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Short Communication

Programmed cell death of retinal cone bipolar cells is independent of afferent or target control

Patrick W. Keeley [a], Nils R. Madsen [a], Ace J. St. John [a], Benjamin E. Reese [a,b,*]

[a] Neuroscience Research Institute, University of California at Santa Barbara, Santa Barbara, CA 93106-5060, USA
[b] Department of Psychological and Brain Sciences, University of California at Santa Barbara, Santa Barbara, CA 93106-9660, USA

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ABSTRACT

Programmed cell death contributes to the histogenesis of the nervous system, and is believed to be modulated through the sustaining effects of afferents and targets during the period of synaptogenesis. Cone bipolar cells undergo programmed cell death during development, and we confirm that the numbers of three different types are increased when the pro-apoptotic *Bax* gene is knocked out. When their cone afferents are selectively eliminated, or when the population of retinal ganglion cells is increased, however, cone bipolar cell number remains unchanged. Programmed cell death of the cone bipolar cell populations, therefore, may be modulated cell-intrinsically rather than via interactions with these synaptic partners.

Introduction

Like many regions within the developing central nervous system, the retina undergoes a period of programmed cell death during histogenesis. In the mouse retina, apoptotic cells are found prenatally in the early neuroblastic layer and within the developing nuclear layers as these emerge postnatally (Péquignot et al., 2003; Young, 1984). The latter wave of programmed cell death has been associated with the period of synaptogenesis as the various types of retinal neurons establish their connectivity with afferents and targets, and may indicate an intercellular dependency with those synaptic partners (Linden, 2000; Linden et al., 2005). This programmed cell death is modulated by the Bcl-2 family of regulatory proteins controlling apoptosis, for when the pro-apoptotic gene, *Bax*, is knocked out, the frequency of dying cells during development is decreased (Mosinger-Ogilvie et al., 1998; Péquignot et al., 2003), and in maturity, the retinal architecture is conspicuously altered. Retinal cell populations in both the ganglion cell layer (GCL) and the inner nuclear layer (INL) are increased in the *Bax* knockout retina, although the outer nuclear layer (ONL) is unaffected (Lee et al., 2011), and comparable effects have been described when the anti-apoptotic *Bcl-2* gene is overexpressed (Strettoi and Volpini, 2002a). The increase in the size of the ganglion cell population is substantial, showing more than a doubling in number (Bonfanti et al., 1996; Mosinger-Ogilvie et al., 1998).

Despite the increase in the thickness of the INL, the specific populations affected and the relative magnitude of rescue for each have gone largely unexplored. Neurons within the INL include the populations of horizontal cells, amacrine cells and bipolar cells. The horizontal cells are not believed to undergo programmed cell death, their numbers being stable during development (Mayordomo, 2001; Raven et al., 2005), and nor are their numbers increased in the *Bcl-2* overexpressing retina (Strettoi and Volpini, 2002a). Some amacrine cell types have been shown to undergo *Bax*-mediated cell death (Whitney et al., 2009), whereas others are unaffected (Whitney et al., 2008). Amongst bipolar cell types, only the rod bipolar cell population, being the largest of all bipolar cell classes, has been shown to be increased, in the *Bcl-2* overexpressing mouse retina (Strettoi and Volpini, 2002a).

Using the *Bax* knockout retina, the present investigation examined three different types of cone bipolar cell (CBC) to ascertain whether other types of bipolar cell exhibit increased cell numbers, and to determine the relative magnitude of programmed cell death impacting each. One attraction of working with CBC populations is that they extend their dendritic endings to receive afferent innervation from the same population of cone photoreceptor terminals in the outer plexiform layer (OPL), while their axons extend into the inner plexiform layer (IPL) where they innervate the dendrites of retinal ganglion cells (Breuninger et al., 2011). We have,

* Corresponding author at: Neuroscience Research Institute, University of California, Santa Barbara CA 93106-5060, USA.
E-mail address: breese@psych.ucsb.edu (B.E. Reese).

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consequently, examined whether programmed cell death amongst CBCs can be modulated by genetically altering the size of these populations of afferents or targets during development.

Materials and methods

Bax knockout mice (*Bax* KO), coneless mutant mice (CL), and *Bax* conditional knockout mice (carrying two floxed *Bax* alleles and a *Brn3b*-cre transgene; *Bax* CKO), and their littermate controls, were sampled at two months of age in wholemount preparations, in order to estimate the total size of three different OFF-CBC types: the Type 2, Type 3b, and Type 4 cells. These three cell types were chosen because there are antibodies available that reliably label these respective populations (Keeley et al., 2014). All mice were given a lethal injection of Euthasol (120 mg/kg sodium pentobarbital, i.p.), and then intracardially perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4 at 20 °C).

Retinas were dissected from the eyes and prepared as wholemounts. Fields were sampled in all four quadrants using an Olympus FV1000 laser scanning confocal microscope with a 40× or 60× oil immersion objective. Retinal quadrants were sampled at both central and peripheral locations (near the optic nerve head and the retinal circumference, respectively), using a field size of either 39,980 or 25,192 sq μm in area, with one exception, in which the Type 2 CBCs in the control and *Bax* KO retinas were sampled only at a single mid-eccentric location in each quadrant (mid-way between the optic nerve head and retinal circumference), using a field size of 28,547 sq μm in area. Because each CBC antibody labels different portions of its respective CBC most efficiently, the axonal stalk was counted for the Type 2 cells, the dendritic stalk as it emerges from the soma for the Type 3b cells, and the soma itself for the Type 4 cells, counting only those lying within the sample field or intersecting the upper or left boundaries. Average densities were determined for each retina, and then multiplied by retinal area to estimate total number. The total number of Brn3b+ RGCs was also determined in *Bax* CKO and control retinas, sampling fields of 25,192 sq μm in area in the center and the periphery of all four retinal quadrants using the Olympus FV1000 laser scanning confocal microscope with a 40× oil immersion objective. The total number of cone opsin+ photoreceptors was determined in the coneless mutant retina and in littermate control retinas by sampling the retina at 1 mm intervals in a square lattice across the entire retina, sampling fields of 10,000 sq μm in area using a Nikon Microphot FXA fluorescence microscope equipped with a 60× oil immersion objective and an Olympus DP11 digital camera. These wholemounts had been briefly post-fixed in 2% glutaraldehyde:2% paraformaldehyde to preserve the structural integrity of the inner segments without compromising immuno-detection of the cone opsin in the outer segments. All counting was conducted blind to condition (with the exception of the cone photoreceptors in the coneless and littermate control retinas, because this difference was obvious). One eye was sampled from each mouse, the n's being indicated in the histograms in each figure. Retinal sections, cut at 150 μm on a Vibratome, were also labeled to reveal the retinal architecture and plexiform layers using Hoechst 33342 and antibodies to CtBP2, being double-immunolabeled to reveal the distribution of cone photoreceptors or Brn3b+ ganglion cells in respective retinas, imaged using the Olympus FV1000 laser scanning confocal microscope. These confocal images were processed for brightness and contrast in Adobe Photoshop CS5. Details of the primary antibodies used in this study are all indicated in Table 1. Student's *t*-test was used for all comparisons, using a p value of <0.05 for statistical significance, indicated by an asterisk in the figures.

Results

The *Bax* knockout retina in maturity shows a substantial increase in the thickness of the INL and GCL (Fig. 1A), without affecting the thickness of the ONL nor the number of cone photoreceptors therein, as recently described (Lee et al., 2011). By labeling the Type 2, Type 3b or Type 4 CBC types, every one of these three cell types was found to be significantly increased in the *Bax* KO retina (Fig. 1B, C, D), although to differing extents. The increase in population size was greatest in the Type 2 CBCs, showing a 64% increase (Fig. 1B; $p=6.8\times10^{-6}$), while the Type 3b cells were increased by 50% (Fig. 1C; $p=1.5\times10^{-3}$). The Type 4 population, by contrast, was only increased by 15% (Fig. 1D; $p=4.7\times10^{-2}$). Preventing *Bax*-mediated cell death during development, therefore, yields increased numbers of all three types of CBCs examined, as has been described for the Type 7 CBCs using a transgenic reporter line (Lee et al., 2011), but as with the other constituents of the INL, varies in magnitude depending upon the cell type.

These three bipolar cell types extend their dendrites to innervate the population of cone pedicles in the OPL, including those of both the M cones and the UV cones (Breuninger et al., 2011). We asked whether CBC number was modulated by the presence of these afferents (Fig. 2A, left), by determining bipolar cell number in the coneless transgenic mouse retina (Soucy et al., 1998). We have confirmed our previous demonstration (Raven and Reese, 2003) that the population of cone photoreceptors is nearly completely eliminated in this coneless mutant retina (Fig. 2B; $p=9.2\times10^{-7}$), due to the expression of an attenuated diphtheria toxin transgene in the cone photoreceptors,

Table 1
Primary antibodies used in the present study.

Antigen	Structure labeled	Immunogen	Type	Supplier	Dilution
C-terminal-binding protein 2 (CtBP2)	Ribbon synapses	Residues 361–445 of mouse CtBP2	Mouse monoclonal	BD Transduction (612044)	1:500
Synaptotagmin-2 (Syt2)	Type 2 cone bipolar cells	Homogenized whole zebrafish	Mouse monoclonal	ZIRC (ZDB-ATB-081002-25)	1:100
cAMP-dependent protein kinase type II-beta regulatory subunit (PKARIIβ)	Type 3b cone bipolar cells	Residues 1–418 of human PKARIIβ	Mouse monoclonal	BD Transduction (610625)	1:1000
Calsenilin (Csen)	Type 4 cone bipolar cells	Full length human Calsenilin/DREAM	Mouse monoclonal	Millipore (05–756)	1:1000
Medium-wave-sensitive opsin 1 (M-cone opsin)	Cone outer segments	Synthetic peptides from mouse green opsin and zebra finch red opsin	Rabbit polyclonal	Millipore (AB5745)	1:1000
Short-wave-sensitive opsin 1 (S-cone opsin)	Cone outer segments	Recombinant human blue opsin	Rabbit polyclonal	Millipore (AB5407)	1:1000
Arrestin-C (mCAR)	Cones	Synthetic linear peptide	Rabbit polyclonal	Millipore (AB15282)	1:5000
Brain-specific homeobox/POU domain transcription factor 3B (Brn3b)	Retinal ganglion cells	C-terminus of human Brn-3b	Goat polyclonal	Santa Cruz Biotechnology (SC-6026)	1:250