Bcl-xL dependency coincides with the onset of neurogenesis in the developing mammalian spinal cord

Lauren C. Fogarty, Beibei Song [1], Yegappan Suppiah, S.M. Mahmud Hasan, Hiliary C. Martin, Sarah E. Hogan, Jieying Xiong, Jacqueline L. Vanderluit *

Division of BioMedical Sciences, Memorial University, 300 Prince Philip Drive, St. John's, NL, Canada, A1B 3V6

ABSTRACT

The *bcl-2* family of survival and death promoting proteins play a key role in regulating cell numbers during nervous system development. Bcl-x$_L$, an anti-apoptotic *bcl-2* family member is highly expressed in the developing nervous system. However; the early embryonic lethality of the *bcl-x* germline null mouse precluded an investigation into its role in nervous system development. To identify the role of *bcl-x* in spinal cord neurogenesis, we generated a central nervous system-specific *bcl-x* conditional knockout (BKO) mouse. Apoptotic cell death in the BKO embryo was initially detected at embryonic day 11 (E11) in the ventrolateral aspect of the spinal cord corresponding to the location of motor neurons. Apoptosis reached its peak at E13 having spread across the ventral and into the dorsal spinal cord. By E18, the wave of apoptosis had passed and only a few apoptotic cells were observed. The duration and direction of spread of apoptosis across the spinal cord is consistent with the spatial and temporal sequence of neuronal differentiation. Motor neurons, the first neurons to become post mitotic in the spinal cord, were also the first apoptotic cells. As neurogenesis spread across the spinal cord, later born neuronal populations such as Lim2$^+$ interneurons were also affected. The onset of apoptosis occurred in cells that had exited the cell cycle within the previous 24 h and initiated neural differentiation as demonstrated by BrdU birthdating and βIII tubulin immunohistochemistry. This data demonstrates that spinal cord neurons become Bcl-x$_L$ dependent at an early post mitotic stage in developmental neurogenesis.

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1. Introduction

Apoptosis is an active form of cell death and an essential component of mammalian central nervous system (CNS) development. The number of neurons in the mature nervous system is determined by a balance between cell proliferation, differentiation and death. The pro- and anti-apoptotic proteins of the *bcl-2* family are key mediators of the apoptotic pathway (Vaux et al., 1988; Youle and Strasser, 2008). Of the pro-survival members, Bcl-x$_L$ plays a pivotal role in post-mitotic neuron survival during brain development. Germline *bcl-x* knockout mice exhibit extensive loss of immature neurons throughout the developing CNS (Motoyama et al., 1995). The confounding cell death in the hematopoietic system in germline *bcl-x* knockouts and the embryonic lethality in the early stages of neurogenesis at embryonic day 13 (E13) has precluded further analysis of the role of *bcl-x* in spinal cord development.

The mouse *bcl-x* gene encodes five distinct isoforms that are generated by alternative splicing transcript (Yang et al., 2002). In the developing mouse CNS, the anti-apoptotic *bcl-x long* (*bcl-x$_L$*) is the predominant isoform (Boise et al., 1993; Gonzalez-Garcia et al., 1994). Bcl-x$_L$ protein expression is first apparent in post-mitotic neurons within the hindbrain and spinal cord at E11, the beginning of the mouse neurogenic period that extends from E10 to E17 (Angevine and Sidman, 1961; Krajewska et al., 2002). By E13.5, Bcl-x$_L$ is widely expressed throughout the CNS in differentiating neurons (Frankowski et al., 1995; Krajewska et al., 2002). Its expression peaks from E13 to post-natal day five, suggesting that Bcl-x$_L$ may have a critical role in the differentiation and survival of maturing neurons (Krajewska et al., 2002). Bcl-x$_L$ protein is located in the mitochondrial outer membrane. Here, it inhibits cell death through interactions with pro-apoptotic Bcl-2 proteins to prevent pore formation in the mitochondrial outer membrane and block progression of the apoptotic cascade.

Germline knockout of the *bcl-x* gene results in early embryonic lethality and has previously limited our ability to assess its role *in vivo* (Motoyama et al., 1995). However, the development of conditional knockout models that restrict gene deletion to specific cell populations has become pivotal in the assessment of various Bcl-2 proteins during development. The necessity of Bcl-x$_L$ in the developing visual system was demonstrated using *bcl-x* conditional knockout models for both rod photoreceptors and retinal ganglion cells (Harder et al., 2012; Zheng et al., 2006). Similarly, conditional deletion of *bcl-x* in catecholaminergic neurons depleted this population by one-third (Savitt et al.,

2005). Importantly, the resulting apoptosis in the absence of *bcl-x* is cell autonomous. This was demonstrated in telencephalic neuronal cultures from *bcl-x* deficient mice which had similar cell death rates in both enriched media and serum-free media (Roth et al., 1996). While these studies indicate the importance of Bcl-x$_L$ as an anti-apoptotic regulator in specific neuronal populations, the role of Bcl-x$_L$ in development of the spinal cord and the cell populations impacted upon its removal have yet to be identified.

Here, we used a nervous system specific *bcl-x* conditional knockout mouse to investigate the role of endogenous Bcl-x$_L$ in embryonic spinal cord development.

2. Materials & Methods

2.1. Mice

Mice were housed on a 12 h light:dark cycle and provided with food and water *ad libitum*. Floxed *bcl-x* (*bcl-x$^{f/f}$*) mice were kindly provided by Dr. E. Rucker (Rucker et al., 2000; Wagner et al., 2000). LoxP sites flank exons 1 and 2 of the *bcl-x* gene and because exon 2 is common to all five *bcl-x* isoforms (Yang et al., 2002) cre-mediated excision effectively removes all five isoforms. To generate CNS-specific *bcl-x* conditional knockout mice (BKO), *bcl-x$^{f/f}$* mice were bred with *nestin:cre* (*Nes$^{cre/+}$*) transgenic mice (Arbour et al., 2008; Berube et al., 2005). Genotyping was performed according to standard protocols with primers for *bcl-x*, and *cre* (Arbour et al., 2008; Wagner et al., 2000). All mice were maintained on a C57Bl/6 background. Heterozygous (Het) (*Nes$^{cre/+}$:bcl-x$^{+/f}$*) and wild type controls (Ctl) (*bcl-x$^{+/f}$* or *Nes$^{cre/+}$* genotypes alone) littermates were used for comparison. During breeding, the observation of a vaginal plug was identified as embryonic day 0.5 (E0.5). All experiments were approved by Memorial University's Animal Care Ethics Committee, adhering to the guidelines set by the Canadian Council on Animal Care.

2.2. Tissue Fixation and Immunohistochemistry

At the time of sacrifice, pregnant dams were euthanized with an intraperitoneal injection of Euthanyl (250 mg/mL sodium pentobarbital-Vètoquinol, IEUS001, Lure cedex, France) followed by cervical dislocation. For the bromodeoxyuridine (BrdU) birthdating, pregnant dams received an intraperitoneal injection of BrdU (100 mg/kg, BrdU Sigma Chemical Co. B5002, Oakville, Ontario, Canada) at 2, 24 or 48 h prior to euthanasia. Following euthanasia of the dam, spinal cords and brainstems were dissected from the embryos, submersion fixed overnight in 4% PFA, and cryoprotected in 30% sucrose prior to freezing. Immunohistochemistry was performed on 14 μm sections collected from the spinal cord and brainstem of Ctl, Het and BKO embryos according to previously published protocols (Hasan et al., 2013; Malone et al., 2012). Primary antibodies included active Caspase-3 (AC3) (BD BioSciences, Cat# 559565, RRID:AB_397274), Bromodeoxyuridine (BrdU) (BD BioSciences, Cat# 347580, RRID:AB_400326), Bcl-xL (Cell Signaling Technology, Cat# 2764, RRID:AB_2228008), Glial fibrillary acidic protein (GFAP, Dako Cat# Z0334, RRID:AB_10013382), Ki67 (Abcam, Cat: Ab16667, RRID:AB_302459), Nestin (Millipore, Cat# MAB353, RRID:AB_94911), O4 (Millipore, Cat# MAB345, RRID:AB_11213138), Nkx6.1 (DSHB, Cat# F55A10, RRID_AB532378)(Pedersen et al., 2006), Lim1/2 (DSHB, Cat# 4F2, RRID:AB_531784), and β-tubulin isoform III (Millipore, Cat# 1637, RRID:AB_2210524) and were followed by the appropriate secondary antibodies anti-rabbit IgG tagged with Alexa Fluor 488 or 594 (Thermo Fisher Scientific, Cat# A21206, RRID:AB_2535792 and Cat# A21207, RRID:AB_141637) and anti-mouse IgG tagged with Alexa Fluor 488 or 594 (Thermo Fisher Scientific, Cat# A21202, RRID:AB_2535788 and Cat# A21203, RRID:AB_2535789). Double BrdU and AC3 immunohistochemistry was performed as previously described (Hasan et al., 2013). Terminal deoxynucleotide nick-end labelling (TUNEL) was performed according to previously published methods and immediately followed by Ki67 immunohistochemistry (Malone et al., 2012).

2.3. Western Blotting

Protein was extracted from dissected E12 brainstem and spinal cord tissue in lysis buffer. Western blotting was performed as previously described in (Vanderluit et al., 2004). Immunoblotting was performed using antibodies to Bcl-xL (Cell Signaling Technology, Cat# 2764, RRID:AB_228008) and β-actin (Sigma-Aldrich, Cat# A5316, RRID:AB_476743) followed by the secondary antibodies horse-radish peroxidase tagged anti-rabbit or anti-mouse (Bio-Rad, Cat# 1706515, RRID:AB_11125142 and Cat# 1706516, RRID:AB_11125547). Blots were developed by chemiluminescence using Western Lightning Plus-ECL (Perkin Elmer, NEL103001EA, Waltham, Massachusetts, United States) according to the manufacturer's instructions. Densitometry of Bcl-xL bands and the corresponding β-actin control bands was performed with ImageJ software according to the protocol outlined in the user guide (ImageJ, RRID:SCR_003070, http://imagej.nih.gov/ij/).

2.4. Microscopy, Cell Counts, Morphometric Measurements and Statistical Analysis

Tissue sections were examined on a Carl Zeiss AxioImager Z.1 (Munich, Germany) microscope with standard fluorescence and brightfield/darkfield settings at ×5 0,25 or ×20 0,50 NA objectives. Images were captured using a Zeiss AxioCam MRM3 camera with Zeiss AxioVision 4.8 software. Figures were compiled using Adobe Photoshop C2 (San Jose, California, United States). Manipulations of brightness and intensity were made equally to all treatment groups.

All cell counts were performed on 14 μm thick coronal sections through the spinal cord or brainstem with 140 μm between each alternating section, to prevent double counting cells. Motor neurons in the E18 spinal cord were quantified from 8 alternating cresyl violet stained sections through the lumbar spinal cord and the means analyzed by one-way ANOVA. Nkx6.1 positive motor neurons in the E15 lumbar spinal cord and E18 facial nucleus were counted on 2 alternating sections and the means analyzed by unpaired *t*-test and one way ANOVA, respectively. The total numbers of Lim2 positive interneurons versus total number of Hoechst positive nuclei were counted within a 139 μm by 139 μm-sized area in both the dorsal and ventral spinal cord. Apoptotic cells were defined as active Caspase-3 positive cells with completely condensed nuclei by Hoechst staining. The number of apoptotic cells and total Hoechst positive nuclei were counted in three representative sections per embryo (Ctl and BKO) at E13 and E15 within a 139 × 139 μm square area in the dorsal and ventral spinal. Means were analyzed by two-way ANOVA followed by Bonferroni post hoc analysis. The cross sectional area of the lumbar spinal cord was measured in two representative sections per embryo and the means analyzed by unpaired t-test. All statistics were performed with GraphPad Prism 5 (La Jolla, California, United States) software, including unpaired t-test and both one and two way analysis of variance (ANOVA). Tukey's post hoc (one-way) or Bonferroni post hoc (two-way) analysis was used to determine differences between genotypes.

3. Results

Due to the early embryonic lethality of germline *bcl-x* knockout mice at E13 (Motoyama et al., 1995), we generated a conditional knockout mouse to study the role of Bcl-x$_L$ in nervous system development. Transgenic mice expressing Cre recombinase from the *nestin* promoter (Arbour et al., 2008) were bred with *bcl-x$^{f/f}$* mice (Wagner et al., 2000) to produce a nervous system-specific *bcl-x* conditional knockout mouse (BKO). Nestin expression begins at E7.5 in neural precursors cells in the CNS (Lendahl et al., 1990), at which point Cre mediated recombination of the *bcl-x* gene occurs throughout the neural precursor