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BCL2L1 (BCL-X) promotes survival of adult and developing retinal ganglion cells ☆

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ABSTRACT

The Bcl-2 family is responsible for regulating cell death pathways in neurons during development, after injury and in disease. The activation of the pro-death family member BAX is often the final step before cell death in neurons. Pro-survival family members such as BCL-X (BCL2L1) act to inhibit BAX activation. Overexpression studies have suggested that BCL-X could play an important physiological role in mediating neuronal viability. Loss-of-function studies performed *in vivo* have implicated BCL-X as a mediator of neuronal survival during the early stages of neurodevelopment. To assess whether BCL-X is needed to promote the survival of neurons in the central nervous system throughout life, *Bcl-x* was conditionally removed from the optic cup or throughout the adult mouse. During development BCL-X was required for the survival of differentiating retinal ganglion cells (RGCs) leading up to their normal window of developmental death. Despite its expression in adult RGCs, BCL-X was not required for maintaining RGC viability in adult retinas. However, the loss of BCL-X in adult RGCs did significantly increase the rate of death of RGCs after axonal injury. Thus, in developing and injured RGCs there appears to be an active cell survival program preventing neuronal death.

Introduction

The Bcl-2 family of genes mediates the intrinsic pathway of apoptosis, which significantly contributes to neuronal death during development, after injury, and in disease. For instance, the pro-death Bcl-2 family member BAX is required for retinal ganglion cell (RGC) death during development, after acute axonal injury, and in ocular hypertensive glaucoma (Li et al., 2000; Libby et al., 2005; Mosinger Ogilvie et al., 1998; Qin et al., 2004; White et al., 1998). BAX activation is controlled by the opposing actions of pro-death and pro-survival members of the Bcl-2 family. During development and after injury RGC apoptosis requires upstream pro-death Bcl-2 family members (Harder and Libby, 2011; McKernan and Cotter, 2007). The physiological role of the pro-survival Bcl-2 family members is less well understood than their pro-death counterparts. Importantly, while *Bcl2* was shown to not have a role in maintaining RGC survival after axonal injury (Dietz et al., 2001), it does help maintain RGC viability in maturing RGCs (Cellerino et al., 1999). Thus, pro-survival Bcl-2 family members can play critical roles in maintaining RGC viability.

There are five pro-survival members of the Bcl-2 family (*Bcl2*, *Bcl-x*, *Bcl-w*, *Bcl2a1a*, and *Mcl1*). Their main function is to prevent BAX (or BAK1) activation and, therefore, cell death (Puthalakath and Strasser, 2002; Strasser, 2005; Willis et al., 2005, 2007). Most of the pro-survival Bcl-2 family members have important roles in neuronal development (Arbour et al., 2008; Crosio et al., 2006; Lukiw et al., 2005; Middleton et al., 2001; Mori et al., 2004; Motoyama et al., 1995; Shacka and Roth, 2006). In fact, antagonization of pro-survival Bcl-2 family members with small molecule inhibitors is enough to trigger neuronal death *in vitro* (Young et al., 2010). BCL-X has been specifically implicated as an important pro-survival factor in neuronal development and disease. Germline deletion of *Bcl-x* leads to death of neurons in the developing central nervous system and embryonic lethality (Motoyama et al., 1995). Conditional deletion of *Bcl-x* in dopaminergic neurons showed that *Bcl-x* is required for the survival of all but a few catecholaminergic cells in the developing substantia nigra (Savitt et al., 2005). Numerous neuroprotective treatments are reported to increase the intracellular ratio of BCL-X to pro-apoptotic members (Kilic et al., 2005; Koh, 2009; Ma et al., 2005; Pike, 1999; Wang et al., 2000) and in injured neurons overexpressing BCL-X can increase survival and sustain neuronal function (Garrity-Moses et al., 2005; Parsadanian et al., 1998; Wiessner et al., 1999). In RGCs *Bcl-x* transcript and protein expression are regulated after injury (Isenmann et al., 1997; Levin et al., 1997; McKernan and Cotter, 2007; Pelzel et al., 2010) and overexpression of BCL-X or BCL2 protects RGCs after axonal injury (Bonfanti et al., 1996; Cenni et al.,

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1996; Chierzi et al., 1999; Malik et al., 2005). Together these studies suggest that BCL-X may play a necessary physiological role in maintaining survival of adult and developing neurons. However, despite the importance of apoptotic cell death during development and in disease, to date there is limited knowledge of how critical physiological levels of pro-survival Bcl-2 family members are in maintaining neuronal survival throughout life (Isenmann et al., 2003). To test the function of an endogenous pro-survival Bcl-2 family member in the central nervous system, the role of *Bcl-x* (*Bcl2l1*) was assessed in developing and adult RGCs *in vivo*.

Results

Immature RGCs require BCL-X for survival

BCL-X is expressed throughout the neuroblastic retina (Fig. 1). At E12.5, RGCs are being born at the ventricular surface (VS) and migrating to the presumptive RGC layer at the inner surface of the retina. BCL-X and the RGC marker βIII-tubulin (TUJ1; Cui et al., 2003) are coexpressed in the GCL at E12.5 and E18.5. The expression pattern of BCL-X indicates it may affect RGC birth and survival. Since germline deletion of *Bcl-x* results in embryonic lethality (Motoyama et al., 1995; Savitt et al., 2005), Six3-cre was used to delete a floxed allele of *Bcl-x* (*Bcl-x^{f}*) from the developing optic cup. RGCs are among the earliest retinal neurons to get specified, represent a large proportion of the first wave of differentiation (Gan et al., 1999), and undergo a significant amount of programmed cell death during development (Mosinger Ogilvie et al., 1998). Based on POU4F2 immunolabeling (an early marker of RGC differentiation; Gan et al., 1999), *Bcl-x* deletion does not alter RGC generation at E12.5 (Figs. 2A,B). Also, the loss of BCL-X does not significantly increase cell death at E12.5, suggesting that both retinal progenitors and newly born RGCs do not require BCL-X for survival (Fig. 2C). Substantial naturally occurring developmental death of RGCs begins around E18.5 (Pequignot et al., 2003). However, without BCL-X large numbers of RGCs are prematurely lost between E12.5 and E18.5. During this time period, ectopic CASP3 activation and thinning of the retina indicate cell death is coincident with the loss of RGCs (Figs. 2C,D). These data indicate that in differentiating RGCs BCL-X is required to prevent apoptotic cell death.

In addition, other types of retinal neurons appear to be susceptible to apoptotic death in the absence of *Bcl-x*. At E18.5, the few surviving RGCs are primarily cells in which *Bcl-x* was not deleted (Fig. 2A arrow), which is consistent with known mosaic expression of Six3-cre, particularly in the retinal margin (Cai et al., 2011; Fuhrmann et al., 2009; Poche et al., 2008). Thus, the large increase in cell death in *Bcl-x$^{f/f}$* Six3-cre^{+} retinas at E18.5 is likely also associated with abnormal cell death of other types of retinal neurons.

The increase in cell death during development produced a smaller adult retina in the *Bcl-x$^{f/f}$* Six3-cre^{+} mice compared to wild type. The *Bcl-x* knockout retina was reduced in thickness (Fig. 3A; *Bcl-x$^{+/?}$* Six3-cre$^{?}$ 180 ± 7 μm, *Bcl-x$^{f/f}$* Six3-cre^{+} 96 ± 7 μm; $P<0.001$, $N=5$ for each genotype) and surface area (Fig. 3B; *Bcl-x$^{+/?}$* Six3-cre$^{?}$ 18 ± 1 mm^2, *Bcl-x$^{f/f}$* Six3-cre^{+} 13 ± 2 mm^2; $P<0.001$, $N=5$ for each genotype). The *Bcl-x$^{f/f}$* Six3-cre^{+} retina consisted of all major cell types, albeit reduced in number, and retained normal gross morphology (Fig. 3A and data not shown). In wild type adult retinas all RGCs express BCL-X, as determined by colabeling with the RGC marker TUJ1 (Fig. 3C). In 6 out of 6 *Bcl-x* knockout retinas all surviving RGCs expressed BCL-X (the Six3-cre is not a complete retinal deleter (Cai et al., 2011; Fuhrmann et al., 2009; Poche et al., 2008)). These results indicate that RGCs require BCL-X for survival during development and suggest that the survival of RGCs in the *Bcl-x$^{f/f}$* Six3-cre retinas is the result of incomplete deletion of *Bcl-x* in the developing retina.

Bcl-x is not required for survival of adult RGCs

The continued expression of BCL-X in adult RGCs and its role as a required survival factor during development raise the question of whether adult RGC viability is also dependent on BCL-X. To address the importance of BCL-X in adult RGCs, *Bcl-x^{f}* was removed in adult mice using a ubiquitously expressed, tamoxifen inducible cre-recombinase (Cre-ER™). Tamoxifen-induced recombination was highly effective, reducing the number of BCL-X positive cells in the RGC layer to 2% of control by 15 days following treatment (Fig. 4A). Loss of BCL-X produced no noticeable change in retinal architecture and did not induce immediate cell death (Figs. 4B, 5A,B) or signs of glial activation (data not shown). In fact, two months after *Bcl-x* deletion there was still normal retinal architecture (Fig. 4B) and normal numbers of RGCs (Fig. 4C).

BCL-X is a pro-survival factor in injured RGCs

Adult RGCs die by apoptotic pathways regulated by the Bcl-2 family after axonal injury (either mechanical or glaucomatous axonal injury; Bahr, 2000; Isenmann et al., 2003; Li et al., 2000; Libby et al., 2005). This cell death involves activation of BAX by other pro-death Bcl-2 family members. Over-expression of BCL-X can protect against cell death following axonal injury (Malik et al., 2005), but it is unknown whether endogenous BCL-X prevents death in injured adult neurons. After acute axonal injury there is near immediate pro-death injury

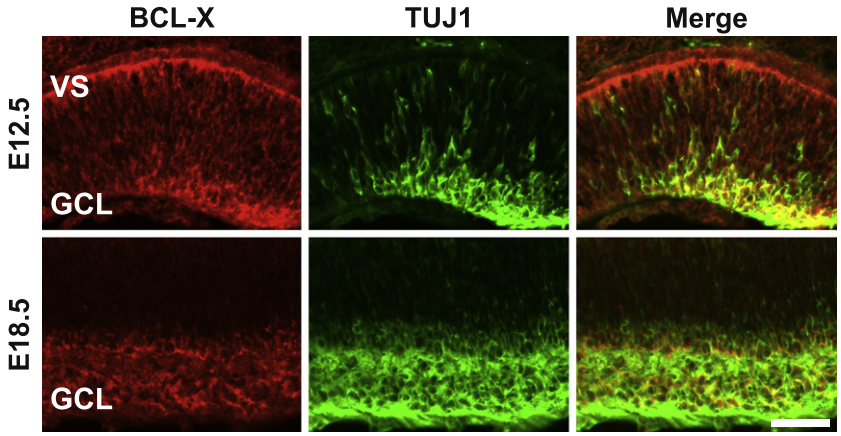

Fig. 1. BCL-X is expressed in differentiating RGCs. BCL-X (red) is expressed by differentiating RGCs (TUJ1+, green) in the retina. At E12.5, RGCs are being born at the ventricular surface (VS) and migrating to the presumptive RGC layer (GCL). BCL-X and TUJ1 are coexpressed (yellow) in the GCL at both E12.5 and E18.5. 4 retinas were examined for each genotype and time point.