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Deficiency in *Bim*, *Bid* and *Bbc3* (*Puma*) do not prevent axonal injury induced death

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Dear Editor,

BAX activation is a critical step in the apoptotic pathway in neurons. BAX activation is multistep process that can be driven by a subset of other pro-apoptotic Bcl-2 family members, specifically the BH3-only proteins BIM, BBC3 (PUMA), and BID.[1] In fact BAX activation appears to require at least one of these pro-apoptotic chaperones during development and after many apoptotic insults.[2] Retinal ganglion cells (RGCs) die by a BAX-dependent mechanism in development and after axonal injury,[3] a key insult in many neurodegenerations. During development BAX-dependent death of RGCs requires BBC3 (Harder and Libby[4] and Supplementary Figure 1A); however, the molecules controlling BAX activation after axonal injury are unknown. We have previously shown that single deficiency in *Bim* or *Bbc3* lessen early RGC death after axonal injury.[4,5] However, by 5 days after injury, RGC death is no longer different in *Bbc3*-deficient mice and there is a significant number of apoptotic RGCs in *Bim*-deficient mice (Supplementary Figure 1B). These data show that both BIM and BBC3 are involved in BAX activation in axonally injured RGCs, but are not solely responsible for RGC death.

As BIM and BBC3 appear to regulate BAX activation in axonally injured RGCs, we tested whether deficiency in both *Bim* and *Bbc3* phenocopied *Bax* deficiency and prevented RGC loss after axonal injury. The amount of protection in *Bim Bbc3* double knockouts was additive and significant compared with wildtype and single mutants as judged by the number of RGCs surviving 21 days after injury (Supplementary Figure 1C). However, apoptotic cell death persisted in the *Bim Bbc3* double mutants after axonal injury, which led to significant RGC loss over time ($P<0.001$; Supplementary Figure 1C). BID has been implicated in neuronal death after many types of insults;[2] therefore, it is possible that it is contributing to RGC death in the absence of BIM and BBC3. *Bid* deficiency alone did not alter the rate or amount of RGC death after injury (Supplementary Figures 1B and C). *Bid* deficiency was also not additive in the context of *Bim* deficiency or *Bbc3* deficiency (Supplementary Figures 1B and C). In order to test whether removing the major direct activators of BAX was necessary to prevent RGC loss, mice deficient in *Bim*, *Bbc3,* and *Bid* were generated. Triple deficiency did not prevent RGC death after axonal injury and did not increase the long term survival of RGCs compared with the protection of *Bim Bbc3* combined deficiency (64 ± 3 and 61 ± 4% of control, respectively; Supplementary Figure 1C). However, triple deficiency did reduce the rate of RGC death compared with *Bim Bbc3* double-deficient animals suggesting that BID can have a role in RGC death after axonal injury (Supplementary Figure 1B). Thus, in RGCs axonal injury induces cell death independently of BIM, BBC3, and BID despite the requirement for BAX.

RGC death in the absence of BIM, BBC3, and BID is consistent with observations suggesting that BAX (and BAK) could be activated independently of these BH3-only proteins.[6] BH3-only proteins with a lower affinity for BAX have been implicated in neuronal death, such as HRK, BMF, and NOXA after neuronal trauma.[7] These molecules may be less efficient at killing cells due to their primary function of antagonizing pro-survival Bcl-2 family members. Interestingly, lowering levels of pro-survival Bcl-2 family members alone can lead to apoptosis.[8] Necroptotic cell death is also dependent on BAX[9] and observed in injured neurons, but may not require BIM, BBC3, and BID. Finally, it is possible that BAX activation could be occurring independently of the Bcl-2 family (e.g. Chipuk *et al.*[10]). Therefore, further defining the mechanism of BAX activation may be important in understanding cell death in neuronal degenerations or after neuronal trauma.

Conflict of Interest

The authors declare no conflict of interest.

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