E2F1 Mediates Death of B-amyloid-treated Cortical Neurons in a Manner Independent of p53 and Dependent on Bax and Caspase 3

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Although B-amyloid (AB) is suggested to play an important role in Alzheimer’s disease, the mechanisms that control AB-evoked toxicity are unclear. We demonstrated previously that the cell cycle-related cyclin-dependent kinase 4/6/retinoblastoma protein pathway is required for AB-mediated death. However, the downstream target(s) of this pathway are unknown. We show here that neurons lacking E2F1, a transcription factor regulated by the retinoblastoma protein, are significantly protected from death evoked by AB. Moreover, p53 deficiency does not protect neurons from death, indicating that E2F1-mediated death occurs independent of p53. Neurons protected by E2F1 deficiency have reduced Bax-dependent caspase 3-like activity. However, protection afforded by E2F1, Bax, or caspase 3 deficiency is transient. In the case of E2F1, but not with Bax or caspase 3 deficiency, delayed death is accompanied by DEVDAFC cleavage activity. Taken together, these results demonstrate the required role of AB1, Bax, and caspase 3 in AB evoked death, but also suggest the participation of elements independent of these apoptosis regulators.

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by memory loss and cognitive impairment (1). Although the etiology of AD is not fully understood, an increasing body of evidence suggests the importance of B-amyloid in the initiation/progression of the disease. B-amyloid (AB), a 39–43-amino acid peptide, assembles into insoluble aggregates forming plaques characteristic of AD (2, 3). AB is formed by alternative proteolysis from an integral membrane-localized B-amyloid precursor protein (4), and its importance in AD is underscored by the identification of mutations in B-amyloid precursor protein in some familial cases of AD (5, 6). In addition, studies with AB1 in vitro cultured neurons treated with toxic forms of aggregated AB protein (7–9) as well as in vivo studies utilizing transgenic mice expressing AB (10) demonstrated neuronal loss by an apoptotic pathway. However, the mechanism by which AB causes neuronal apoptosis is not well understood.

Recent evidence has suggested that cell cycle molecules that normally control cell proliferation play an important required role in some forms of neuronal death. Of particular relevance to AD, numerous reports demonstrate abnormal up-regulation of a variety of cell cycle proteins/activity in brains from AD patients. These include cyclin B, D, and E, cdc2, and Cdk4 (11–13). In addition, phosphoepitopes, including phosphorylated tau, common to both mitotic cells and degenerating AD neurons, have been described (12). In support of the potential importance of cell cycle in AD, we have shown that flavopiridol, a pharmacological inhibitor of Cdns, as well as expression of dominant negative Cdk4/6 but not 2 or 3, protects cultured cortical neurons from death evoked by AB (14). This evidence supports the idea that Cdk4/6 activation plays an obligatory role in death of neurons evoked by AB.

These previous observations raise the question of the relevant target of Cdk4/6 in neurons and whether the activity of such targets is required for neuronal death. The only known substrates for Cdk4/6 are members of the retinoblastoma protein (pRb) family (15–17). Although the function of Rb family members is likely complex, its role in cell cycle regulation by interaction with E2F family members is best characterized (18). In this regard, phosphorylation of Rb family members by Cdns results in release and activation of the transcription factor E2F, which then activates genes required for S phase transition. Transcriptional activation by E2F requires association with its obligate binding partner DP1/2 (19–21). Interestingly, the role of Rb and E2F in control of apoptosis has also been suggested. In numerous cases, expression of Rb is protective against death (22–24), whereas expression of E2F1 has been shown to evoke death (25, 26). In addition, Rb null mice show neurological deficits and neuronal apoptosis (23, 27, 29). Finally, we reported previously that Rb becomes phosphorylated during AB-evoked death and that expression of dominant negative DP1 is neuroprotective (14). Taken together, these results suggest that E2F family members may play a required role in death of neurons evoked by AB.

Accordingly, we examined whether E2F1 deficiency is protective against AB-evoked death. In addition, we explored the potential role of three common apoptotic elements, the tumor suppressor p53, the BCL2 family member Bax, and the cytokine protease caspase 3, and determined how E2F1 may be ordered in relation to these apoptotic elements. We show here that E2F1 transiently protects cortical neurons from AB-evoked death in a p53-independent manner. In addition, we show that E2F1 acts upstream of
Bax-dependent caspase 3 activation required for acute, but not delayed, neuronal death.

**EXPERIMENTAL PROCEDURES**

**Materials**

Flavopiridol ([L86–8275, ([–] cis-5,7-dihydroxy-2-(2-chlorophenyl)-8-(3-hydroxy-1-methyl)piperidinyl)-4H-benzopyran-4-one]) was a generous gift from Dr. Peter J. Worland (National Cancer Institute). Camptothecin was obtained from Sigma Chemical Co. AB (1–40) was a generous gift from Dr. Peter J. Worland (National Cancer Institute). BAF and DEVD-AFC were purchased from Enzyme Systems Products (Dublin, CA).

**Knockout Mice**

p53, Bax, and caspase 3-deficient (C57BL/6 background) neurons were obtained from embryos derived from heterozygous pairings. E2F1 (B6/129 background)-deficient neurons were obtained from embryos of E2F1 (−/−) × E2F1 (−/−) breedings. For each experiment, neurons obtained from heterozygote or wild type littermate embryos were used as controls. Genotyping of each individual embryo was performed by PCR as follows.

p53 was genotyped using GATCTTGAGAAGGCAGAC (O-p53–1) and TGTACTTGAGTGATGTTGG (O-p53–2) primers to detect the wild type allele (450 bp) and TATACCTCGACGGCGCT (O-p53–X7) and TCTCTGGTGTTCAGGTTATC (O-neo-2) primers to detect the targeted allele (535 bp). PCR conditions are as follows: 94 °C, 5 min (1 cycle); 94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min (30 cycles); 72 °C, 10 min.

E2F1 was genotyped using GGATAGTTCTGAGCCTTCTGG (E2F1–5′), CTAAATGACCAACCAAGG (E2F1–3′), and CAAGTGCCAGCGGGCTGCTAAAG (PGKB) primers. The primers were used in one PCR to amplify an untargeted 170-bp fragment and a targeted 230-bp fragment. PCR conditions are as follows: 94 °C, 1 min (1 cycle); 94 °C, 5 s; 92 °C, 4 s; 55 °C, 1 min; 72 °C, 2 min (30 cycles); 72 °C, 5 min.

Bax was genotyped using GTTGGCATCTTGAGG (BaxIN5R), CGGTCTCCATGTCTCAGCCG (NEOR), and TACGATGCGACCATCATG (BaxEX5F) primers. BaxIN5R and BaxEX5F primers were used to amplify a 304-bp fragment from the wild type Bax allele. BaxIN5R and NEOR were used to amplify a 504-bp fragment from the targeted Bax allele. PCR conditions are as follows: 94 °C, 5 min (1 cycle); 94 °C, 1 min; 62 °C, 1 min; 72 °C, 1.5 min (30 cycles); 72 °C, 7 min.

Caspase 3 was genotyped using GTGGCATCTTCTGCTTGAGG (LZ1), ATGAAAGGCGCAGCATGACT (F108), and CTAAGTTAAAACAAATGACCGTCCGA (WCPR21) primers. F108 and WCPR21 were used to amplify a 1,733-bp fragment from the wild type caspase 3 allele. F108 and LZ1 were used to amplify a 1,605-bp fragment from the targeted caspase 3 allele. PCR conditions are as follows: 94 °C, 2 min (1 cycle); 94 °C, 30 s; 60 °C, 1 min; 72 °C, 1 min (30 cycles); 72 °C, 5 min.

**Culture and Survival of Cortical Neurons**

Mouse cortical neurons were cultured from embryonic day 16 mice as described previously (30). For the knockout studies, each individual embryo was dissected and plated individually. The neurons were plated into 24-well dishes (approximately 200,000 cells/well) coated with poly-L-lysine (100 μg/ml) in serum-free medium (N2: Dulbecco’s modified Eagle’s medium (1:1) supplemented with 6 mg/ml n-glucose, 100 μg/ml transferrin, 25 μg/ml insulin, 20 nM progesterone, 60 μM putrescine, 30 nM selenium). Under these conditions, cultures typically contain more than 99% neurons as assessed by staining with antibody directed against phosphorylated neurofilament (RT97, Developmental Studies Hybridoma Bank, University of Iowa). 2–3 days after initial plating, the medium was exchanged with serum-free medium supplemented with preaggregated AB (50 μg/ml), or camptothecin (10 μM) and flavopiridol (1 μM) where appropriate. AB was preaggregated by incubation in serum-free medium at a concentration of 0.2 mg/ml at 37 °C overnight. At appropriate times of culture under the conditions described, cells were lysed, and the numbers of viable cells were evaluated as described previously (30). Briefly, cells were lysed in 200 μl of cell lysis buffer (0.1 × phosphate-buffered saline, pH 7.4 containing 0.5% Triton X-100, 2 mM MgCl2, and cetlyldimethylhexylammonium bromide (0.5 g/100 ml)), which disrupts cells but leaves the nuclei intact. Numbers of healthy nuclei were evaluated by phase microscopy. Nuclei that displayed characteristics of blebbing, disruption of nuclear membrane,
phase bright apoptotic bodies, and chromatin margination were excluded. All experimental points are expressed as a percentage of cells present at day of treatment and are reported as mean ± S.E. (n = 3).

**Western Blot Analyses**

Cortical neurons were dissociated and cultured as described above. Neurons were harvested, and 10 μg of protein was loaded onto SDS-polyacrylamide gels and transferred onto nitrocellulose membrane. Blots were probed with anti-Bax (Santa Cruz), anti-p53 (Pharmingen), or anti-actin (Sigma) antibodies as indicated.

**TUNEL Analyses**

TUNEL analyses were performed using the Roche Molecular Biochemicals kit. Briefly, cortical neurons were fixed in 1% glutaraldehyde for 15 min. The cells were permeabilized in 1:1 methanol:aceton for 10 min. After washing with phosphate-buffered saline, the cells were incubated with Hoechst 33258 (0.5 ng/ml) in phosphate-buffered saline for 10 min. TUNEL labeling was performed per the manufacturer’s instructions.

**Caspase Activity**

Cortical neurons were harvested at the indicated times for caspase activity. Briefly, cells were washed three times in phosphate-buffered saline and collected in caspase lysis buffer as described previously (31). The cells were then incubated on ice for 20 min and sonicated briefly for 3 s. The extract was then centrifuged for 15 min at 12,000 rpm on an Eppendorf tabletop centrifuge. The supernatant was then collected and assayed for protein by Bradford (Bio-Rad), and 5 μg of protein was incubated with DEVD-AFC. The increase in fluorescence was measured using a fluorometer (400 nm excitation, 505 nm emission) as described previously (31).

**Reverse Transcription-PCR**

Total RNA was extracted from rat brains using TRizol reagent (Life Technologies, Inc.). First strand cDNAs were reverse transcribed from 4 μg of total RNA. The same amounts of cDNAs were subsequently used for PCR amplification for a total of 25 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min of each cycle. These optimal amplification conditions and cycle numbers were determined experimentally to ensure specific and linear signal generation, and expression of B-actin mRNA was used as a standard to quantify the relative amount of cRNA expression of E2F1 as described previously (32). The mouse specific E2F1 PCR primers (GACTGTGACTTTGGG ACC and TGTTCCATTTCACTTCCC) and B-actin primers (AACACCACACCATGACATGAG and GTGTGGGCATAGAGGTCTTTACGG) were used to generate an E2F1 PCR product of 434 bp and B-actin fragment of 509 bp. The PCR products were fractionated on a 2% agarose gel, photographed, and quantitated by scanning laser densitometry. The authenticity of the PCR products was confirmed by cloning into a pCR2.1 vector (Invitrogen) and sequencing using an automated Applied Biosystems 373A DNA sequencer.

**RESULTS**

**Role of E2F1 in Neuronal Death—** Our previous results implicated the Cdk4/6/Rb pathway as an obligate component for death of cortical neurons evoked by AB (14). Our finding that dominant negative DP1 also protected neurons from death suggested that E2F members may play a required role in signaling death under these circumstances. Accordingly, we examined whether E2F1, the E2F member most associated with pro-apoptotic activity, plays a role in death of cortical neurons exposed to AB. As shown in Fig. 1A, treatment with preaggregated AB results in approximately 60% death of embryonic cortical neurons cultured from mice heterozygous deficient for E2F1 (+/+) at 24 h and 80% death of neurons at 48 h. In contrast, significant protection is observed in AB-treated neurons cultured from E2F1 knockout (-/-) littermates (approximately 75% survival after a 24-h AB treatment). This protection is transient and reduced, but significant protection is still observed at 48 h (40% versus 20% in the controls). As shown in Fig. 2, more healthy neurons with intact processes are present in cultures of E2F1 (−/−) neurons exposed to AB than in identically treated control cultures that mainly contain apoptotic bodies and degenerating neuritic processes.

Death of cortical neurons evoked by AB treatment is accompanied by an increase in caspase 3-like activity as reflected by DEVD-AFC cleavage activity, which peaks 12–24 h after AB treatment (Fig. 1B). As shown in Fig. 1B, DEVD-AFC cleavage activity in AB-treated E2F1 (−/−) neurons is significantly lower than in E2F1 (+/+) neurons 12 and 24 h after AB treatment. As with survival, however, the differences in DEVD-AFC cleavage activity in E2F1 (−/−) neurons and E2F1 (+/−) controls diminish 48 h after AB treatment. The decrease in DEVD-AFC cleavage activity observed in AB-treated E2F1 (−/−) neurons places E2F1 involvement biochemically upstream of caspase 3-like activity. It is important to emphasize, however, that although a reduction in DEVD-AFC cleavage activity is observed in the E2F1 knockouts, it is not eliminated; only a 50–55% reduction in DEVD-AFC cleavage activity is observed at 12 and 24 h. Strikingly, inhibition of death by the Cdk inhibitor flavopiridol results in neuroprotection (14) accompanied by nearly complete reduction in DEVD-AFC cleavage activity 18 h after AB treatment (data not shown). These data suggest that Cdk activation after AB treat-
ment activates both E2F1-dependent and -independent pathways of caspase activation. Finally, an increase in E2F1 transcript levels, as measured by reverse transcription-PCR, was observed 3 h after AB treatment (Fig. 3). Because E2F1 is known to regulate its own transcription (16), this evidence

**Fig. 4.** p53 (−/−) neurons are not resistant to death and do not show reduced caspase 3-like activity when exposed to AB. Cortical neurons from p53 (−/−) or p53 (−/+ E16 embryos were treated with AB (50 μg/ml) or as a positive control, camptothecin (10 μM). Panel A, viability was assessed by nuclear counts. Each point is the mean ± S.E. of data from three cultures. Similar results were obtained in at least three independent trials. * indicates significance (p < 0.01, as derived from Student’s t test) compared with p53 (−/−) neurons treated with camptothecin for 12 h. Panel B, DEVD-AFC cleavage activity was assessed as described under “Experimental Procedures.” Each data point is the mean ± S.E. of data from three cultures, each from independent embryos. * and ** indicate significance (p < 0.02, as derived from Student’s t test) compared with survival of caspase 3 (−/−) cultures at 24 h.

**Fig. 5.** AB-treated caspase 3 (−/−) neurons are resistant to death and have no caspase 3-like activity compared with control cultures. Cortical neurons from caspase 3 (−/−), caspase 3 (−/+), or caspase 3 (+/+) E16 embryos were treated with AB (50 μg/ml). Panel A, viability was assessed by nuclear counts of caspase 3 (−/−) (filled circles), caspase 3 (−/+)(open diamonds), or caspase 3 (+/+)(open squares) neurons. Each point is the mean ± S.E. of data from three cultures. Similar results were obtained in at least three independent trials. * indicates significance (p < 0.02 as derived from Student’s t test) compared with survival of caspase 3 (−/−) cultures at 24 h. Panel B, DEVD-AFC cleavage activity was assessed as described under “Experimental Procedures.” Each data point is the mean ± S.E. of data from three cultures, each from independent embryos. * and ** indicate significance (p < 0.02, as derived from Student’s t test) compared with caspase 3 (−/−) neurons treated with AB for 24 and 48 h, respectively.
provides further support for the role of E2F1 in death. Taken together, these observations indicate that E2F1 plays an important role in neuronal death and caspase-3-like activation. However, it shows that signals downstream of Cdk activity other than E2F1 also participate in death evoked by AB.

Role of p53 in Cortical Neuronal Death—Previous studies demonstrated that E2F-mediated apoptosis can be p53-dependent or -independent (25, 33, 34). Accordingly, we examined whether p53 deficiency would lead to protection from AB-evoked death. As shown in Fig. 4A, cortices cultured from p53−/− embryos are not significantly protected from AB treatment compared with littermate controls. Similarly, no difference in cleavage activity is observed in p53−/− neurons treated with AB (Fig. 4B). As a control, p53-deficient cultures treated with the DNA-damaging agent camptothecin showed dramatic protection from death and no DEVD-AFC activity by the DNA-damaging agent camptothecin as reported previously (Fig. 4A). Finally, p53 protein was undetectable in AB-treated cortical neurons (data not shown). These results demonstrate that the actions of E2F1 in AB-evoked neuronal death occur in a p53-independent manner.

Role of Caspase 3 Death of Neurons Evoked by AB—Our present results indicated that E2F1 acts upstream of DEVD-AFC cleavage reflective of caspase-3-like activity. This observation led to the question of whether caspase-3-like activity was required for AB-induced neuron death. As shown in Fig. 5A, caspase 3−/− neurons are resistant to AB-evoked death (60% survival in caspase 3−/− neurons versus 35% in the controls at 24 h). Neurons from caspase 3−/− or caspase 3−/+ neurons show low background levels of TUNEL labeling and healthy nuclei as observed by Hoechst staining. In contrast, numerous caspase 3−/+ neurons but not caspase 3−/− neurons treated with AB for 24 h show bright TUNEL labeling and condensed shrunken nuclei as observed by Hoechst. AB-treated neurons from caspase 3−/− mice also lack DEVD-AFC cleavage activity compared with littermate controls (Fig. 5B). This indicates that the DEVD-AFC cleavage activity observed upon AB treatment is caused by caspase 3 or caspases downstream of caspase 3. As with E2F1 deficiency, the protection observed in caspase 3−/− neurons is transient, and no significant protection is observed at 48 h after AB treatment. Interestingly, prolonged survival is not observed in...
AB-treated neurons cotreated with the general caspase inhibitor BAF (data not shown). These results suggest that caspase 3 is functionally important for AB-induced neuronal death. In addition, the delayed death that occurs in caspase 3-deficient neurons occurs in a manner independent of caspases.

Role of Bax in Death of Neurons Evoked by AB—How is caspase 3 activated in cortical neurons treated with AB? We show here that Bax is absolutely required for this process. Unlike with E2F1-deficient neurons, AB-treated Bax (−/−) neurons show no induction of DEVD-AFC cleavage activity even after 48 h of treatment when nearly 50% of the neurons have died (Fig. 7). Interestingly, Bax (−/−) neurons lack even background levels of DEVD-AFC cleavage activity normally observed in neurons from control littermates (Fig. 7). These protected neurons display healthy soma and intact processes (Fig. 8). However, just as with protection afforded by E2F1 deficiency, protection from AB-evoked death in Bax (−/−) neurons is transient and diminishes at 48 h (approximately 50% survival versus 20% survival in the controls). Finally, although Bax is required for death evoked by AB, no increase in Bax levels is observed with AB treatment (Fig. 9), demonstrating that its involvement is not caused by general changes in protein levels.

Our previous findings indicated that delayed death of cortical neurons evoked by DNA damage in the presence of general caspase inhibitors is not accompanied by nuclear manifestations characteristic of apoptosis (36). Accordingly, we examined whether nuclear morphology would be altered during delayed death of Bax (−/−) neurons evoked by AB treatment. As shown in Fig. 10, AB treatment of Bax (−/+) neurons results in increasing percentage of condensed (60% at 48 h) and fragmented nuclei (27% at 48 h) present in neuronal cultures. During delayed death of Bax (−/−) neurons, however, fragmented nuclei are rarely detected (2% at 48 h). Condensed nuclei, however, are abundant (70% at 48 h). In contrast, delayed death of E2F1-deficient neurons is accompanied by significant nuclear fragmentation (Fig. 10C). These results demonstrate that delayed death of Bax (−/−) neurons is morphologically different from that observed in E2F1-deficient and control cultures and suggest that the mechanism of delayed death in Bax- and E2F1-deficient neurons differs.

Taken together our results suggest that E2F1, in a p53-independent manner, acts upstream of bax mediated caspase 3 activation and that this pathway contributes to the death of cortical neurons evoked by AB. However, delayed death which occurs in the absence or Bax/caspase 3 occurs independently of this signaling pathway.

**Fig. 8.** Phase-contrast micrographs of Bax (−/+)(panels A and B) or Bax (−/−)(panels C and D) cortical neurons untreated (panels A and C) or treated with AB (panels B and D) for 24 h.

**Fig. 9.** Induction of Bax protein levels is not observed during AB treatment. Western blot analyses of cortical neurons treated with AB are shown.

**DISCUSSION**

Although recent evidence suggests the importance of AB in AD-related neuronal death, the signaling mechanisms controlling this event are unclear. Previous results had indicated that Cdk activation and consequent phosphorylation and inactivation of pRb may signal for death (14). To delineate further the Cdk/Rb pathway, we explored the requirement for E2F1, a transcription factor regulated by pRb, and examined the potential involvement/relationship of this pathway with the select apoptotic elements p53, Bax, and caspase 3.

**Role of E2F1 in Neuronal Death Evoked by AB—**We show here that neurons deficient in E2F1 are transiently protected from death evoked by AB. The finding that E2F1 transcripts increase during AB-induced neuronal death is consistent with this result. In addition, these results are in accordance with observations that E2F1 expression itself is sufficient to induce death of cortical neurons in the absence of any apoptotic stimuli and that expression of dominant negative DP1 protects neurons from AB (14). Taken together, along with findings that Cdk4/6 activity is required for death of AB-treated cortical neurons, these results suggest a model by which Cdk4/6 activation, consequent phosphorylation/inactivation of pRb, and activation of E2F1-containing complexes contribute to the death signal evoked by AB treatment. Most importantly, our results suggest that the up-regulation of cell cycle signals reported in AD brains such as cyclin D1 and Cdk4 (11–13) plays a functional role in signaling of neuronal death.

Several lines of evidence indicate that E2F1 does not act alone to mediate cell cycle involvement in this paradigm, however. First, protection afforded by E2F1 deficiency is only transient. Second, inhibition of caspase activity in E2F1 (−/−) AB-treated cortical neurons is incomplete. Third, in contrast, the Cdk inhibitor flavopiridol almost completely inhibits DEVD-AFC cleavage activity. These results suggest that multiple proteins, including E2F1, mediate the cell cycle-related death signal and indicate the existence of other Rb-regulated death signaling pathways or functional redundancies with other E2F members. Pertinent to this hypothesis is the observation that Rb members interact with numerous other proteins. For example, Rb binds to and modulates activity of signaling molecules such as c-Abl, a tyrosine kinase thought to be required for death evoked by DNA damage (37, 38). In addition, multiple E2F members are reported to mediate death (39, 40).

The manner in which E2F1 promotes apoptosis is not clear. Although it is thought that E2F5s may regulate transcription of “death genes,” such candidates have not been reported. In addition, overexpression of transactivation mutants of E2F1 can still induce apoptosis (41). As well, previous work in proliferating cells has suggested that E2F1-mediated death occurs via p53-dependent and -independent mechanisms (25, 33, 34).

In the former case, it is thought that E2F1 up-regulates p19 ARF which then stabilizes p53 levels, possibly through a direct or indirect function of MDM2 (42, 43). Interestingly, our data indicate that p53 is not required for the death of cortical neurons evoked by AB. This is in contrast to other models of

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neuronal death, such as DNA damage, where p53 plays a critical role in promoting neuronal apoptosis (44). These results also demonstrate that the requirement of p53 differs with different death stimuli and that E2F1 involvement in AB-evoked neuronal death is a p53-independent process. It is currently unknown whether additional p53 family members serve to regulate this death paradigm.

Role of Caspase 3 and Bax in Neuronal Death by AB—Our results with E2F1-deficient neurons indicate that E2F1 acts prior to Bax-dependent caspase 3-like activation. Importantly, both Bax- and caspase 3-deficient neurons are protected from death evoked by AB treatment. As is the case for E2F1-deficient neurons, however, protection afforded by caspase 3 deficiency is transient. Unlike with E2F1 deficiency, the delayed death observed in Bax- and/or caspase 3-deficient neurons occurs in the absence of further DEVD-AFC cleavage activity and nuclear fragmentation, suggesting a differing mechanism of delayed death. The lack of long term protection in E2F1-deficient neurons is likely because of incomplete caspase inhibition. The observation of nuclear fragmentation that accompanies delayed death in E2F1-deficient neurons supports this hypothesis. In contrast, the mechanism of Bax/caspase 3-independent delayed death is likely complex. Bax translocation to the mitochondria has been reported in many cell types including neurons and is thought to lead to mitochondrial dysfunction and release of cytochrome c (45–47). It is presumed

Bax (−/−) neurons (A–C) or Bax (−/−) neurons (D–F) were untreated (A and D) or treated with AB for 24 h (B and E) or 48 h (C and F). Neurons were fixed, stained with Hoechst, and visualized by fluorescence. Panels B and C, quantification of healthy (stippled), condensed (hatched), and fragmented (black) nuclei in Bax (panel B) or E2F1 cultures (panel C) was assessed by counting three random fields/culture (minimum of 100 cells counted/culture). Each data point is mean ± S.E. of data from three cultures, each from separate embryos.
that such translocation is a requirement for caspase activation through the Apaf1-caspase 9 complex (28, 48). Our results are consistent with this model. Accordingly, we hypothesized previously that mitochondrial dysfunction after Bax-mediated cytochrome c release was critical for caspase-independent death (36). In support of this, we have demonstrated previously that cytochrome c release is not inhibited in dying caspase 3-deficient neurons or neurons treated with general caspase inhibitors (36, 49). However, our current results indicate that delayed death occurs even in the absence of Bax. These results suggest mitochondrial dysfunction independent of Bax or a mitochondrial-dependent form of delayed death evoked by AB. In contrast, Bax deficiency leads to significantly longer protection in neurons deprived of trophic support or exposed to DNA damage (35, 44). Although the reason for this is unclear, it suggests that the presence of several alternative death pathways that depend upon the death insult.

Taken together, the results presented here provide a plausible mechanism by which AB evokes death of cortical neurons. In this model (see Fig. 11), AB treatment evokes cyclin D1/Cdk4/6 activity and Rb phosphorylation and inactivation. As a consequence, multiple factors that interact with Rb members, including E2F1, act through Bax to activate caspases, including caspase 3. Activation of the caspases then leads to apoptotic death of neurons. Inhibition of E2F1, Bax, or caspases leads to transient protection but ultimately results in delayed death (although perhaps through differing mechanisms). The delayed death observed in E2F1-deficient neurons may likely occur as a result of other Rb-mediated signals resulting in caspase activation and death. Inhibition of Bax, however, leads to a pathway of delayed death independent of caspase 3-like activity.

Finally, our observations of delayed neuronal death have important implications for the development of neuroprotective strategies for the treatment of chronic neurodegenerative conditions. Neuroprotective regimens that target early death signaling events activated during AB treatment, such as Cdk activation, may be more effective than inhibition of more downstream molecules, such as E2F1, Bax, or caspases. The latter targets may be less effective in maintaining longer term survival and functional integrity because of the presence of alternative death pathways.

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