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Letter to the Editor

The proapoptotic proteins Bax and Bak are not involved in Wallerian degeneration

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Dear Editor,

Neurons appear to have at least two self-destruct programs.[1] In common with other types of cell, they have an intracellular suicide program responsible for apoptosis that is triggered when they are damaged, infected, or present in greater numbers than required. Additionally, they appear to have a second, molecularly distinct, self-destruct program in their axons,[2] which can be activated locally, in response to transection for example. Activation following transection leads to rapid degeneration of the isolated part of the cut axon, a process termed Wallerian degeneration (WD).[3] At present, it is unclear whether any of the regulatory proteins of the apoptotic pathway play a role in regulating WD or other types of localized axonal degeneration. Here, we show that two proteins, Bax and Bak, which are known to be central to the activation of most forms of apoptosis,[4] are not involved in WD.

During vertebrate development, many types of neurons are produced in excess and then culled by apoptosis. In addition, some developing neurons produce excess axonal branches that are subsequently lost, in some cases at least, by degeneration.[1] Both neuronal apoptosis and localized axonal degeneration have also been implicated in neurological disorders.[1] While genes that encode the major mediators (caspases) and regulators (Bcl-2 family members) of apoptosis have been implicated in normal neuronal cell death, it is less clear whether the same genes are involved in the localized axonal degeneration associated with either development or disease. In some studies, localized axonal degeneration has been reported to be associated with caspase activation and/or to be blocked by caspase inhibitors.[5] In other studies, however, the axonal degeneration caused by transection (WD) or local trophic factor withdrawal was not associated with caspase activation and was not blocked by caspase inhibitors.[2] Here, we show that the Bcl-2 family proteins Bax and Bak, proteins that play a critical role in activating many forms of apoptosis,[4] are not involved in WD.

Proteins of the Bcl-2 family regulate the cell intrinsic pathway of apoptosis.[6] Antiapoptotic family members such as Bcl-2 and Bcl-X_L maintain the normal function of the mitochondrial outer membrane and prevent cells from dying.[6] When activated, the proapoptotic family members Bax and Bak alter the mitochondrial outer membrane and release cytochrome *c* and other proteins from the intermembrane space that help initiate the caspase cascade and contribute to apoptosis in other ways.[6] Mice lacking both Bax and Bak (*Bax*$^{-/-}$*Bak*$^{-/-}$) have reduced normal cell death, and most die around birth.[4]

Mitochondria are important components of axons, as high levels of ATP production are required to maintain the membrane potential and allow the propagation of action potentials. To test the possibility that mitochondria might contribute to localized axonal degeneration through a Bax/Bak-dependant mechanism, we studied WD in cultured explants of sciatic and optic nerves isolated from *Bax*$^{-/-}$ *Bak*$^{-/-}$ mice. For comparison, we also studied WD in the

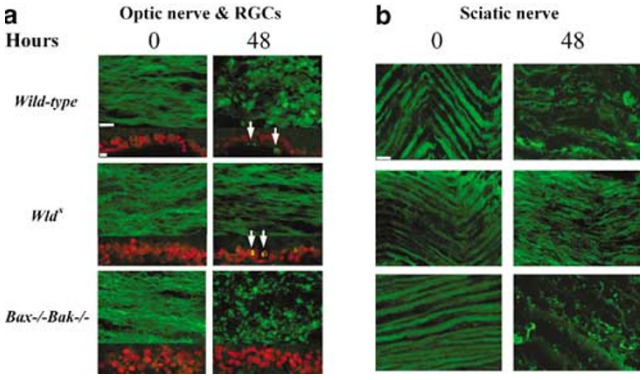

Figure 1 Fluorescence micrographs of frozen sections of explants of optic nerve and retina **(a)** or sciatic nerve **(b)** after 2 d in culture. At least three explants from different mice were examined in each case with similar results. The *Bax*$^{-/-}$ *Bak*$^{-/-}$ explants were compared with wild-type explants from the same litter or from mice of the same age or with *Wld*^{s} explants from mice of the same age. The explant culture method has been described in detail previously.[2] Briefly, optic nerves, sciatic nerves, and retinas were dissected and either fixed immediately in 2% paraformaldehyde in PBS for 4 h at 4°C or placed in sterile Millipore culture plate inserts (Millicell-CM, 0.4 μm 12 mm diameter, Millipore Corporation, Bedford, MA 01730, USA) and cultured in six-well culture plates at 37°C. Culture medium consisted of Neurobasal A, supplemented with B27 (GIBCO BRL, Life Technologies Ltd, Paisley, UK) and penicillin/streptomycin/amphotericin B (Sigma, UK). Explanted tissue was cultured for up to 48 h before being fixed as above. Explants were then cryoprotected in 30% sucrose/PBS before freezing in a 1 : 1 mixture of 30% sucrose/PBS : OCT (TissueTek, Sakura, Netherlands), sectioning at 15 μm, immunolabeling, and imaging in a Biorad Radiance scanning confocal microscope (Biorad, Hercules, CA, USA). In **(a)**, mice were 14–21 days old (although similar results were obtained from a *Bax*$^{-/-}$*Bak*$^{-/-}$ animal examined on the day of birth). A longitudinal section of optic nerve is shown at the top of each panel, and a cross section of the RGC layer of the corresponding retina is shown at the bottom. Retinal cell nuclei were labeled with propidium iodide (red) and apoptotic nuclei (green) were labeled by the TUNEL technique[8] (Apoptag, Intergen, Oxford, UK). Neurofilaments in the axons of the optic nerves were labeled with a mixture of rabbit antineurofilament antibodies (NA1297, Affiniti Research Ltd, Exeter, UK), followed by fluorescein-conjugated goat anti-rabbit immunoglobulin antibodies (Jackson ImmunoResearch, Pennsylvania, USA), as previously described.[2] Note that apoptotic RGCs (arrows) are present in both the wild-type and *Wld*^{s} retinal explants but not in the *Bax*$^{-/-}$ *Bak*$^{-/-}$ retinal explants, while fragmented neurofilaments are present in the wild-type and *Bax*$^{-/-}$*Bak*$^{-/-}$ optic nerve explants but not in *Wld*^{s} optic nerve explants. In **(b)**, mice were 1–2 months old, and the neurofilaments in longitudinal sections of sciatic nerve explants were labeled as in **(a)**. The results are similar to those in the optic nerve explants in **(a)**. Scale bars =10 μm

Wallerian Degeneration Slow (*Wld*^{s}) mutant mouse, a naturally occurring mutant in which WD is greatly slowed.[7]

As shown in Figure 1, within 2 days, sciatic and optic nerve axons in explants from wild-type mice underwent WD (as assessed by the breakdown of neurofilaments), and retinal ganglion cells (RGCs) in retinal explants underwent apoptosis (as assessed by the TUNEL assay).[8] In explants from *Bax*$^{-/-}$*Bak*$^{-/-}$ mice, the sciatic and optic nerve axons also underwent WD within 2 days, but the RGCs in corresponding retinal explants were completely protected from apoptosis. By contrast, in explants from *Wld*^{s} mice, the sciatic and optic nerve axons were protected from WD, whereas the RGCs were not protected from apoptosis. Thus, *Bax*$^{-/-}$*Bak*$^{-/-}$ RGCs are protected from apoptosis but not from WD, whereas the reverse was the case for *Wld*^{s} RGCs.

These results indicate that WD does not depend on Bax or Bak, distinguishing it from most forms of apoptosis and some nonapoptotic forms of cell death.[9] Thus, the mechanism of WD, like that of other forms of localized axonal degeneration, remains as mysterious as ever.

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