation that can be mediated by a variety of transcription factors [17, 33–35]. We therefore next examined whether caspase-2 might function upstream of transcription factor activation. Here, we focused on c-Jun which is reported to be elevated in neurons from AD patients and which is activated in response to a cascade of phosphorylation events set in motion by $A\beta_{1-42}$ treatment [11] or by NGF deprivation [17, 33–35]. Inhibition of this phosphorylation cascade blocks both $A\beta_{1-42}$ and NGF deprivation–mediated Bim induction as well as neuron death [11, 17].

To assess the potential role of caspase-2 in c-Jun activation, we treated hippocampal neurons with $A\beta_{1-42}$ for 8 hrs in the presence or absence of Pen1-siCaspase-2 and carried out western immunoblots to detect phospho-c-Jun. $A\beta_{1-42}$ caused robust phosphorylation of c-Jun on serine 63 leading to nuclear translocation, which was significantly inhibited by Pen1-siCaspase-2 (Figure 6A), and by Pen1-siRAIDD (Figure 6B). Complementary experiments were conducted to assess the effects of Pen1-siCaspase-2 and Pen1-siRAIDD on $A\beta_{1-42}$ induction of nuclear phospho-c-Jun. Hippocampal neuron cultures were treated with or without the two Pen-1 siRNAs and with or without $A\beta_{1-42}$ for 8 hrs, immunostained with anti-phospho-c-Jun and then blindly assessed for proportions of neurons with strong nuclear staining for phospho-c-Jun. $A\beta_{1-42}$ alone elicited a large increase in proportions of neurons exhibiting strong nuclear phospho-c-Jun immunostaining, which was fully blocked in the presence of Pen1-siCaspase-2 or Pen1-siRAIDD (Figure 6C). To assess the effect of Pen1-siCaspase-2 on the appearance of nuclear phospho-c-Jun in response to NGF deprivation, sympathetic neuronal cultures were treated with or without Pen1-siCaspase-2 and maintained with or without NGF. The cultures were then analyzed for strong nuclear phospho-c-Jun immunostaining as described above for $A\beta_{1-42}$ treatments. NGF deprivation induced a large increase in proportions of neurons with strong nuclear phospho-c-Jun immunostaining and this was fully blocked in the presence of Pen1-siCaspase-2 (Figure 6D). These findings indicate that caspase-2 expression, as well as caspase-2 activation, is required for c-Jun phosphorylation/activation and nuclear localization in response to $A\beta_{1-42}$ and to NGF deprivation. These observations illustrate at least one mechanism by which caspase-2 promotes Bim induction, and demonstrate that activated caspase-2 participates in c-Jun activation in response to apoptotic stimuli.

Caspase-2 is required for Bim induction in an animal model of AD pathology

To extend our *in vitro* studies, we determined whether caspase-2 also regulates Bim in an *in vivo* animal model of AD pathology. Previous studies have shown that infusion of $A\beta_{1-42}$ into the hippocampus causes neurodegeneration by 2 weeks [36]. We infused $A\beta_{1-42}$ (0.4 nmoles) or vehicle alone via convection enhanced delivery (CED) (Figure 7A) [24] into the right hippocampi of 16-month-old wild-type (n=4) and caspase-2 null mice (n=4). The animals were sacrificed two weeks later and the brains prepared for Fluoro-Jade B and Bim immunohistochemical staining. Consistent with the previously described role of caspase-2 in neuron death caused by $A\beta_{1-42}$ *in vitro* [10], Fluoro-Jade B staining revealed evidence of neurodegeneration in wild-type but not caspase-2 null brains; staining increased 15-fold in wild-type mouse brains injected with $A\beta_{1-42}$, but there was no change in caspase-2 null

brains (Figure 7B). Immunostaining of brains from wild-type mice showed a three-fold increase in Bim expression within cortical neurons near the site of infusion for animals receiving $A\beta_{1\text{-}42}$ compared with those infused with vehicle alone (Figure 7C). No such increase occurred in brains of caspase-2 null mice infused with $A\beta_{1\text{-}42}$ (Figure 7C). Taken together, these findings confirm Bim induction in an animal model of AD pathology and indicate that in this model, caspase-2 and Bim up-regulation are required for neurodegeneration.

DISCUSSION

In this study we examined the mechanism by which caspase-2 participates in neuron death and its position in the hierarchy of apoptotic signaling cascades triggered by β-amyloid exposure and NGF deprivation. Surprisingly, our findings place caspase-2 upstream of the transcription-dependent induction of Bim, a BH3-only protein that participates in neuron death in a variety of apoptotic paradigms (Figure 8) [17, 37–41].

Although gene silencing and knockout studies have strongly supported the participation of caspase-2 in multiple apoptotic paradigms in both neuronal and non-neuronal cells, the absence of a strong developmental phenotype in caspase-2 null mice has led to questions about its physiological functions *in vivo* [5]. However, compensatory changes in gene regulation have been observed when apoptosis-related genes are deleted [42] and in this regard, we have reported that both caspase-9 and Diablo/Smac levels are elevated in neurons isolated from caspase-2 knockout animals [11]. Moreover, recent studies with caspase-2 null animals show significant protection of neurons from apoptotic stresses including optic nerve transection, hypoxia-ischemia, excitotoxic stress, and MPTP treatment [14, 15]. We additionally report here that neurons in caspase-2 null mice are protected from *in vivo* β-amyloid exposure.

Several different types of evidence presented here support the positioning of caspase-2 upstream of Bim induction. First, bVAD trapping shows rapid activation of caspase-2 by apoptotic stimuli and at least in the β-amyloid model this occurs well before induction of Bim mRNA or protein. Second, addition of bVAD before apoptotic stimulation, which will both trap and inhibit initiator caspases, blocks Bim induction. Third, Bim induction by apoptotic stimuli is abolished in neurons in which caspase-2 is either silenced or deleted. Fourth, knockdown of RAIDD, an adapter protein required for caspase-2 activation and neuron death in the apoptotic paradigms that we employed, suppresses Bim induction. Fifth, silencing of either caspase-2 or RAIDD inhibits phosphorylation/activation and nuclear translocation of c-Jun, a transcription factor required for efficient induction of Bim in the apoptotic paradigms that we studied. Lastly, neurons of caspase-2 null mice are resistant to Bim induction and to degeneration promoted *in vivo* by infusion of β-amyloid.

In addition to placing caspase-2 upstream of Bim induction, our findings indicate that this is a cell autonomous mechanism. The Penetratin-linked siRNAs that we used to silence caspase-2 and RAIDD efficiently enter essentially all treated cells [26], thus arguing against cell-extrinsic and bystander mechanisms.

Our bVAD capture experiments indicate that caspase-2 is activated soon after neurons encounter apoptotic stimuli – within 30 min for β-amyloid treatment and 2 hrs for NGF deprivation. This is well before any evident sign of neurodegeneration occurs (8–12 hrs) and is consistent with the role of caspase-2 as an apical regulator of a pathway that requires downstream activation of genes such as Bim. The rapidity of caspase-2 activation places this event close to the initial responses of neurons to apoptotic stimuli. In this regard, it may be relevant that capase-2 activity can be regulated by phosphorylation/dephosphorylation [43].

Since Bim has been described to act upstream of a caspase cascade [19, 37], we also considered the possibility that Bim might function upstream of caspase-2 activation. However, knockdown of Bim, which is protective in the apoptotic paradigms used here, had no apparent effect on caspase-2 activation as monitored by bVAD capture.

Positioning caspase-2 to a proximal role in the apoptotic cascade has raised the major question of how it might influence mitochondrial release of apoptotic substances. Our observation that caspase-2 activation leads to Bim induction addresses this question. An alternative mechanism that has been raised by which activated caspase-2 may affect mitochondria is by cleavage of the BH3-only protein Bid [44–46]. When cleaved, the truncated form of Bid (tBid) contributes to cell death by promoting mitochondrial release of apoptosis-inducing proteins such as cytochrome c [47]. However, it has been questioned whether the efficiency with which caspase-2 cleaves Bid would be sufficient to promote death [44]. In any case, it remains to be seen whether caspase-2-dependent Bid cleavage occurs in the systems studied here, and whether this plays a role in the cell death process along with elevated Bim.

In many death paradigms, including NGF deprivation and β-amyloid exposure, apoptotic stimuli initiate signaling pathways that culminate in activation of transcription factors, leading to the induction of death promoting genes such as Bim. A number of these transcription factors have been described as influencing Bim expression including CHOP, FoxO, c-Jun, B- and C-myb, NF-Y, and Egr1 [16, 17, 33–35, 48]. Here, we focused on c-Jun which is phosphorylated/activated in response to NGF deprivation and β-amyloid treatment and is required for Bim induction and neuron death in both paradigms [11, 18]. We found that β-amyloid and NGF deprivation stimulated c-Jun phosphorylation/activation and nuclear translocation and that these effects were suppressed by knockdown of either caspase-2 or RAIDD. Thus, it appears that at least one upstream mechanism by which caspase-2 mediates Bim induction is by stimulating c-Jun transcriptional activity. An important next step is to determine whether caspase-2 is involved in regulating additional transcription factors including those listed above.

The mechanism by which activated caspase-2 promotes c-Jun phosphorylation is currently unknown. Apoptotic regulation of c-Jun in neurons requires a cascade of events that includes successive activation of upstream protein kinases including MLKs, MKKs and JNKs as well as the involvement of scaffold proteins such as POSH and JIPs [49, 50]. A parsimonious model for stimulation of this pathway by activated caspase-2 is that caspase-2 cleaves a suitable substrate. This cleavage may lead to either activation of a required initiator of the pathway or removal of an inhibitory regulator. In this light, it has been reported that caspase-2 participates in doxorubicin-induced apoptotic death of non-neuronal cells by cleaving/activating PKCdelta that in turn stimulates JNK signaling and c-Jun phosphorylation [51]. However, we were unable to detect any cleavage of PKCdelta in either of our apoptotic paradigms (data not shown).

c-Jun has been shown to regulate several other proteins in addition to Bim that mediate death of neurons including the BH3-only protein DP5/HRK [52] and the prolyl hydroxylase SM20/EGLN3 [53]. It thus seems possible that induction of these and potentially other apoptotic proteins will also require caspase-2 activation. Furthermore, JNK/c-Jun signaling and Bim induction have been implicated in a variety of cell death paradigms in addition to those examined here [54, 55], raising the possibility of caspase-2 involvement in a wide range of apoptotic conditions.

Previous studies have indicated that caspase-2 expression and activation, JNK/c-Jun signaling and Bim induction are required for neuron death promoted by NGF deprivation

and β-amyloid exposure [10, 16–18]. We further show here that these are hierarchically and causally linked in the same apoptotic pathway. Bim induction and neuron death were also found here to be downstream of caspase-2 in an *in vivo* model of Alzheimer's disease (AD). These findings may be directly relevant to AD in which β-amyloid [56, 57] and defective trophic factor signaling [58] have been implicated in the neurodegeneration and death that are characteristic of this disorder. We observed that both Bim and caspase-2 are elevated and co-expressed in a vulnerable population of neurons in post-mortem brains of AD patients. Immunostaining for c-Jun [59] and phospho-c-Jun [60] is also enriched in AD neurons. Taken together, these observations indicate that each of the elements of the apoptotic pathway described here are present and abnormally expressed in AD neurons. Given the apical position of caspase-2 in this pathway and its apparently limited role in unstressed cells, this caspase and its activation mechanism may be suitable targets for clinical intervention in AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

AD	Alzheimer's Disease
Aβ	β-amyloid
BH3	Bcl-homology domain 3
biotin-VAD-FMK	biotin-Val-Ala-DL-Asp-fluoromethylketone
CED	convection enhanced delivery
ERK	extracellular-signal-regulated kinase
JNK	Jun kinase
NGF	nerve growth factor
Pen1	Penetratin1
RAIDD	RIP (receptor-interacting protein)-associated ICH-1 [ICE (interleukin-1β-converting enzyme)/CED-3 homologue 1] protein with a Death Domain
RIP	receptor-interacting protein
SCG	superior cervical ganglia
siRNA	small interfering RNA

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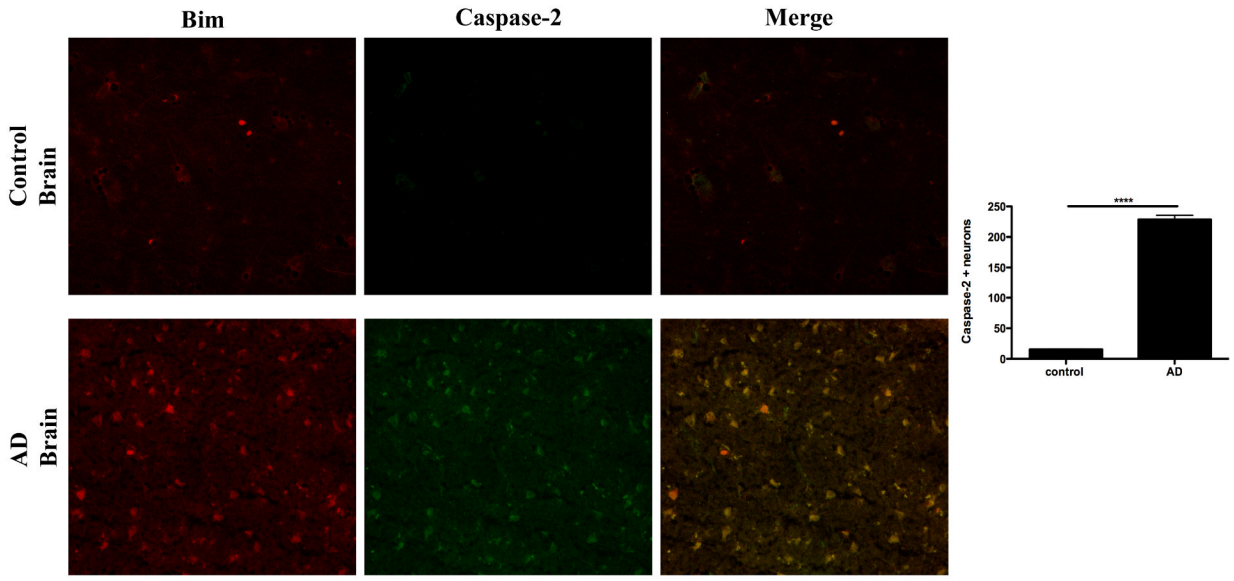

Figure 1. Entorhinal cortical neurons of post-mortem AD brains with elevated Bim immunostaining also show elevated caspase-2 immunostaining

Sections of entorhinal cortex from six AD and six age-matched controls were co-immunostained for Bim (red) and caspase-2 (green). Representative images were taken for each case by using an inverted fluorescent microscope and camera set to the same exposure time. Images were taken at 20X. Scale bar = 50 μm. Quantification of caspase-2 positive neurons in control and AD brains is shown in bar graph, **** indicates $p<0.0001$, indicating AD mean value is significantly different from control (N=6). Co-localization measures showed that 93–100% co-localization of caspase-2 and Bim in AD brains.

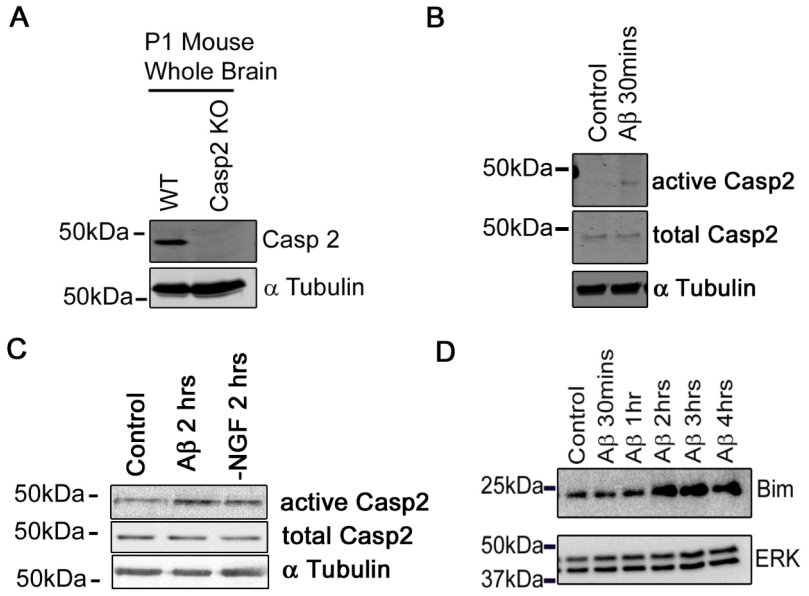

Figure 2. $A\beta_{1\text{-}42}$ and NGF deprivation promote rapid activation of caspase-2 and $A\beta_{1\text{-}42}$ activates caspase-2 before Bim induction

(A) Specificity of anti-caspase-2 antiserum in western immunoblotting. Whole brain extracts from postnatal day 1 (P1) wild-type and caspase-2 knockout mice were used to assess the specificity of the affinity purified rabbit polyclonal caspase-2 antibody.

(B) $A\beta_{1\text{-}42}$ induces rapid activation of caspase-2 in hippocampal neurons. Hippocampal cultures were treated with 50 μM bVAD-fmk for 2 hrs and then with or without 3 μM Aβ for an additional 30 min. Activated caspase-2 was pulled-down with streptavidin beads and identified by western immunoblot analysis using affinity purified polyclonal caspase-2 antibody. Representative blots are shown, (N=3). Quantification in Supplemental Figure 1.

(C) $A\beta_{1\text{-}42}$ treatment and NGF deprivation induce rapid activation of caspase-2 in sympathetic neurons. Sympathetic cultures were treated with 50 μM bVAD-fmk for 2 hrs prior to and then with or without 3 μM $A\beta_{1\text{-}42}$ treatment or NGF-deprivation (-NGF) for 2 hrs. Active caspase-2 was pulled-down by streptavidin beads and identified by western immunoblot analysis using polyclonal caspase-2 antibody. Representative blots are shown, (N=3). Quantification in Supplemental Figure 1.

(D) Elevation of Bim in response to $A\beta_{1\text{-}42}$ occurs later than caspase-2 activation. Hippocampal cultures were treated with or without 3 μM $A\beta_{1\text{-}42}$ for the indicated times and Bim levels were assessed by western immunoblotting. ERK was used as an internal loading control.

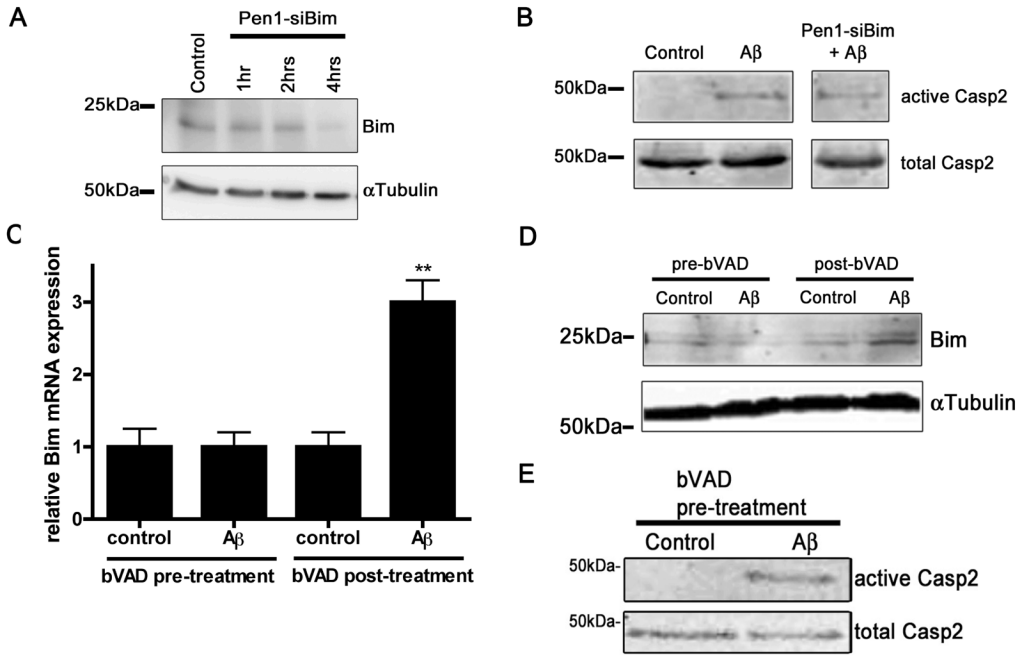

Figure 3. Bim induction requires caspase activity

(A) Pen-siBim effectively knocks down Bim expression. Hippocampal cultures were treated with or without Pen1-siBim (80 nM) for the indicated times. Cultures were analyzed by western blot for Bim protein expression; α-tubulin was used as a loading control.

(B) Knockdown of Bim does not compromise activation of caspase-2 by $A\beta_{1\text{-}42}$ treatment. Hippocampal cultures were treated with bVAD-fmk (50 μM) and with or without Pen1-siBim (80 nM) for 3 hrs and then with or without 3 μM $A\beta_{1\text{-}42}$ for an additional 2 hrs. Active caspase-2 was pulled down using streptavidin beads and identified by western blot analysis with a polyclonal caspase-2 specific antibody. Representative blots are shown, (N=3). Quantification in Supplemental Figure 2.

(C) bVAD-fmk pretreament blocks Bim mRNA induction by $A\beta_{1\text{-}42}$. Hippocampal cultures were treated with 50 μM bVAD-fmk for 2 hrs prior to and then with or without 3 μM $A\beta_{1\text{-}42}$ for an additional 4 hrs (bVAD-fmk pre-treatment) or with 3 μM $A_{1\text{-}42}$ for a total of 4 hrs with 50 μM bVAD-fmk added during the last 2 hrs of incubation (bVAD-fmk post-treatment). Cultures were analyzed by qPCR for Bim expression. Bim mRNA levels were normalized to β-tubulin mRNA expression and are expressed as mean values ± SEM. ** indicates that mean value is significantly different from control ($p<0.003$) (N=3).

(D) bVAD-fmk pretreament blocks Bim protein induction by $A\beta_{1\text{-}42}$. Hippocampal cultures were treated as in C and analyzed by western blot for Bim protein expression; α-tubulin was used as a loading control. A representative blot is shown, (N=3). Quantification shown in Supplemental Fig. 2.

(E) bVAD-fmk pretreatment captures caspase-2 activated by $A\beta_{1\text{-}42}$ treatment. Hippocampal neuron cultures were pre-treated with bVAD-fmk as in 2B, Activated caspase-2 was pulled down using streptavidin beads and identified by western blot analysis with a polyclonal caspase-2 specific antibody. A representative blot is shown, (N=3). Quantification in Supplemental Figure 2.

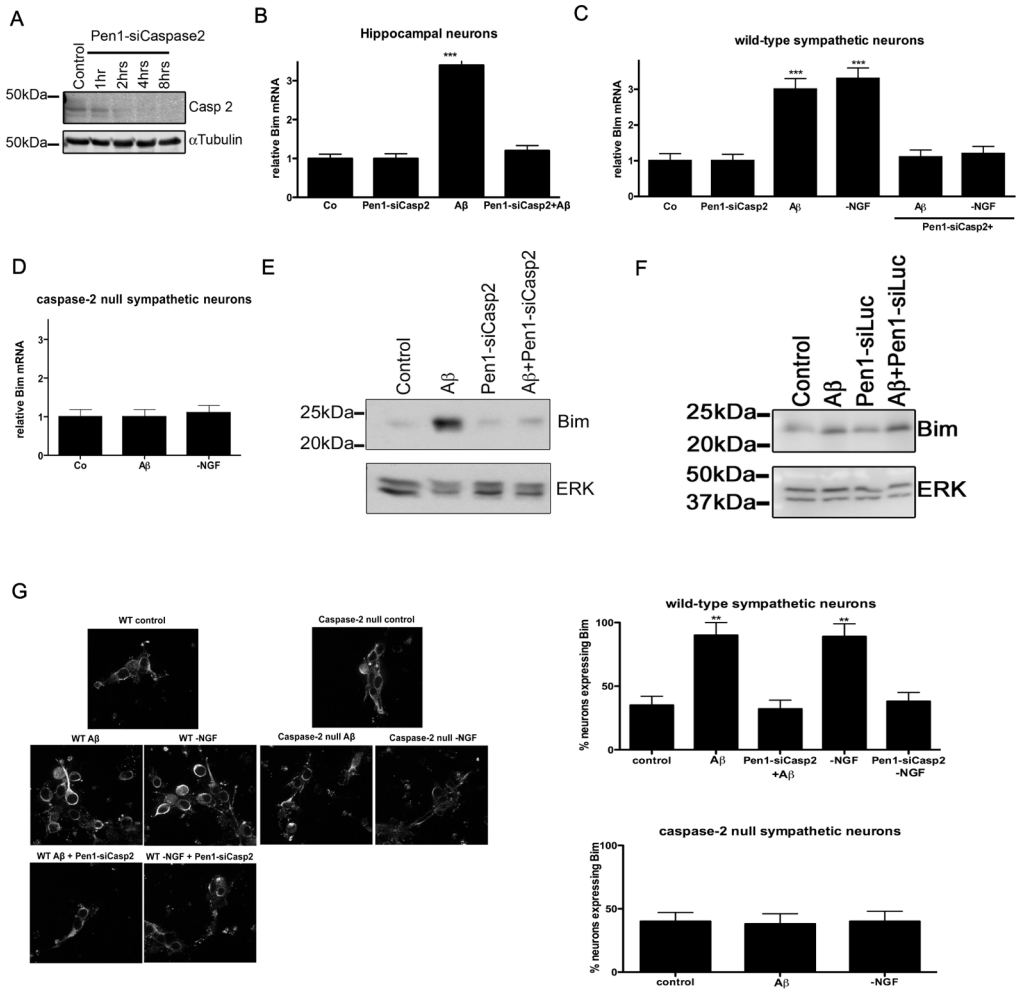

Figure 4. Caspase-2 is required for induction of Bim protein levels by apoptotic stimuli in hippocampal neurons and sympathetic neurons

(A) Pen-siCaspase-2 rapidly knocks down caspase-2. Hippocampal cultures were treated with or without Pen1-siCaspase-2 (80 nM) for the indicated times and analyzed by western blot with a polyclonal caspase-2 specific antibody; α-tubulin was used as a loading control.

(B) Knockdown of caspase-2 inhibits induction of Bim mRNA by $A\beta_{1-42}$ in hippocampal cultures. Hippocampal cultures were treated for 2 hrs with Pen1-Caspase-2 (80 nM) and then treated with or without 3 μM $A\beta_{1-42}$ for 4 hrs and analyzed by qPCR for Bim expression. Bim mRNA levels were normalized to β-tubulin mRNA. ** indicates that mean value is significantly different from control ($p<0.002$), (N=3).

(C) Knockdown of caspase-2 inhibits induction of Bim mRNA by $A\beta_{1-42}$ or NGF-deprivation in sympathetic neurons cultured from wild-type mice. Wild-type sympathetic cultures were treated for 2 hrs with Pen1-siCaspase-2 and then with or without 3 μM $A\beta_{1-42}$ or NGF deprivation for 4 hrs. Culture extracts were analyzed by qPCR for Bim expression and Bim mRNA levels were normalized to β-tubulin mRNA expression and are expressed as mean values ± SEM. *** indicates that mean value is significantly different from control ($p<0.0001$), by unpaired Student's t-test (N=3).

(D) Bim mRNA is not induced by $A\beta_{1-42}$ or NGF deprivation in sympathetic neuron cultured from caspase-2 null mice. Caspase-2 null sympathetic cultures were treated with or without 3 μM $A\beta_{1-42}$ or NGF deprivation for 4 hrs and analyzed by qPCR for Bim mRNA

expression. Relative Bim mRNA levels were normalized to β-tubulin mRNA expression and values given as means ± SEM. There are no significant differences from control, (N=3).
(E) Knockdown of caspase-2 inhibits induction of Bim protein in hippocampal neurons by $A\beta_{1\text{-}42}$. Hippocampal cultures were treated for 8 hrs with or without 3 μM $A\beta_{1\text{-}42}$ in the presence or absence of Pen1-siCaspase-2 (80 nM). Bim levels were determined by western blotting with normalization to ERK. A representative blot is shown, (N=7). Quantification in Supplemental Figure 3.
(F) $A\beta_{1\text{-}42}$ induced Bim up-regulation is not affected by a non-related siRNA. Hippocampal cultures were treated for 2 hrs with or without 3 μM $A\beta_{1\text{-}42}$ and with or without Pen1-siLuciferase (80 nM). Bim levels were determined by western blotting.
(G) Apoptotic stimuli induce Bim expression in sympathetic neurons cultured from wild-type, but not caspase-2 null mice. Sympathetic cultures from P1 wild-type and caspase-2 null mice were treated for 6 hrs with or without 3 μM Aβ or subjected to NGF-deprivation for 6 hrs. Wild-type cultures were also pretreated with Pen1-siCaspase-2 for 2 hrs as indicated. Bim was visualized by immunocytochemistry using a PerkinElmer spinning disc confocal, 60X magnification and cultures were blindly scored as previously described [18] for proportions of neurons with high Bim staining. Left-hand panels show photos of representative cultures under indicated conditions. Right hand panels show quantification of proportions of neurons with high Bim expression under each condition. Values are means ± SEM. ** indicates that mean value is significantly different from control ($p<0.0015$), by unpaired Student's t-test (N=3). Scale bar = 20μm.

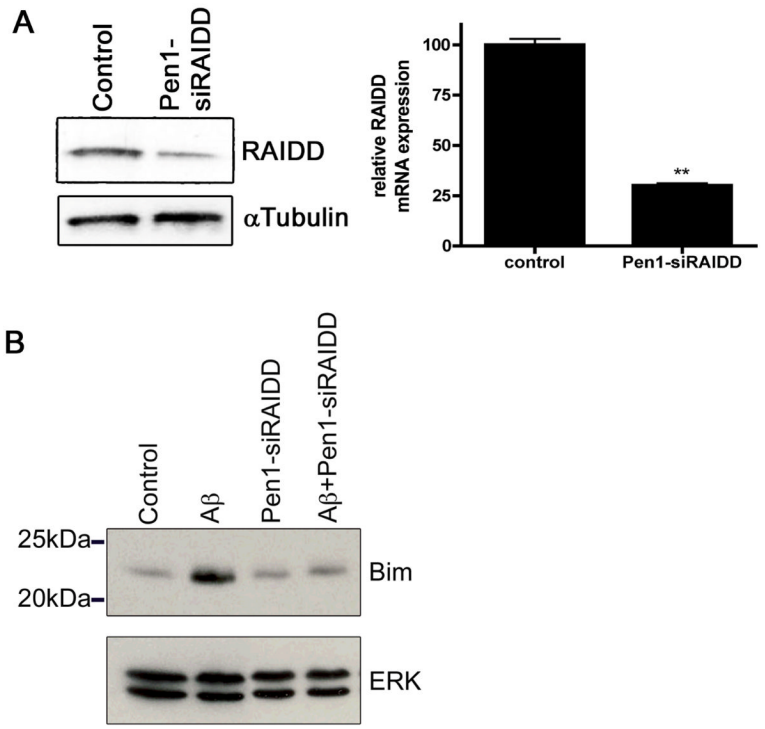

Figure 5. RAIDD is required for Bim protein induction in hippocampal neurons by $A\beta_{1\text{-}42}$

(A) Pen1-siRAIDD knocks down RAIDD expression. Hippocampal neuron cultures were treated for 4 hrs with Pen1-siRAIDD (80 nM). RAIDD protein and mRNA levels were assessed by western immunoblotting (left) and qPCR (right), respectively. RAIDD mRNA levels were normalized to β-tubulin mRNA expression. ** indicates that mean value is significantly different from control ($p<0.0015$), (N=3).

(B) Knockdown of RAIDD blocks Bim protein induction by $A\beta_{1\text{-}42}$. Hippocampal neuron cultures were treated for 8 hrs with or without 3 μM $A\beta_{1\text{-}42}$ in the presence or absence of Pen1-siRAIDD (80 nM). Bim levels were assessed by western blotting. A representative blot is shown, (N=6). Quantification in Supplemental Figure 4.

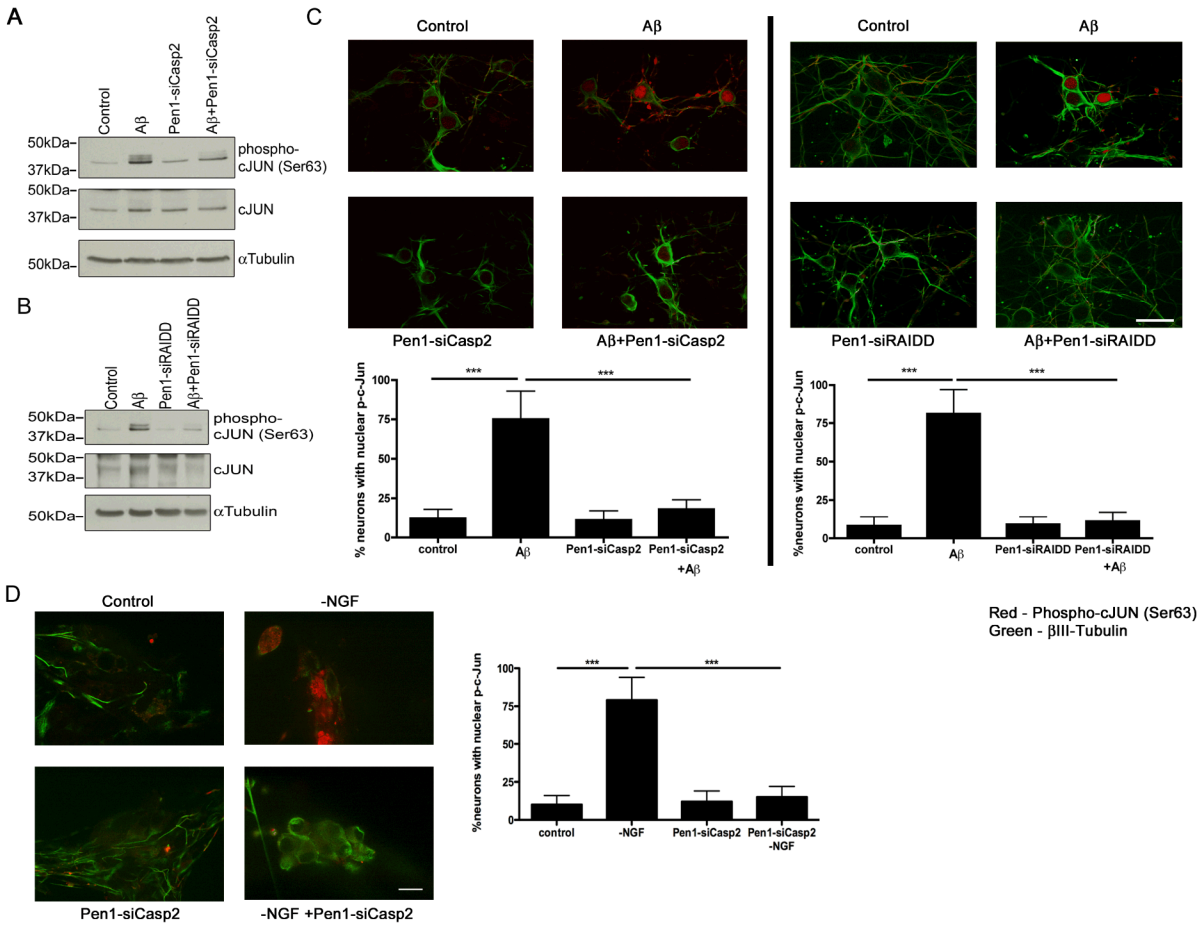

Figure 6. Both caspase-2 and RAIDD are required for $A\beta_{1\text{-}42}$-induced cJun phosphorylation and nuclear localization in hippocampal neurons

(A) Caspase-2 is required for elevation of cJun phosphorylation in response to $A\beta_{1\text{-}42}$. Hippocampal neurons were treated for 8 hrs with or without 3 μM $A\beta_{1\text{-}42}$ in the presence or absence of Pen1-siCaspase-2 (80 nM). Phospho-cJun (Ser 63) levels were assessed by western immunoblotting. Blots from a representative experiment are shown, (N=5). Quantification in Supplemental Figure 5.

(B) RAIDD is required for elevation of cJun phosphorylation in response to $A\beta_{1\text{-}42}$. Hippocampal cultures were treated for 8 hrs with or without 3 μM $A\beta_{1\text{-}42}$ in the presence or absence of Pen1-siRAIDD (80 nM). Phospho-cJun (Ser 63) levels were assessed by western immunoblotting. Blots from a representative experiment are shown, (N=4).

(C) Both caspase-2 and RAIDD are required for elevated nuclear localization of phospho-cJun in hippocampal neurons in response to $A\beta_{1\text{-}42}$ treatment. Hippocampal cultures were treated for 8 hrs with or without 3 μM $A\beta_{1\text{-}42}$ in the presence or absence of Pen1-siCaspase-2 or Pen1-siRAIDD (80 nM). Phospho-cJun (Ser 63) was visualized by immunocytochemistry using PerkinElmer spinning disc confocal, 60X magnification. Representative images are shown for each condition. Blinded counts were carried out to assess the proportions of neurons under each condition with strong nuclear staining of phospho-cJun (Ser 63). *** indicates that mean value is significantly different from control ($p<0.0008$); *** indicates that mean value is significantly different from $A_{1\text{-}42}$ ($p<0.001$) by ANOVA, Bonferroni post-hoc test (N=3). Scale bar = 20 μm.

(D) Caspase-2 is required for elevated nuclear localization of phospho-cJun in sympathetic neurons in response to NGF deprivation. Sympathetic cultures were treated for 6 hrs with or

without anti-NGF in the presence or absence of Pen1-siCaspase-2 (80 nM). Phospho-cJun (Ser 63) was visualized by immunocytochemistry using a PerkinElmer spinning disc confocal microscope, 60X magnification. Representative images are shown for each condition. Blinded counts were carried out to assess the proportions of neurons under each condition with strong nuclear staining of phospho-cJun (Ser 63). *** indicates that mean value is significantly different from control ($p<0.0008$); *** indicates that mean value is significantly different from -NGF ($p<0.001$) by ANOVA, Bonferroni post-hoc test (N=3). Scale bar = 20 μm.

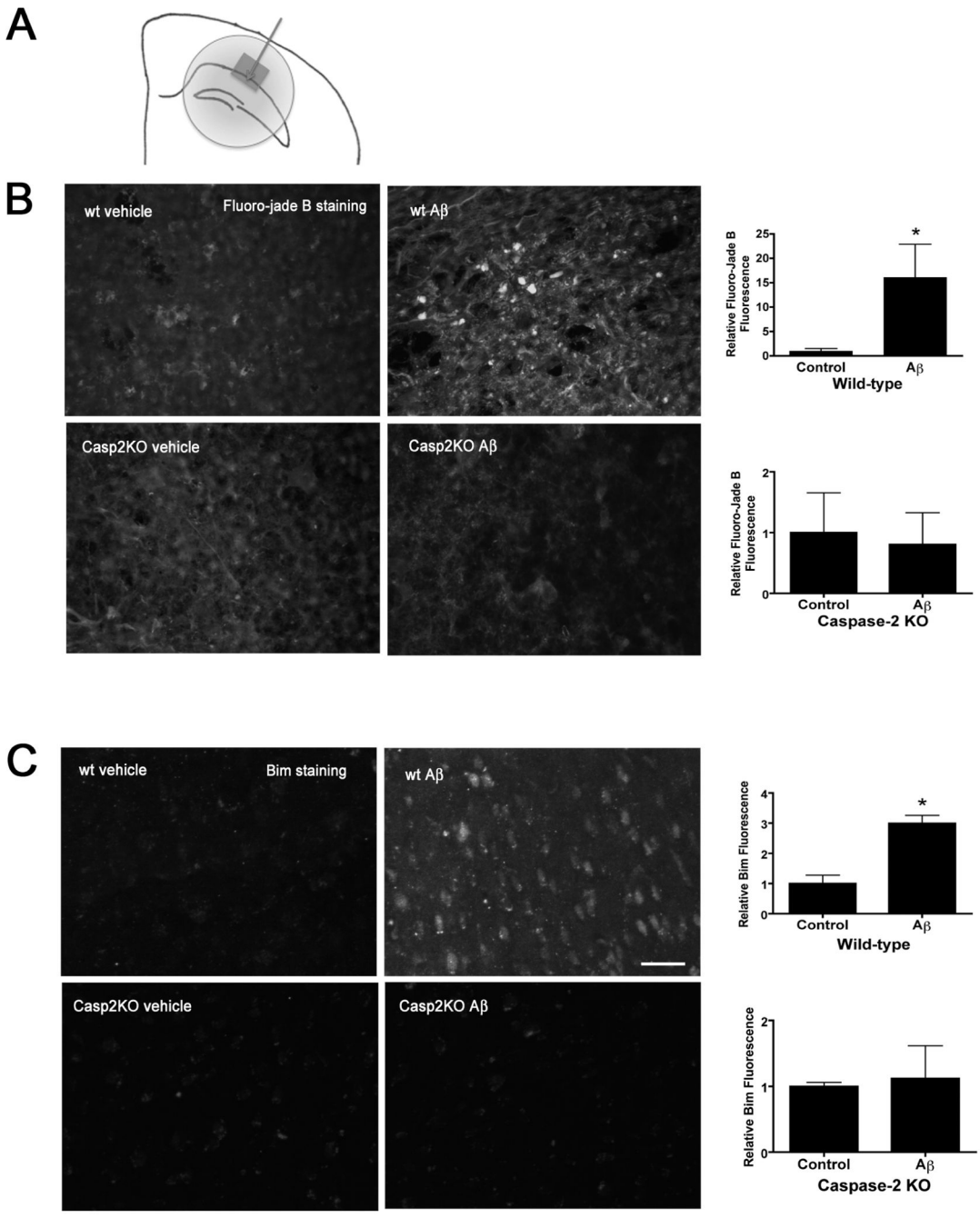

Figure 7. Caspase-2 is required for Bim induction in an *in vivo* model of AD pathology

$A\beta_{1\text{-}42}$ (0.4 nmoles) or vehicle was infused into the right hippocampi of 16-month-old wild-type (n=8 mice, 4 vehicle, 4 $A\beta_{1\text{-}42}$) and caspase-2 null mice (n=8, 4 vehicle, 4 $A\beta_{1\text{-}42}$). 2 weeks later the animals were sacrificed and the brains processed for immunohistochemistry. **(A)** Schematic indicating where $A\beta_{1\text{-}42}$ was delivered (arrow indicates site of delivery) and approximate spread, based on staining with an antibody specific for oligomerized $A\beta_{1\text{-}42}$, of $A\beta_{1\text{-}42}$ in the brain. The rectangle indicates the area imaged for B and C.

(B) Fluoro-Jade B staining. Coronal sections from each cohort of animals were stained with Fluoro-Jade B and imaged with a PerkinElmer spinning disc confocal microscope, 40X

magnification. 4 animals were treated per condition, representative images are shown. Adjacent graphs show quantification of relative fluorescence intensity normalized to area, using ImageJ, for each genotype and treatment, * indicates that mean value is significantly different from control ($p<0.05$), N=4.

(C) Bim immunostaining. Sections adjacent to those used for B were immunostained for Bim and imaged with a PerkinElmer spinning disc confocal microscope, 40X magnification. 4 animals were treated per condition, representative images are shown. Adjacent graphs show quantification of relative fluorescence intensity normalized to area, using Image J, for each genotype and treatment, * indicates that mean value is significantly different from control ($p<0.05$), N=4.

Scale bar = 20 μm.

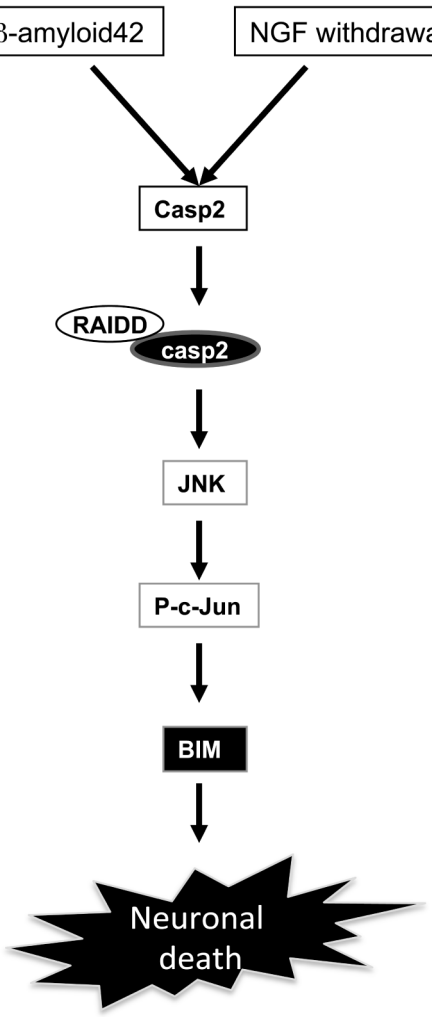

Figure 8.
Caspase-2 death signaling pathway