B-cell Lymphoma-2 (Bcl-2) is an Essential Regulator of Adult Hippocampal Neurogenesis

By

Maheen Ceizar

This thesis is submitted as a partial fulfillment of the M.Sc. program in Neuroscience

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

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ABSTRACT

Of the thousands of dividing progenitor cells (PCs) generated daily in the adult brain only a very small proportion survive to become mature neurons through the process of neurogenesis. Identification of the mechanisms that regulate cell death associated with neurogenesis would aid in harnessing the potential therapeutic value of PCs. Apoptosis, or programmed cell death, is suggested to regulate death of PCs in the adult brain as overexpression of B-cell lymphoma 2 (Bcl-2), an anti-apoptotic protein, enhances the survival of new neurons. To directly assess if Bcl-2 is a regulator of apoptosis in PCs, this study examined the outcome of removal of Bcl-2 from the developing PCs in the adult mouse brain. Retroviral mediated gene transfer of Cre into adult floxed Bcl-2 mice eliminated Bcl-2 from developing PCs and resulted in the complete absence of new neurons at 30 days post viral injection. Similarly, Bcl-2 removal through the use of nestin-induced conditional knockout mice resulted in reduced number of mature neurons. The function of Bcl-2 in the PCs was also dependent on Bcl-2-associated X (BAX) protein, as demonstrated by an increase in new neurons formed following viral-mediated removal of Bcl-2 in BAX knockout mice. Together these findings demonstrate that Bcl-2 is an essential regulator of neurogenesis in the adult hippocampus.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABC	Avidin-Biotin Complex
AC3	Activated Caspase 3
ANOVA	Analysis of Variance
APAF1	Apoptosis Activating Factor 1
BAD	Bcl-2 Associated Death Promoter
BAK	Bcl-2 Antagonist or Killer
BAX	Bcl-2 Associated X
Bcl-2	B-cell Lymphoma 2
Bcl-xl	B-cell Lymphoma-Extra Large
BID	BH3 Interacting-Down Death Agonist
BLBP	Brain Lipid-Binding Protein
BrdU	Bromodeoxyuridine
CNS	Central Nervous System
DAB	3,3'-Diaminobenzidinetetrahydrochloride
DAPI	4’,6-diamidino-2-phenylindole dihydrochloride
DCX	Doublecortin
DG	Dentate Gyrus
DNA	Deoxyribonucleic Acid
Dpi	Days Post Infection
FACS	Fluorescence Activated Cell Sorting
fBcl-2	Floxed Bcl-2
GABA	Gamma-Aminobutyric Acid
GCL	Granule Cell Layer

GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
H_20_2	Hydrogen Peroxide
HCl	Hydrogen Chloride
IAP	Inhibitors of Apoptosis Proteins
IFU	Infectious Units
IHC	Immunohistochemistry
Ip	Intraperitoneal Injection
KO	Knockout
Mcl-1	Myeloid Leukemia Cell Differentiation
MOMP	Mitochondrial Outer Member Permeabilization
NaN_3	Sodium Azide
NDS	Normal Donkey Serum
NeuN	Neuron Nuclei
NSE	Neuron Specific Enolase
OB	Olfactory Bulb
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCs	Precursor Cells
PET	Polyethylenimine
PSA-NCAM	Polysialyated-Neural Cell Adhesion Molecule
PUMA	P53 Upregulated Modulator of Apoptosis
RGC	Retinoic Ganglionic Cells
SEM	Standard Error of Mean

SGZ	Subgranular Zone
SOX2	Sex Determining Region Y-Box 2
SVZ	Subventricular Zone
TAM	Tamoxifen
TBS	Tris-Buffered Saline
TNF	Tumor Necrosis Factor
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
WT	Wild-Type
YFP	Yellow Fluorescent Protein

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1. INTRODUCTION

1.1 Adult Neurogenesis

The discovery that the adult human brain has the ability to generate new neurons (Eriksson et al., 1998) overturned the traditional view that neurons cannot form after embryonic development. Although discovered earlier in other species (Altman and Das, 1965; Paton and Nottebohm, 1984), the discovery of adult neurogenesis in humans generated a lot of attention and hope to identify novel therapies that could enhance adult neurogenesis as a treatment for disorders associated with neuronal loss, such as Alzheimer's disease. In the last decade, methodological enhancements in birth-dating and labeling of progenitor cells (PCs) through the use of Bromodeoxyuridine (BrdU) and histological markers has allowed visualization of PCs as they develop into neurons. These advances have allowed the field to make great progress in identifying the cellular mechanisms that underlie the dynamic process of adult neurogenesis. For example, there are many intrinsic cell signalling pathways and external regulators such as aging, that have been identified to alter the various stages of cell development including proliferation, migration, differentiation and integration of mature neurons into the neuronal circuitry (Alvarez-Buylla and Lim, 2004; Zhao et al., 2008; Ming and Song, 2011). Analogous to the elimination of cells during developmental neurogenesis many of new cells die during adult neurogenesis. One research question that remains is the cellular mechanism(s) that is responsible for the elimination of cells during adult neurogenesis. Although the

cells die during their development due to apoptosis, how and which apoptotic pathways are regulated has only begun to be identified. Identification of the mechanisms regulating PCs death needs to be dissected to harness the possible therapeutic potential of enhancing neurogenesis. This thesis addresses this need by examining the role of the anti-apoptotic protein, B-cell Lymphoma-2 (Bcl-2) in adult neurogenesis and defining at what stages during cellular development Bcl-2 is required for cell survival.

1.2 Progenitor Cell (PC) Development in the Hippocampus

The process of adult neurogenesis consists of a series of progressive developmental stages that are required for the formation of new neurons (Ming and Song, 2011). This process occurs in the adult brain in two discrete regions: (1) the subventricular zone (SVZ) of the lateral ventricles where mature neurons integrate into the olfactory bulb (OB) and (2) the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG), where mature neurons integrate into the granule cell layer (GCL) **(Figure 1)**. The identification of PCs in the adult brain within these two regions was first discovered through the use of tritiated thymidine which incorporates into the S phase of the cell cycle as PCs divide and can be revealed by autoradiography (Altman, 1963). This work was subsequently followed by the use of BrdU, a thymidine analog, to birth-date the PCs (Dayer et al., 2003), as well as immunohistochemical detection of endogenous proteins that have been identified to be transiently expressed in PCs during different developmental stages of neurogenesis. Although various terminologies have

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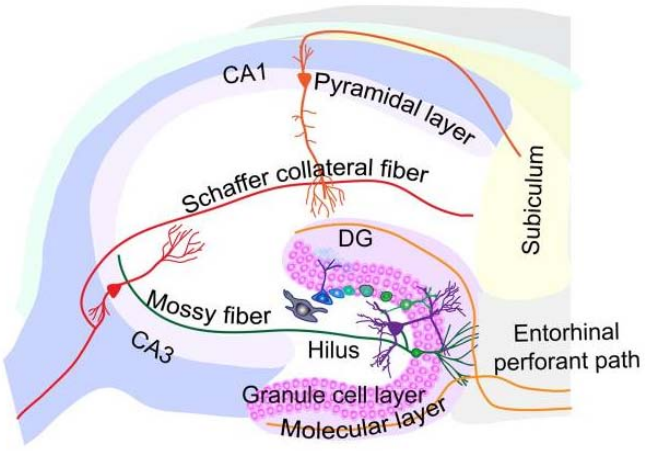

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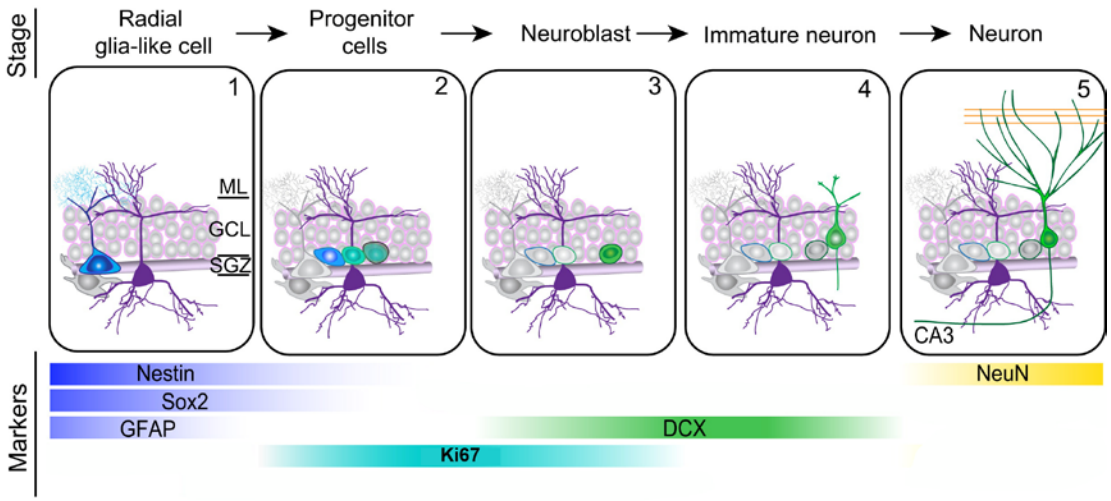


Modified from Ming and Song, 2011.

Figure 1. The Process of Adult Neurogenesis.

One region in which adult neurogenesis occurs is the **(A)** SGZ of the DG in which PCs can be identified **(B)** through their developmental stages and with phenotypic markers**.**

been used to classify PCs at different developmental stages, this thesis will utilize one of the most commonly used nomenclatures first proposed by Gerd Kempermann (Kempermann et al., 2004). As shown in **Figure 1**, in the SGZ this classification has the steps of cell maturation labeled from the type-1 stem cell to the newly formed mature neuron. Similarly, in the SVZ-RMS-OB pathway as cells mature they are labeled as type B which is most similar to type-1 cells in the SGZ and ends with mature neurons integrating into the OB (Alvarez-Buylla et al., 2001). The remainder of this thesis will be focused on the hippocampus; therefore, a more in depth discussion of the cells in this region is warranted.

The dividing PCs in the hippocampus originate from “type-1 cells” and are often referred as “stem” or “radial glia” cells. The type-1 cells have been labeled as stem cells since they posses properties of self-renewability and multipotency in a primary cell culture neurosphere assay (Palmer et al., 1997). However, whether the hippocampus has an inherent stem cell population is an unresolved debate that has arisen due to variability in methods to isolate the cells for *in vitro* analysis first established by Reynolds and Weiss (Reynolds, 1992; Seaberg and van der Kooy, 2002b; Bull and Bartlett, 2005). Unpublished data from our laboratory also supports that cells derived from the hippocampus do not display the self-renewability in culture, thus for the remainder of this thesis, these cells are called type-1 stem-like cells.

In vivo, the type-1 cells have a short tree-like process that projects into the GCL and a triangle shaped soma, as first identified through use of nestin-GFP

reporter mice (Yamaguchi et al., 2000). The radial glial phenotype of stem cells in development possess astrocyctic characteristics such as vascular end feet and expression of glial fibrillary acidic protein (GFAP) (Filippov, 2003). In addition to expressing nestin and GFAP, type-1 cells have also been identified to express brain lipid-binding protein (BLBP) and/or sex determining region Y- box 2 (SOX2) (Ming and Song, 2011). Although these endogenous proteins are expressed in these cells, these proteins are not exclusive to type-1 cells, and thus it is necessary to identify the type-1 cells through a combination of ubiquitous markers. Furthermore, since the type-1 cells have a long cell cycle length they are less likely to be labeled with a single *in vivo* BrdU injection since the BrdU will be incorporated into the type-2 PCs that are rapidly dividing and highly outnumber their predecessors, the type-1 cells.

The type-2 PCs are called “transiently amplifying” or “lineage determined progenitors”, since these cells are rapidly proliferating with a cell cycle length of approximately ~12-24 hours (Mandyam et al., 2007). Morphologically, type-2 cells have an irregularly shaped dense nucleus, no obvious processes extending from the cell body and are located specifically within the SGZ close to the hilus (Kempermann et al., 2004). Electrophysiologists have demonstrated that at type-2 stage in development, the PCs are responsive to gamma-aminobutyric acid (GABA) synaptic input in an excitatory fashion identified through the expression of GABAa transporters which allow the influx of Cl^- ions into the cell(Ge et al., 2006). The number of type-2 cells have also been shown to increase in

response to external stimuli such as exercise (Kronenberg et al., 2006) and drugs such as fluoxetine (Encinas et al., 2006). These studies have used a variety of methods to identify the type-2 cells including use of BrdU and retroviruses which will label this rapidly dividing population, as well as histology to detect proteins that are specifically expressed in type-2 cells. The absence and presence of immature neuronal marker doublecortin (DCX) is used, for example, to further classify the type-2 cells into type-2a and type-2b, respectively. The type-2b PCs can also be identified through their expression of proteins such as polysialyated-neural cell adhesion molecule (PSA-NCAM), and NeuroD and hence are the first cells to express markers of neuronal lineage commitment.

The final stage of PC development is termed type-3 cell or “neuroblasts” (Kempermann et al., 2004). Unlike the type 2 cells, the type-3 cells no longer express nestin. Type-3 is the final stage in which cells are still proliferative (can be labeled with BrdU or retrovirus) and mature to become post-mitotic. PCs that have become post-mitotic continue to express DCX and cells can reach this stage as early as 3 days (d) after their first division (Kempermann et al., 2004). Type-3 cells that are at an early post-mitotic stage can be further identified by the expression of calretinin (Brandt et al., 2003). Morphologically, as the type-3 cells mature they change from a rounded nucleus to a triangular nucleus and form an apical dendrite.

The transition of type-3 cells to a new granular neuron that expresses NeuN can occur as early as 1d following the BrdU treatment *in vivo* in the rodent (Kempermann et al., 2003). The functional integration of the PCs to mature neurons is accompanied by electrophysiological changes including no longer using GABA as an excitatory input. In the type-3 cells GABA is an inhibitory input and there is the codevelopment of a glutamatergic excitatory phenotype (Tozuka et al., 2005). The new mature neuron receives input from the entorhinal cortex and sends their axonal projections into the CA3 region of the hippocampus where they provide excitatory input to pyramidal cells of CA3 region (Vicini, 2008). Remarkably, during this whole process from type-1 cells to new neurons, there is a massive death of cells which results in a small percentage of the dividing PCs becoming incorporated into the hippocampus (Hayes and Nowakowski, 2002).

1.3 Cell Death Occurring During Adult Neurogenesis

Early studies examining adult neurogenesis using thymidine analogs to label the dividing cells illustrated that the number of PCs that survive to become mature neurons is much lower than the number of PCs initially labeled when dividing (Biebl et al., 2000). These findings lead to the hypothesis that PCs are eliminated via apoptosis during their development, similar to the process that occurs during embryonic neurogenesis (Oppenheim, 1991). This hypothesis was supported by many studies that used BrdU to label the dividing cells and found a sharp decline in the initial number of PCs labeled compared to those identified to

become mature neurons (Biebl et al., 2000; Dayer et al., 2003; Kempermann, 2004; Kuhn et al., 2005; Mandyam et al., 2007). These studies further identified that the mechanism that is responsible for the death of PCs during their development is apoptosis since the dying PCs could be identified with the expression of apoptotic cell death markers such as activated caspase 3 (AC3) or were labeled using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Kuhn et al., 2005; Tashiro et al., 2006a; Sierra et al., 2010).

Examination of the timeline for death of PCs in adult neurogenesis has led many to suggest that PCs at the immature neuron stage (type-2b, type-3) are most susceptible to apoptosis (Brandt et al., 2003; Brown, 2003; Kuhn et al., 2005; Sierra et al., 2010). These findings were predominantly identified through colabeling studies that showed enhanced cell death as assessed by AC3 or TUNEL in the DCX expressing (DCX+) PCs when compared to the non-DCX+ PCs (Plümpe et al., 2006; Sierra et al., 2010). In the DCX+ PCs it has further been shown that the NMDA receptor subunit NR1 is an important mediator of apoptosis at approximately the 3rd week of cell life (Tashiro et al., 2006b). Viral-mediated removal of NR1 was associated with a decline in number of surviving PCs and a significant proportion of DCX+ NR1-null PCs were apoptotic as shown by AC3 expression. This work, together with others (Kuhn et al., 2005; Sierra et al., 2010) all suggest PCs are susceptible to cell death at a critical time point when they express DCX.

The hypothesis that apoptosis in adult neurogenesis only occurs when cells are immature neurons has recently been challenged by the work of Sierra et al. (2010) who has suggested that PCs also die at a very early stage in development before expression of DCX. This study demonstrated that the majority of PCs undergo apoptosis within 1-4 days of birth as determined through the clearance of apoptotic cells phagocytized by microglia. Although this work has yet to be replicated, it raises an interesting hypothesis that suggests that either at early (1-4 days) and later time points during maturation (between ~2-4 week old) PCs are sensitive to apoptosis mediated cell death **(Figure 2)**. This raises the subsequent question about whether a similar or different apoptotic mediated process is regulating the death of PCs that can occur during these 2 different phases of their development in the adult brain.

1.4 Apoptosis

The term apoptosis was initially coined by Kerr, Wyllie and Currie (1972) as a process that plays "a complementary but opposite role to mitosis in the regulation of animal cell populations". Since then apoptosis has been recognized to be a complex programmed process that can cause cell death and is essential and advantageous during both development and aging(Martinou and Youle, 2011). For example, apoptosis is responsible for the differentiation of fingers and toes in a developing embryo (Brill et al., 1999). Deficits in apoptosis are also implicated in numerous forms of pathology, including excessive apoptosis being associated with atrophy and insufficient amounts of apoptosis contributing to excessive

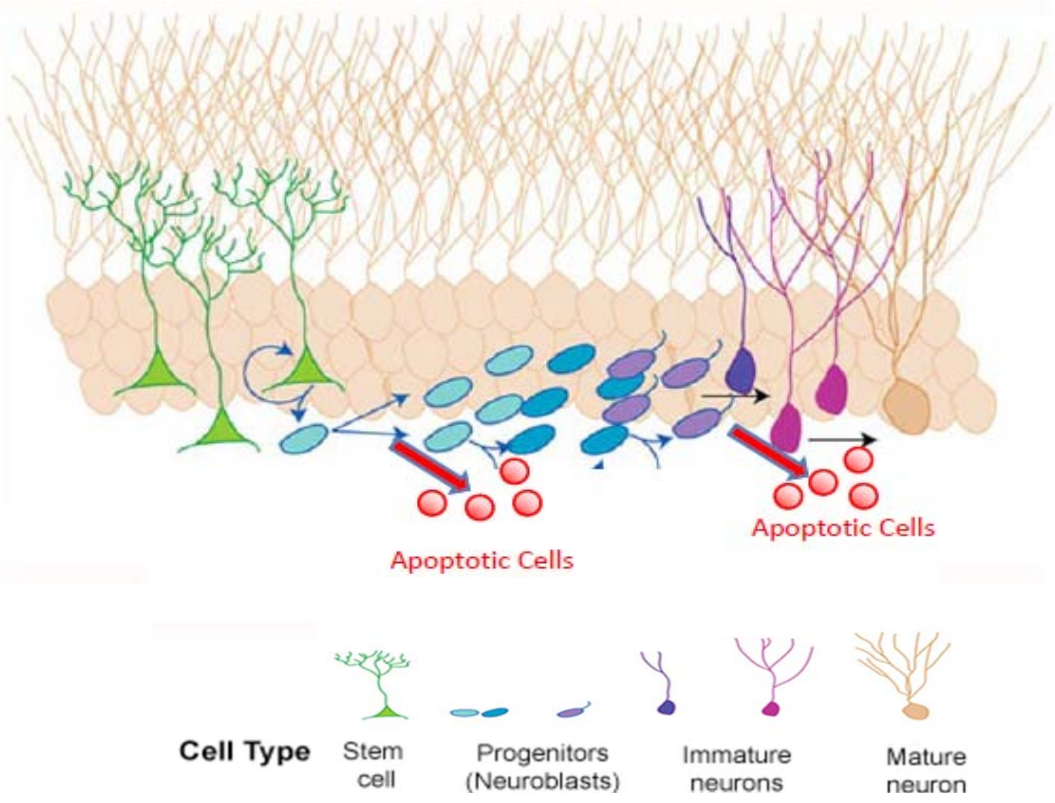


Figure 2. Two Proposed Time Points Over the Course of Neurogenesis During which Cells are Suggested to be Susceptible to Apoptosis.

Schematic diagram illustrating that cells during the process of adult neurogenesis can die from apoptosis at either an early time point (~1-4 days old) or during their development to immature neurons (~2 weeks old).

proliferation, as observed in neurodegenerative diseases and cancer, respectively (Lowe and Lin, 2000; Mattson, 2000). Therefore, given the importance of apoptosis, the biochemical events that characterize the process, as well as the morphological changes that occur in cells during apoptosis have been well characterized.

In the early stages of apoptosis, use of the electron and light microscopy have identified that pyknosis and cell shrinkage occur as two hallmarks of apoptosis (Johnson et al., 2000; Taatjes et al., 2008). Pyknosis is caused by the condensation of chromatin and has been identified as a key characteristic of cell death. Cell shrinkage is visualized through important cues such as dense cytoplasm, and tightly packed organelles. These processes are followed by subcellular changes such as electron dense nucleus, which tends to aggregate and leads to the cell fragmentation in apoptotic bodies. Unlike necrosis, apoptosis does not result in altering organelle integrity as apoptotic bodies have the ability to enclose themselves and are phagocytosed by macrophages (Häcker, 2000). The apoptotic process has been characterized to be initiated by either an intrinsic or extrinsic pathway that ultimately convene on caspases that execute cell death (Tait and Green, 2010).

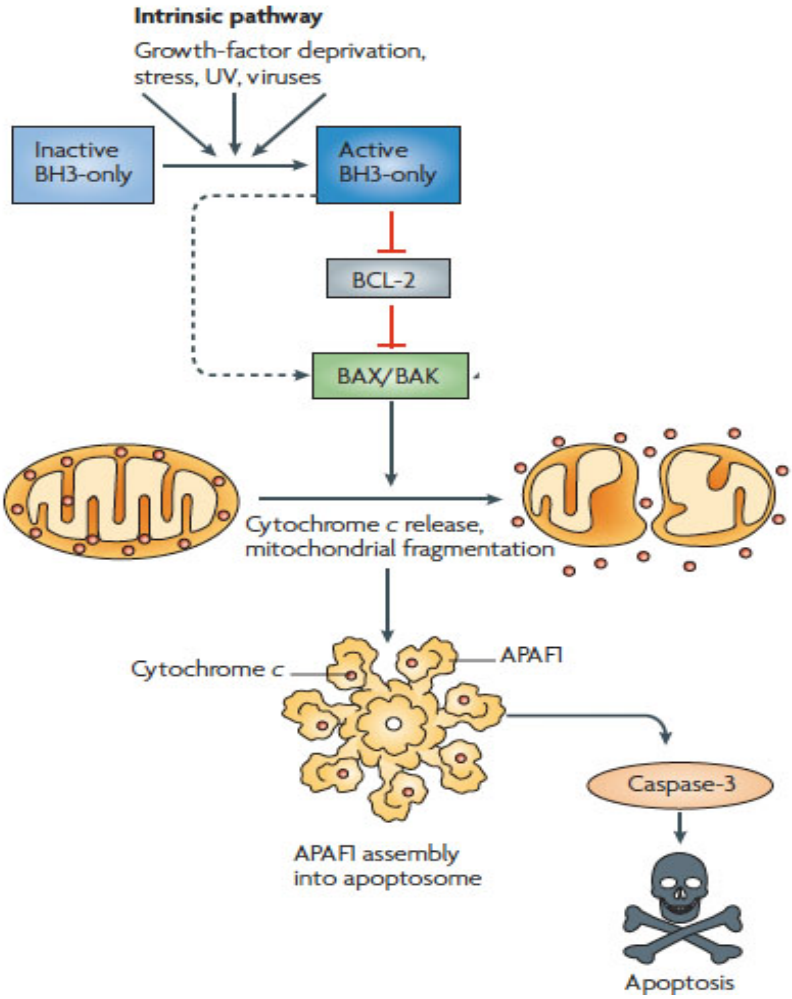
The extrinsic apoptotic pathway is initiated from outside the cell when signals from outside the cells identify a cell must die (Reed, 2000). The extrinsic apoptotic pathway involves the transmembrane receptor interactions such as the tumor necrosis factor (TNF) family of death receptors which transmit signals from

the cell surface to intracellular signalling pathways (Elmore, 2007). The extrinsic cell death pathway has not been widely studied in adult neurogenesis since dying PCs in the SGZ have been shown to express markers of the intrinsic mitochondrial pathway, but not the extrinsic death-receptor pathway (Ekdahl et al., 2003).

1.5 The Intrinsic Apoptotic Pathway

The intrinsic apoptotic pathway is activated through intracellular signals that converge on mitochondrial mediated events (Elmore, 2007) **(Figure 3)**. The intracellular signals can be activated by variety of stimuli such as radiation, hyperthermia, viral infections, and lack of hormones, cytokines or growth factors. These stimuli cause the activation of proteins such as members of the apoptotic protein family, Bcl-2 that have the ability to permeate mitochondrial outer membrane permeabilization (MOMP) which leads to the release of pro-apoptotic signals such as cytochrome *c* from the mitochondria.

Literature has suggested the Bcl-2 family of proteins are key regulators of the apoptotic pathway (Veis et al., 1993; Soane and Fiskum, 2005; Xu et al., 2007; Bunk et al., 2010) **(Figure 3)**. Initial overexpression studies found Bcl-2, an anti-apoptotic protein, to inhibit cell death as opposed to the previously discovered oncogenes that promote cell proliferation (Vaux et al., 1988). The Bcl-2 family consists of 25 proteins divided into three groups based on homology and function (Soane and Fiskum, 2005; Cheung et al., 2008; Youle and Strasser, 2008). The first group is the anti-apoptotic proteins which include members such as Bcl-2, B-



Modified from Youle and Strasser, 2008.

Figure 3. Role of Bcl-2 Family Proteins in the Intrinsic Pathway of Apoptosis.

Bcl-2 can inhibit the function of BAX/BAK to prevent mitochondrial fragmentation and cell death. Inhibition of Bcl-2 will allow BAX/BAK to signal for cytochrome *c* release and downstream activation of caspases, ultimately leading to cell death.

cell lymphoma-extra large (Bcl-xl), and myeloid leukemia cell differentiation (Mcl-1). In contrast, the second group is the pro-apoptotic proteins including for example the Bcl-2 associated X (BAX), and Bcl-2 antagonist or killer (BAK). The third group of the Bcl-2 family is the BH3-only proteins which have a conserved BH3 domain homology and primarily function to interact with and regulate the core pro and anti-apoptotic family members to promote apoptosis.

The Bcl-2 family of proteins control the intrinsic mitochondrial apoptotic pathway by governing the MOMP. The activation of apoptosis in the mitochondrial apoptotic pathway is controlled primarily by the Bcl-2 family members, Bcl-2-associated X protein (BAX) or Bcl-2 antagonist or killer (BAK). However, BAX and BAK can be inhibited through the activation of anti-apoptotic protein, Bcl-2 (Youle and Strasser, 2008; Tait and Green, 2010). BAX/BAK undergo conformational changes that allow for the formation of oligomers that stably insert into the outer mitochondrial membrane (Chipuk et al., 2006). The MOMP signals for the formation of the pores in the mitochondria and causes the release of cytochrome c, and Smac/DIABLO into the cytoplasm, which can activate caspase dependent apoptosis. More specifically, cytochrome *c* can bind to apoptosis activating factor 1 (AFAF1) and initiator caspase-9 and triggers the formation of the apoptosome and allows downstream activation of caspase 3. On the other hand, Smac/DIABLO interacts with inhibitors of apoptosis proteins (IAPs) to inhibit their function, this in turn enhances the activation of caspase 3(Wu et al., 2000). Essentially the release of either cytochrome *c* or

Smac/DIABLO has been shown contribute to caspase 3 dependent cell death. Ultimately, the activation of caspase 3 is synonymous to activation of cell execution functions which includes events such as endonuclease activation, degradation of chromosomal DNA, cytomorphological changes and ultimately formation of apoptotic bodies which have the ability to phagocytose the cell (Elmore, 2007).

1.6 Players Involved with Apoptosis in Adult Neurogenesis

In comparison to the numerous studies that have identified apoptotic neurogenic cells in the adult neurogenic cells (Biebl et al., 2000; Dayer et al., 2003; Tashiro et al., 2006a; Lagace, 2008), few studies have identified mechanisms responsible for the induction and execution of apoptosis in the context of adult neurogenesis. More specifically, Bcl-2 and BAX have been targeted to enhance neurogenesis and thus have indirectly been implicated in regulating apoptosis in adult generated cells in the brain (Kuhn et al., 2005; Sahay et al., 2011).

Kuhn et al. (2005) first evaluated whether an increase in neurogenesis would occur in NSE-huBcl-2 mice that were created to overexpress human Bcl-2 under a neuron-specific enolase (NSE) promoter. Adult (8 week old) NSE-huBcl-2 mice had a decrease in the number of dying cells as measured by the TUNEL assay. Also, there was an increase in neurogenesis as demonstrated by increase in the number of BrdU+ cells that were also NeuN+ 4 weeks after initial BrdU injection. Based on these findings this study suggested that the effects of

overexpressing Bcl-2 occur at a late maturation stage of PCs. However this interpretation could be confounded by the fact that the NSE driver only targets the maturing or mature neurons in the developing and adult brain (Bernard et al., 1997) and thus this may not be an ideal model to assess the role of Bcl-2 throughout the lifespan of the PC development.

A key player known to interact with Bcl-2 in regulating the apoptotic pathway is BAX (Youle and Strasser, 2008). Embryonic knockout (KO) mice for BAX were used to demonstrate that apoptosis occurring embryonic neuronal development is mediated by BAX (Deckwerth et al., 1996; White et al., 1998; Sun and Oppenheim, 2003). These mice were also used to demonstrated that a knockout of BAX results in an increase in number of neurons in the hippocampus (Sun et al., 2004b). Most recently, Sahay et al., (2011) created an nBAX inducible transgenic mouse model in which BAX can be conditionally removed from nestin-expressing PCs and their progeny. The nBAX mice have a significant increase in the number of mature neurons in the DG 8 weeks after removal of BAX. Since the mice had this significant enhancement in neurogenesis, this study went on to assess if an increase in neurogenesis was associated with improvement in hippocampal dependent learning and memory. In support of new neurons in the DG having a functional role, the nBAX mice were identified to have a significantly higher ability to discriminate two similar contexts. Thus overall, this study made 2 significant advances by finding that BAX is an essential regulator of adult neurogenesis and improvements in cognitive function can be obtained through

increasing neurogenesis.

OBJECTIVES AND STATEMENT OF HYPOTHESIS

Objective:

In order to enhance neurogenesis it is important to identify the intrinsic cellular mechanisms that regulate apoptosis during PC development in the adult brain. Bcl-2 regulates the intrinsic apoptotic pathway and indirect evidence suggests Bcl-2 has an essential role in regulating the survival of PCs (Kuhn et al., 2005). The objective of this thesis is to determine whether Bcl-2 is required during adult hippocampal neurogenesis.

Hypothesis:

Bcl-2 is a required intrinsic regulator of PCs during their development in the adult naïve brain.

Aims:

1. To determine if the removal of Bcl-2 from dividing PCs alters PC maturation.
2. To remove Bcl-2 from nestin-expressing PCs and their progeny by creating an inducible triple transgenic Bcl-2 knockout mouse and determine if this alters PCs development.

2. MATERIALS AND METHODS

2.1 Animals

Animal procedures were performed with approval from University of Ottawa Animal Care Committee and adhered to the Guidelines of the Canadian Council on Animal Care. Mice lines used include: floxed Bcl-2 mice (created by Thorp et al., 2009, obtained from Jackson Laboratory); BAX knockout mice (created by Knudson et al., 1995, obtained from Dr. Ruth Slack, University of Ottawa); inducible Nestin-CreER^{T2} mice (Lagace et al., 2007b, obtained from Dr. Amelia Eisch laboratory, University of Texas Southwestern); reporter R26R-eYFP mice (Srinivas et al., 2001,obtained from Jackson Laboratory). The nBcl-2 mice were created through breeding Nestin-CreER^{T2} with fBcl-2 and R26R-eYFP mice. Both male and female mice were used and all strains were maintained on C57bl/6J background. Animals were group housed (~4-5 per cage) in standard laboratory cages and were kept on a 12 hour night/day cycle with free access to food & water.

2.2 Genotyping

Animals were genotyped to determine the zygosity of each of the four transgenes. Tail clippings (~1 mm) were taken from ~3 week old mice. DNA was extracted using a HotSHOT DNA extraction method (Truett et al., 2000). Briefly, the tail clipping is incubated at 95°C for 30 minutes in 75 µl of Alkaline Lysis Buffer (25 mM NaOH and 0.2 mM Na_2EDTA) followed by addition of 75 µl of the Neutralization solution (40 mM Tris-HCl). The genotype of the mice was

determined using Polymerase Chain Reaction (PCR) based on previously published protocols for floxed Bcl-2 (Thorp et al., 2009), BAX (Knudson et al., 1995), Cre (Indra et al., 1999), and YFP (Sorlano et al., 1999). The PCR products were separated by size on a 2% agarose gel with ethidium bromide using electrophoresis and visualized using UV radiation. PCR product size was estimated based on comparison with a DNA ladder (100 bp ladder; DM001-R500M, Frogga Inc.). **Table 1** provides a list of the PCR primers and expected product size used to determine zygosity.

2.3 Retroviral Vectors & Injections

Retroviruses were prepared as previously published using the plasmids received from Dr. Fred Gage (Salk Institute of Biological Sciences) with minor modifications (Tashiro et al., 2006a ,Nature Protocol). Briefly, 293T cells were plated (~$8x10^6$ cells / 150 mm) and the following day the cells were co-transfected with the retroviral plasmid (CAG-GFP/Cre or CAG-RFP), packing plasmid (CMV-Gag-Pol), and envelope plasmid (CMV-VSV-G) in a 3:2:1 ratio respectively, using polyethylenimine (PET, Polyscience Cat# 23966). At both 48 and 72 hours (hr) after transfection the supernatant containing the virus was harvested. The virus was concentrated by two rounds of ultracentrifugation (20,000 rpm, 2 hr at 4°C) with 20% sucrose cushion, dissolved in phosphate buffered saline (PBS) and aliquoted and stored at -80°C.To determine the titres of the virus, 100 μl of diluted virus (10^4 dilution from stock) were added into each well of a 24-well plate that was seeded, the day before, with 1.25 x 10^5 cells of

Table 1. Table of Primers and PCR Products Used for Genotyping of Mice

Transgene		5’ Primer	3’ Primer	PCR Product Size (bp)
fBcl-2	Transgene	P22: 5’-GCC CAC CAT CTA AAG AGC AA-3’	P23: 5’-GCC CAC CAT CTA AAG AGC AA-3’	296
	Transgene	P23: 5’-GCC CAC CAT CTA AAG AGC AA-3’	P22: 5’-GCC CAC CAT CTA AAG AGC AA-3’	350
BAX	WT	P41: 5’-GTTGACCAGAGTGGCGTAGG-3’	P43: 5’-GAGCTGATCAGAACCATCATG-3’	304
	Transgene	P41: 5’-GTTGACCAGAGTGGCGTAGG-3’	P42: 5’-CCGCTTCCATTGCTCAGCGG-3’	507
$CreER^{T2}$	+Control	P26: 5’–CTAGGCCACAGAATTGAAAGATCT–3’	P27: 5’–GTAGGTGGAAATTCTAGCATCATCC–3’	324
	Transgene	P24: 5’–GCGGTCTGGCAGTAAAAACTATC–3’	P25: 5’–GTGAAACAGCATTGCTGTCACTT–3’	100
YFP	WT	P21: 5’–GGAGCGGGAGAAATGGATATG–3’	P20: 5’–GCGAAGAGTTTGTCCTCAACC–3’	560
	Transgene	P21: 5’–GGAGCGGGAGAAATGGATATG–3’	P 19: 5’–AAAGTCGCTCTGAGTTGTTAT–3’	310

293T cells in each well. Live fluorescence-positive cells were counted after 48 hr with a 20X objective. The infectious viral particle (IVF) per ml for each well was calculated as the number of infected cells per viewing field multiplied by 243.22 (total area of the well) x 10^5 (dilution factor). The virus titre was determined by the mean of IVF for each well and ranged between 1-3x10^8 IVF/per ml.

The retroviruses were injected by a microsyringe into the dentate gyrus during stereotaxic surgery. Three doses of the required analgesic, Buprenorphine, at a dosage of 0.05mg/kg were administered via subcutaneous injections to the mice: the first was given one hour before surgery and the last two were given 6 and 12 hr after the initial injection. Mice were anesthetized with concentration of 2% isoflurane and injected bilaterally into the dentate gyrus with 1:1 ratio of 1.5 µl of CAG-RFP and CAG-GFP/Cre retrovirus. The virus was administered at the rate of 0.2 µl/min for a total of 7.5 minutes and the needle was removed 5 min after the injection was completed. The coordinates used for the dentate gyrus were -1.7 mm anterior/posterior, ±1.2 mm medial/lateral from bregma, and -2.4 mm dorsal/ventral from the skull. The mice recovered in a 37°C incubator until they awakened from the anesthesia. For all experimental time points (3, 7, 30 days post injection) a minimum of 3 animals per genotype were used.

2.4 Tamoxifen Treatments

Tamoxifen (TAM, dissolved in 90% sunflower seed oil and 10% EtOH) was administered to 5 week old nBcl-2 KO mice via intraperitoneal (ip) injections for 5 consecutive days based on Lagace et al., (2007b). TAM was used at a dosage of 160mg/kg since use of TAM at 180mg/kg was associated with a high mortality rate of ~20% in the nBcl-2 KO mice. For all experimental time points (12 days and 30 days post TAM) a minimum of 8 animals per genotype were analyzed.

2.5 Perfusion and Tissue Collection

Animals were anesthetized with Euthanyl (90mg/kg) ip and tanscardially perfused with cold PBS (pH=7.4) at a rate of 7 ml/min for 6 min, followed by 4% paraformaldehyde in PBS for 15 minutes at the same rate. Brains were removed and post-fixed for 1 hour in 4% PFA and then cryoprotected in 30% sucrose with 0.1% sodium azide (NaN3) in PBS until sectioning. Brains were sectioned on a freezing microtome (Leica SM 2000R) coronally into 30µm slices and stored in PBS with 0.01% NaN3 at 4°C.

2.6 Immunohistochemistry

Immunohistochemistry (IHC) was performed on free-floating sections in a carrier solution (0.1% Tween, 0.1% Triton-X in 1XPBS) with the primary antibodies listed in **Table 2**. Primary antibodies were diluted in carrier and incubated at 4°C overnight on a shaker. Staining was visualized with either a Cy2, Cy3, and Cy5 labeled secondary antibody (Table 2, Jackson laboratory) used at a dilution of 1:500. Sections were then mounted onto charged slides and

Table 2. List of Primary and Secondary Antibodies

Antibody	Company	Catalog #	Concentration
Chicken-Anti-GFP (Green Fluorescent Protein)	Aves	GFP-1020	1:5000 (Free-Floating), 1:20000 (Slide-Mounted)
Rabbit-Living Colors DsRed Polyclonal Antibody (100ul)	Clontech	632496	1:5000
Goat-anti-DCX (C-18)	Santa Cruz	sc8066	1:500
Rabbit-Monoclonal anti Ki-67	Medicorp	275R-14	1:100
Mouse-A-Glial fibrillary acidic protein (GFAP)	Millipore	MAB3402X	1:250
Goat- SOX-2	Santa Cruz	SC-17320	1:250
Mouse-A-NeuN Clone A60	Millipore	MAB377	1:500
Rabbit- Cleaved Caspase-3 (Asp175) (5A1E) mAb - 100ul	Cedar Lane	9664S	1:250
Secondary Antibody			
DyLight 488 AffiniPure Donkey Anti-Chicken IgY	Jackson Laboratory	703-485-155	1:500(Free-Floating)1:200 (Slide-Mounted)
Cy2-AffiniPure Donkey Anti-Rabbit IgG	Jackson Laboratory	711-225-152	1:500
Cy3-AffiniPure Donkey Anti-Rabbit IgG	Jackson Laboratory	711-165-152	1:500
Cy5-AffiniPure Donkey Anti-Rabbit IgG	Jackson Laboratory	711-175-152	1:500
Biotin-SP-AffiniPure Donkey Anti-Rabbit	Jackson Laboratory	705-485-147	1:200
Cy3-AffiniPure Donkey Anti-Goat IgG	Jackson Laboratory	705-165-147	1:500
Cy3-AffiniPure Donkey Anti-Mouse IgG	Jackson Laboratory	715-495-150	1:500
DyLight 649 AffiniPure Donkey Anti-Mouse IgG	Jackson Laboratory	715-495-150	1:500

dried for 10-15 min before being cover-slipped with Immumount mounting media (Immu-mount; 2860060, Fisher Scientific).

IHC detection of GFP cells (GFP+) in the TAM treated mice, as well as activated caspase 3 (AC3) cells, was performed on tissue that was slide-mounted. Briefly, sections were mounted on charged slides, dried overnight and then antigen retrieval was performed using 0.1M citric acid (pH=6) at 95°C for 15 min. For AC3 staining, sections were also incubated in 0.1% trypsin (Trypsin, T7409-10G, Sigma) followed by 2N HCl (Hydrochloric Acid, 258148-2.5L, Sigma). To prevent non-specific binding of the antibody, all tissue was blocked in 3% Normal Donkey Serum (NDS; 017-000-121, Jackson Immuno Research Laboratory) for 1 hr at room temperature (RT) prior to being incubated overnight either with GFP or AC3 primary antibody (Table 2) diluted in 3% NDS, 0.3% Triton-X in 1X Tris-buffered saline (TBS). Biotinylated attached secondary antibodies (Table 2) were incubated at RT for 1 hr, followed by 30 min incubation in 0.3% H_20_2 to quench endogenous peroxidases and 60 minutes incubation in Avidin-Biotin Complex (Elite Kit, PK-6100, Vector Laboratories). YFP+ cells were labeled with fluorescein using TSA FluoresceinTyramide Reagent (PSAT701, Perkin Elmer Life and Analytical Sciences, 1:50, 10 min) and counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; 11836170001, Roche, 1:10000, 5 min). AC3+ cells were labeled with 3,3'-Diaminobenzidinetetrahydrochloride (Metal enhanced DAB kit; PI34065, Fisher), and counterstained with fast red nuclear stain (Nuclear Fast Red; H3403,

Cederlane). Dehydration was performed through immersion of the slides in series of 95% to 100% EtOH, 100% EtOH, followed by Citro-Solv (Citrosolve clearing agent 1G,22143975, Fisher) consecutively for 20 sec, 1 min, 5 min. Slides were cover slipped with DPX (DPX mountant; 44581, Sigma) mounting media.

2.7 Quantification Using Microscopy

The number of immunoreactively labeled cells (GFP+, RFP+) were counted manually in sections through every 9th section in the dentate gyrus using modified stereology (Mandyam et al., 2007). All counts were performed using the Olympus BX51 fluorescent microscope at 50X magnification by an observer blind to experimental condition. The total number of GFP+ cells, RFP+ cells and GFP+RFP+ were counted in all animals to determine if there was a difference in the Bcl-2 KO population in the retrovirus experiments, whereas in the nBcl-2 KO mice, the total number of recombined GFP+ cells was counted. Quantification of histological analysis was also verified through a 2nd assessment of counts that was performed blindly. Variability between ratings was accepted at <10%.

All colabeled cells were assessed using a Zeiss LSM510-META confocal microscope with the 40X oil immersion objective at emission wavelength of 488,543 and 633. Minimum of 20 cells per animal were measured for all colabeling analysis. Optical z-plane sectioning was used to evaluate if the cells were colabeled using ZEN 2009 acquisition software from Zeiss.

2.8 Statistical Analysis

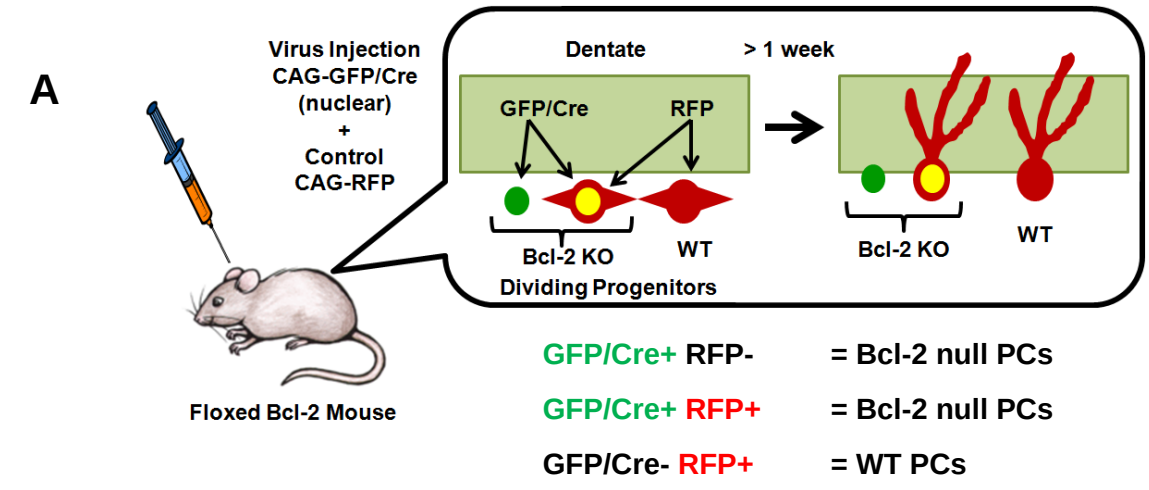
Outcomes are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism. A two-tailed independent t-test was used for analysis of two groups and analysis of variance (ANOVA) followed by Bonferroni posthoc was used for analysis of more than 2 groups. Statistical significance was set *a priori* at $p<0.05$.

3. RESULTS

3.1 Retroviral-Mediated Removal of Bcl-2 Prevents the Survival of Newborn Neurons

Bcl-2 $^{-/-}$ mice are viable to birth but die postnatally due to polycistronic kidney disease (Veis et al., 1993)**.** Therefore to evaluate the role of Bcl-2 in adult neurogenesis we inducibly removed Bcl-2 from the adult mice using fBcl-2 mice (Thorp et al., 2009) that have exon 2 flanked between two loxP sites. To target the removal of Bcl-2 from the PCs, we used a retroviral approach to deliver Cre recombinase to the dividing PCs and allow for the permanent removal of Bcl-2 and labeling of PCs and their progeny. A dual-labeling retrovirus system was implemented as previously published which allows for stereotaxic injection of a 1) nuclear CAG-GFP/Cre retrovirus that removes exon 2 from fBcl-2 mice and labels infected cells with GFP; and 2) an internal control CAG-RFP retrovirus, which does not alter Bcl-2 expression and labels the infected cells with RFP (Tashiro et al., 2006b) (**Figure 4A)**. Therefore all infected cells that are either GFP+ (green) and GFP+RFP+ (yellow) have Bcl-2 removed, while all cells that are only RFP+ (red) are WT for Bcl-2. The use of this dual-labeling retrovirus system enables us to have two independent control groups: (1) RFP+ PCs in the fBcl-2 mice and (2) WT littermates. Animals were sacrificed at 3, 7, 30 days post infection (dpi) **(Figure 4B)**.

There was a significant difference in the number of GFP+ infected cells between days post infection ($F(2,20)=16.80$, $p<0.001$), between fBcl-2 and WT



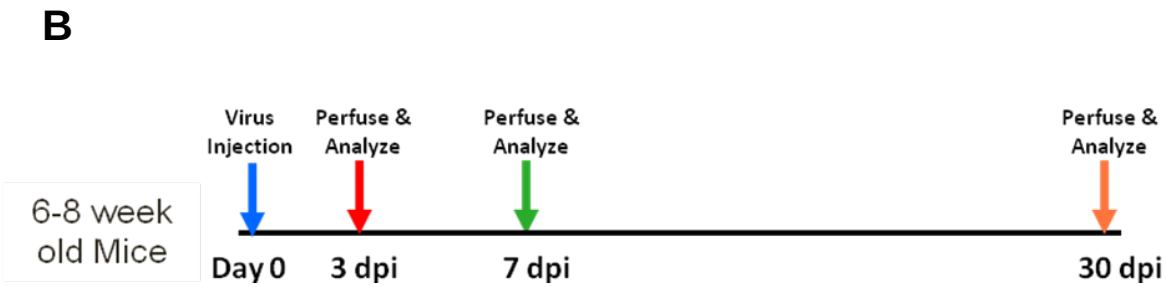


Figure 4. Dual-labeling Retroviral System and Experimental Design for Use of Retrovirus in Floxed Bcl-2 Mice.

A) Illustration of the dual labeling system which allows for examination of PCs with and without Bcl-2 in the same animal. Retrovirus are stereotaxically injected into fBcl-2 mice and the Bcl-2 null PCs are either GFP/Cre+RFP- or GFP/Cre+RFP+ and the WT PCs are GFP/Cre-RFP+. **B)** Experimental timeline for virus administration and subsequent analysis post removal of Bcl-2.

mice ($F(1,20)=6.87$, $p<0.05$), as well as a significant interaction between day and genotype ($F(2,20)=3.67$, $p<0.05$) **(Figure 6B).** Posthoc analysis demonstrated that at 7 dpi there was a significant reduction in the number of GFP+ cells in the fBcl-2 mice compared to WT controls. However, with the use of the Bonferroni post hoc analysis no significant difference was identified at 30 dpi yet there was a striking effect with no GFP+ cells present in fBcl-2 mice.

The reduction in number of GFP+ cells in the fBcl-2 mice did not appear to be a nonspecific effect of viral infection, since there was no significant difference in the number of labelled RFP cells between the fBcl-2 and WT mice **(Figure 6A)**. As expected there was a significant reduction in the number of RFP+ cells in days post infection ($F(2,20)=22.68$, $p<0.001$). This is expected since the majority of PCs are unable to survive due to cell death during their development (Biebl et al., 2000; Sierra et al., 2010).

Since the number of RFP cells changes over time, it is important to examine the relative ratio of survival of infected cells lacking Bcl-2 (GFP+RFP+) compared to those infected but do not lack Bcl-2 (RFP+). As illustrated in **Figure 5** and quantified in **Figure 6C** this ratio also demonstrated that over time there was no significant effect at 3dpi, however there was a significant difference between WT and fBcl-2 mice ($F(1,20)=41.14$, $p<0.001$), as well as an interaction between time and genotype ($F(2,20)=10.78$, $p<0.01$).The fBcl-2 mice had a significantly lower ratio of surviving cells at both 7 and 30 dpi compared to WT mice. These results

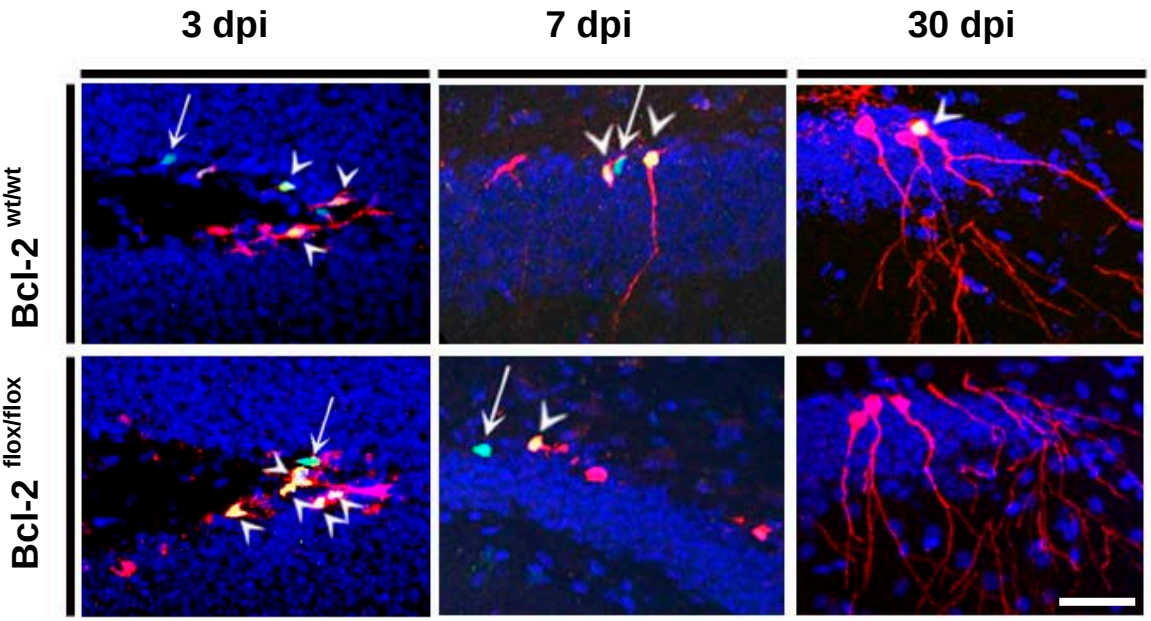


Figure 5. Removal of Bcl-2 Affects the Survival of Newborn Dentate Gyrus Neurons

Representative images of cells in fBcl-2 and WT animals at 3,7, and 30 dpi. Arrowheads represent double labeled PCs while arrows represent PCs that are GFP+ only.

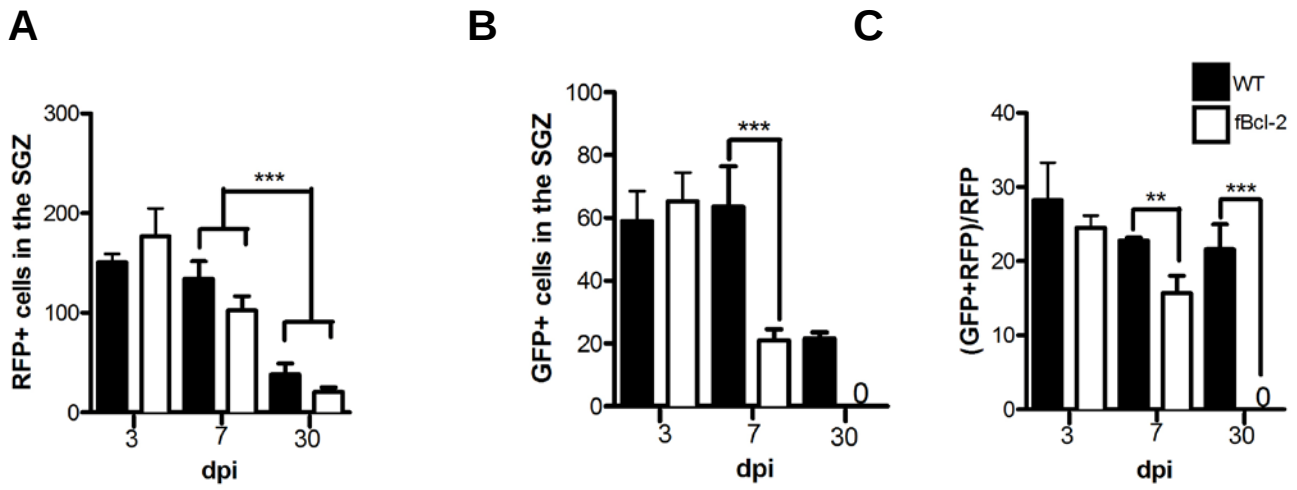


Figure 6. Removal of Bcl-2 Affects the Survival of Newborn Dentate Gyrus Neurons

Quantification of **(A)** total GFP+ cells **(B)** RFP+ cells and **(C)** ratio of RFP+ GFP+ over total RFP+ cells illustrates there is a significant interaction between time and genotype in both GFP+ cells and ratio of Bcl-2 null PCs. Post-hoc test indicates there is significant reduction in fBcl-2 compared to WT mice in both the number of Bcl-2 null (GFP+) cells and ratio of GFP+RFP+/RFP+ cells surviving at 7 and 30 days respectively but no difference in WT (RFP+) PCs.

suggest that Bcl-2 is an essential regulator for the survival of PCs within the dentate gyrus of the adult brain.

PCs are suggested to be susceptible to apoptosis at both an early (~1-4 days) and late (~10 days) stage of development (Kuhn et al., 2005; Sierra et al., 2010). Retroviruses mainly infect a later stage-terminally dividing population, therefore I hypothesized removal of Bcl-2 from dividing PCs would reduce the number of immature neurons that express DCX. In WT mice at 7 dpi over 80% of the GFP+ infected cells had expressed DCX **(Figure 7A-B)**, which is in agreement with Jagasia et al.(2009) that demonstrate that 85% express DCX at 7 dpi. Compared to the WT mice, the fBcl-2 mice had a significant decrease in the number of GFP+DCX+ cells at 7 days after removal of Bcl-2**.** There was no difference in the percent of RFP+ cells that expressed DCX **(Figure 7C).** These data suggest that Bcl-2 is important in the formation of the DCX+ immature neurons.

3.2 Bcl-2 Functions in the Adult Hippocampus in a BAX Dependent Manner

In the mitochondrial apoptotic pathway Bcl-2 can heterodimerize with BAX to prevent BAX from forming pores in the mitochondria and ultimately prevent the activation of the apoptosis pathway **(Figure 2)** (Youle and Strasser, 2008). Embryonic deletion of BAX increases the survival of PCs (Sun et al., 2004a) and recently an inducible transgenic mouse that has BAX deleted from nestin-expressing PCs and their progeny was shown to have enhanced adult

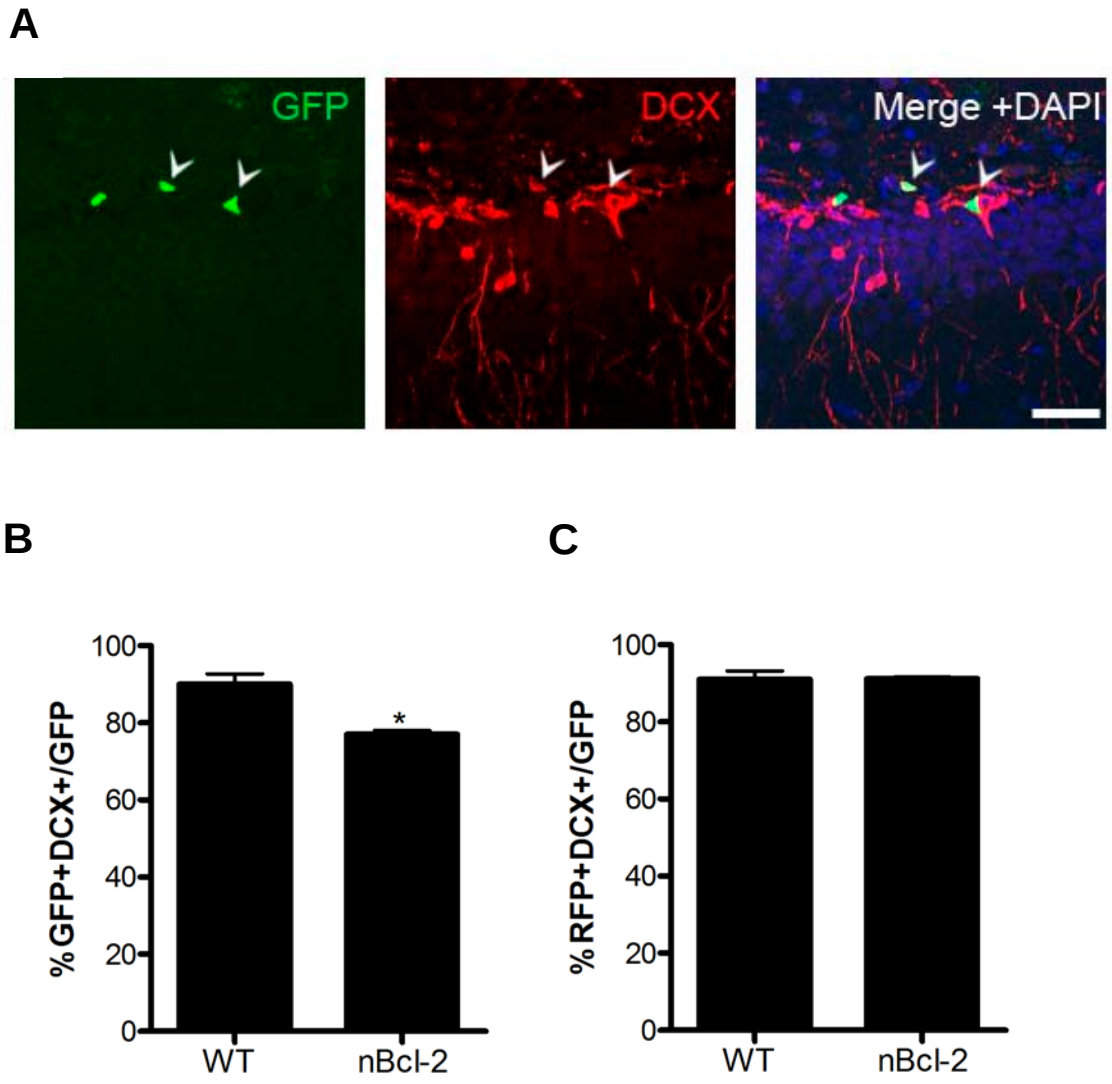


Figure 7. Reduction in Percentage of Immature Neurons at 7 Days Post Removal of Bcl-2 via Retrovirus.

Representative Images **(A)** and quantification **(B-C)** of GFP+DCX+ and RFP+DCX+ after the removal of Bcl-2. There is a reduction in GFP+DCX+ (arrowhead) in fBcl-2 animals compared to WT littermates but no difference in the proportion of RFP+DCX+ cells.

neurogenesis (Sahay et al., 2011). This evidence supports that BAX is important for adult neurogenesis leading to the question of whether Bcl-2 function is dependent on BAX in adult hippocampal neurogenesis.

In order to address this question, fBcl-2 mice were bred with embryonic BAX KO mice to create fBcl-2/BAX KO mice that were infected with the dual-labeling retrovirus to remove Bcl-2 **(Figure 8A)**. BAX KO mice are viable however homozygous males were sterile therefore we had to use homozygous females to breed since the males are sterile due to lineage-specific abnormalities in cell death (Korsmeyer et al., 1993). Control littermates included Bcl-2 KO mice (data not shown) and the Bcl-2 WT/BAX KO mice. At 7 dpi, there was no significant difference in number of infected GFP+ cells, RFP+ cells, or the ratio of GFP+RFP+/RFP+ cells in the fBcl-2/BAX KO and WT mice **(Figure 8B-D).** These results suggest Bcl-2 functions in a BAX dependent manner, and the removal of BAX is able to rescue the effect observed following removal of Bcl-2.

3.3 Generation of the Inducible Triple Transgenic Bcl-2 Knockout Mouse

In order to examine whether Bcl-2 altered PCs when they are at an early stage of development we created an inducible triple-transgenic mouse called nBcl-2 KO that allows for Bcl-2 to be removed from the nestin-expressing PCs and their progeny. Nestin is a filament protein that is transiently expressed in the early stem-like and intermediate stage of PC development and becomes down-regulated as PCs reach a more mature stage (Hendrickson et al., 2011). The nBcl-2 KO mice was made by breeding Nestin-CreER^{T2} mice that uses 5.8 kb of

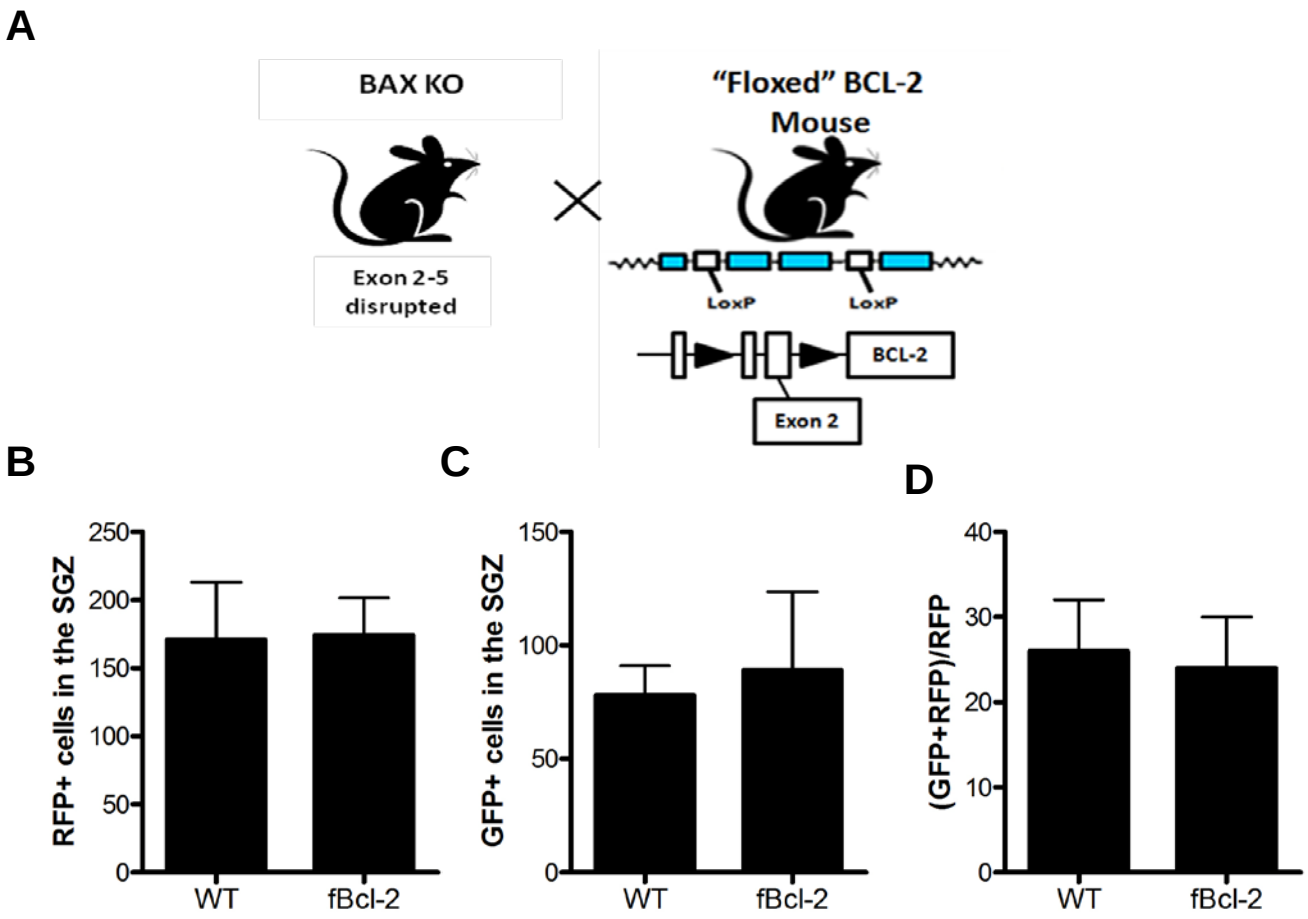


Figure 8. In BAX Knockout Mice Removal of Bcl-2 Does Not Change the Ratio of GFP+RFP+ over total RFP+ cells.

A) Creation of Bcl-2 conditional and BAX germline KO mice to determine if Bcl-2 is BAX dependent by removing Bcl-2 using retrovirus. Quantification of **(B)** RFP+ **(C)** GFP+ and **(D)** ratio of GFP+ cells over total RFP+ cells after removal of Bcl-2 in a BAX dependent line after 7 dpi showed no difference in the Bcl-2 KO compared to the WT littermates.

the nestin promoter and exons 1-3 of the nestin gene to drive CreER^{T2} protein expression in the adult PCs, with fBcl-2 mice, and R26R-eYFP reporter mice **(Figure 9)**. Administration of tamoxifen (TAM) to the nBcl-2 KO mice allows for TAM to bind to the estrogen receptor on Cre, which permits CreER^{T2} to translocate to the nucleus, cut at the loxP sites to remove exon 2 of Bcl-2 and the stop codon in front of the yellow fluorescent protein (YFP+) **(Figure 10A).** Mice were then sacrificed at 12 and 30 days post TAM **(Figure 10B).**

3.4 Removal of Bcl-2 in Nestin-Expressing Cells and their Progeny Reduces the Number of Stem-Like PCs and Immature Neurons at 12 Days

At 12 days post TAM there was a significant 2-fold decrease in the number of recombined YFP+ PCs in the nBcl-2 KO mice compared to WT littermates ($t(14)=6.6, p<0.001$) **(Figure 11)**. The diversity in the morphology of the YFP+ cells at 12 days post TAM was in agreement with previous reports (Lagace et al., 2007b; Lagace et al., 2008; Gao et al., 2009) and is expected since this model allows for recombination in stem-like PCs and their progeny. The reduced number of Bcl-2 null PCs therefore could be due to an increase in death of PCs and/or a reduction in the number of dividing PCs.

To determine if there were any changes in the number of dying cells in nBcl-2 KO compared to WT mice, cells expressing AC3+ cells were counted. AC3 has been widely used in numerous adult neurogenesis studies to evaluate cell death and is also downstream of Bcl-2 in the mitochondrial apoptotic pathway hence provides us with a good evaluation on PC death in this conditional KO model

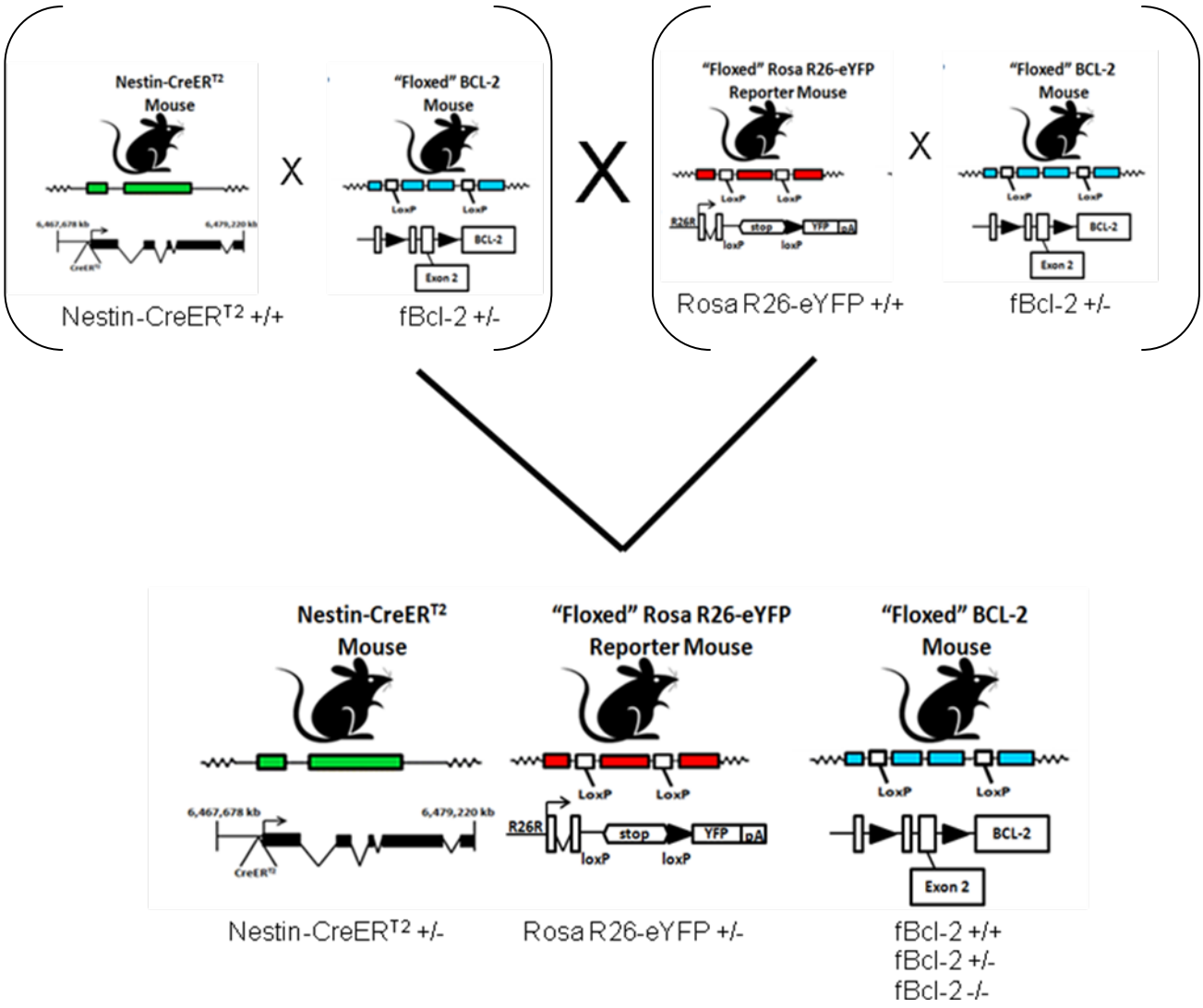
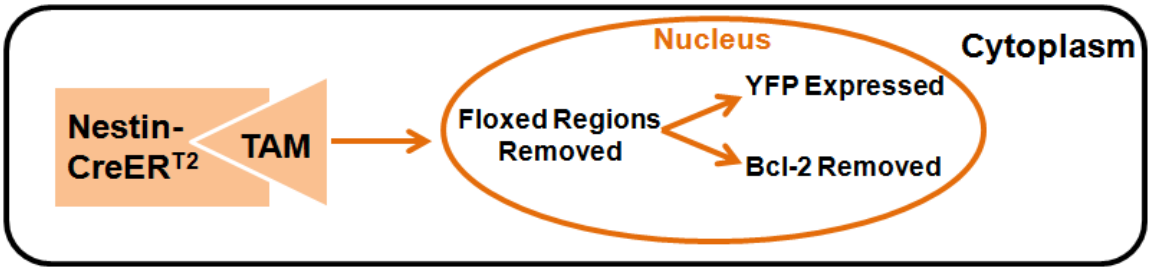


Figure 9. Breeding Strategy Used to Obtain nBcl-2 KO Mice.

Nestin-CreERT2 homozygous/fBcl-2 heterozygous were crossed with Rosa R26-eYFP homozygous/fBcl-2 heterozygous. This pairing produced mice that were all heterozygous for Nestin-CreERT2 and Rosa R26-eYFP and were either homozygous, heterozygous or WT for fBcl-2. Mice that were used for experiments were homozygous or WT for fBcl-2.

A



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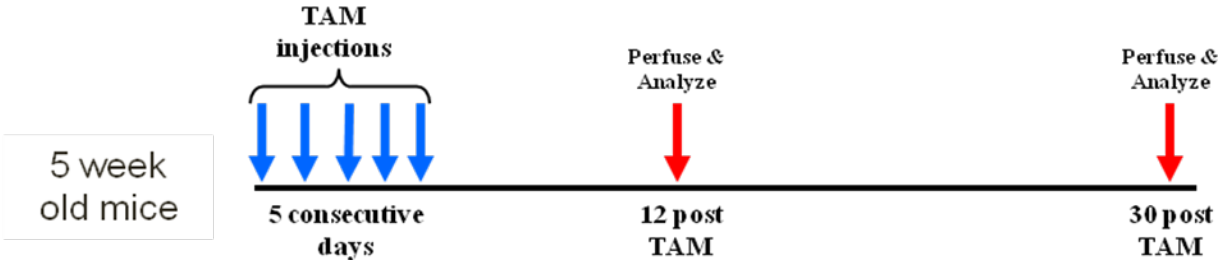

Figure 10. Inducible Nestin-CreERT2/R26R-eYFP/fBcl-2 (nBcl-2) Mice Allows for Removal of Bcl-2 and Specific Recombination in Nestin-expressing PCs and their Progeny.

A) nBcl-2 mice are administered the estrogen ligand Tamoxifen (TAM) to allow CreER^{T2} to recombine DNA at loxP sites, removing a STOP codon in R26R-eYFP mice and Exon 2 in fBcl-2, allowing YFP expression and removal of Bcl-2 in recombined PCs. **B)** nBcl-2 and WT littermates are administered TAM for 5 consecutive days and sacrificed at 3, 12, and 30 day after their last day of TAM treatment.

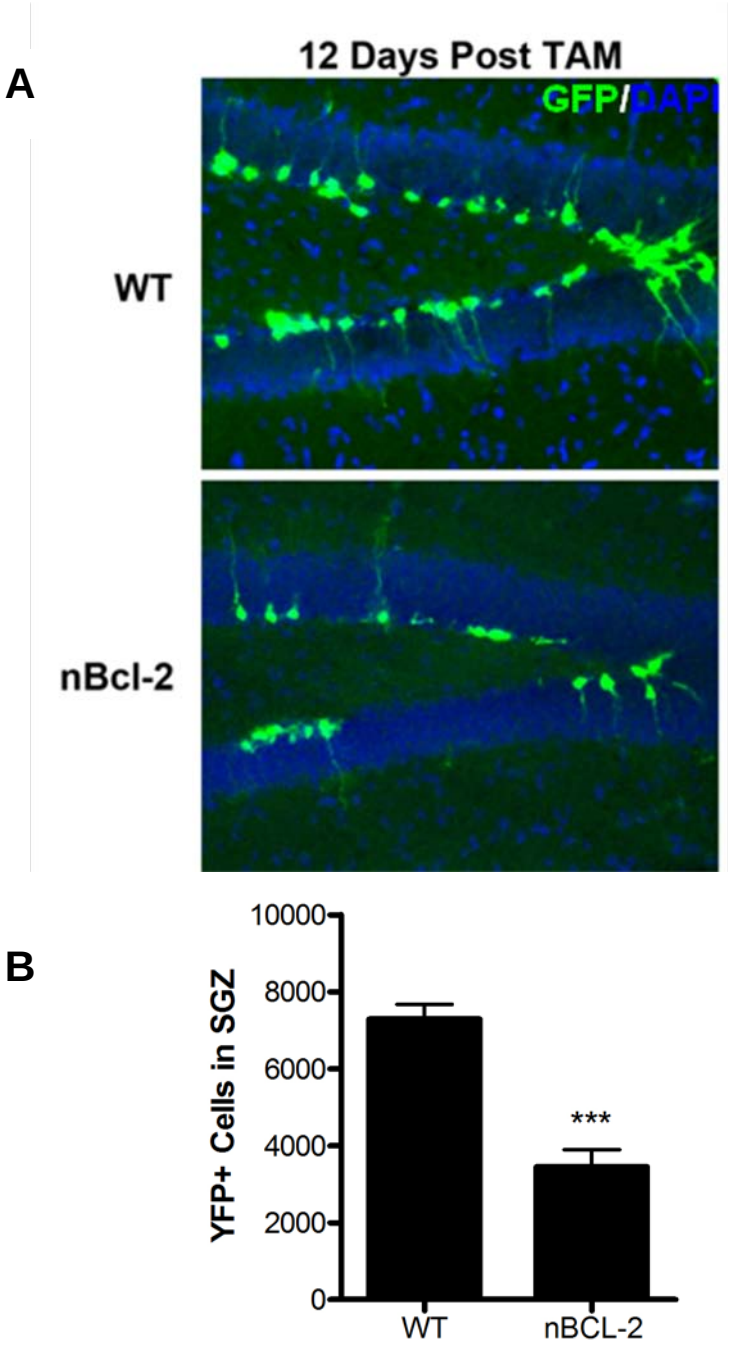

Figure 11. The nBcl-2 KO Mice Have a Significant Reduction in Number of Recombined Cells Compared to WT Littermates at 12 days after TAM.

Representative images **(A)** and quantification **(B)** of GFP+ PCs in WT versus nBcl-2 KO mice treated at 12 days after TAM.

(Tashiro et al., 2006b; Lagace, 2008). In agreement with our retroviral results, there was increased cell death in the nBcl-2 KO animals ($t(14)=2.7$, $p<0.05$) **(Figure 12)** suggesting that removal of Bcl-2 is contributing to the reduced number of PCs and their progeny in the nBcl-2 KO mice.

In order to determine at what stage during PC development Bcl-2 is functionally important, the recombined PCs were analyzed for expression of proteins that label stem cells, dividing cells, and immature neurons. There is no single marker that can detect stem-like cells, therefore we analyzed the PCs for expression of GFAP and Sox2 in order to classify the cells as stem-like (Lagace et al., 2007b). At 12 day post TAM, nBcl-2 KO had a significantly reduced proportion of PCs that had a stem cell-like phenotype when compared to WT mice ($t(8)=2.51$, $p<0.05$) **(Figure 13).**

The proportion of proliferating PCs in the nBcl-2 KO and WT mice was analyzed through examination of the recombined cells for expression of Ki67, which is a nuclear protein expressed in G1, G2 and S phase of the cell cycle (Mandyam et al., 2007). Unlike the significant reduction in stem-like cells in the nBcl-2 KO mice, there was no difference in the proportion of cycling (Ki67+) PCs in the nBcl-2 KO and WT mice **(Figure 14).**

Lastly, the proportion of the PCs that had an immature neuronal phenotype was classified by expression of DCX as was previously done with retroviral experiments. This analysis revealed a significant reduction in proportion of Bcl-2

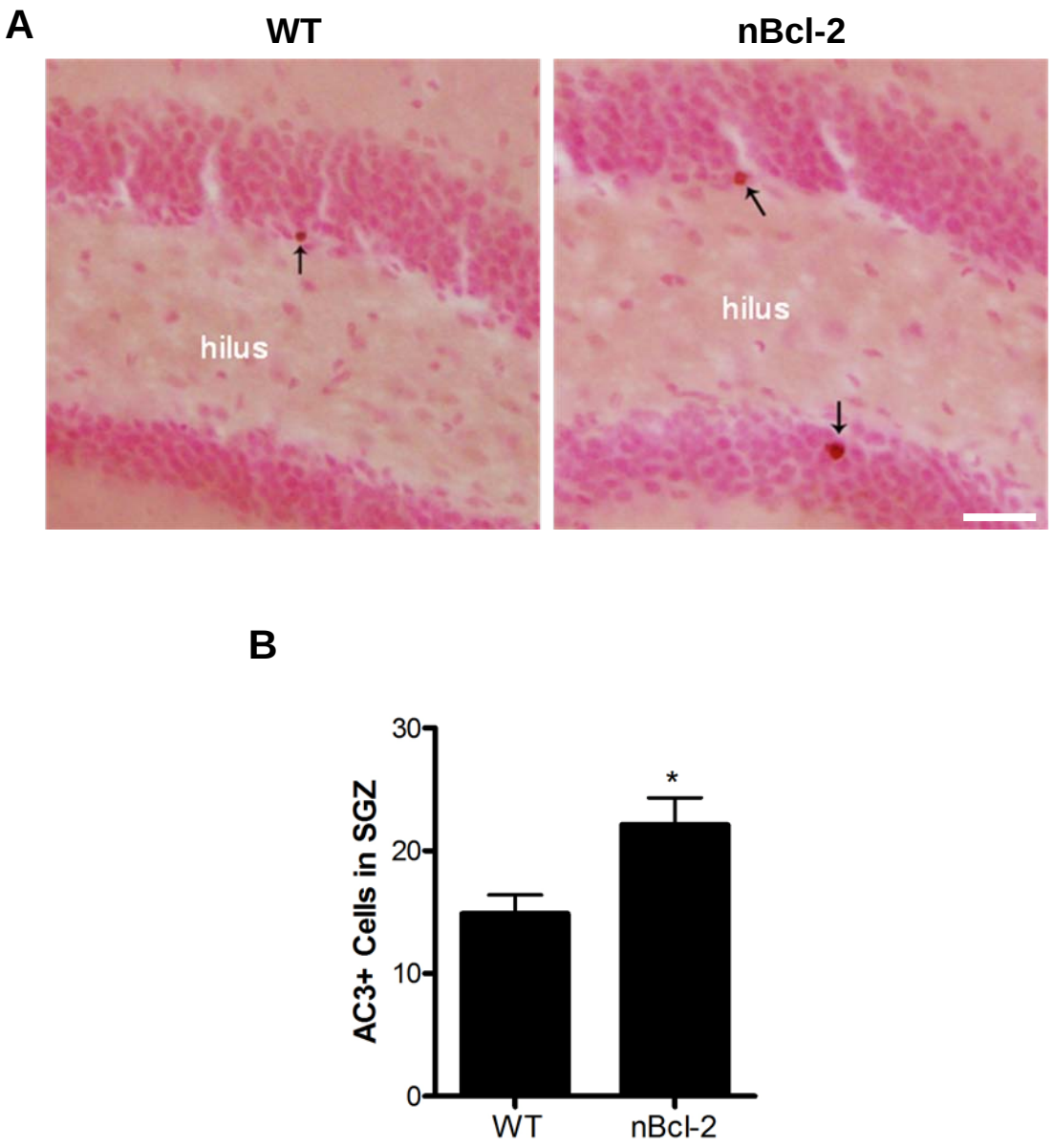

Figure 12. The nBcl-2 KO Mice Have Increased Cell Death at 12 days after TAM

A) Representative Image and **B)** quantification of total number of activated caspase 3 (AC3) cells shows a significant difference at 12 days post TAM between WT and nBcl-2 KO mice.

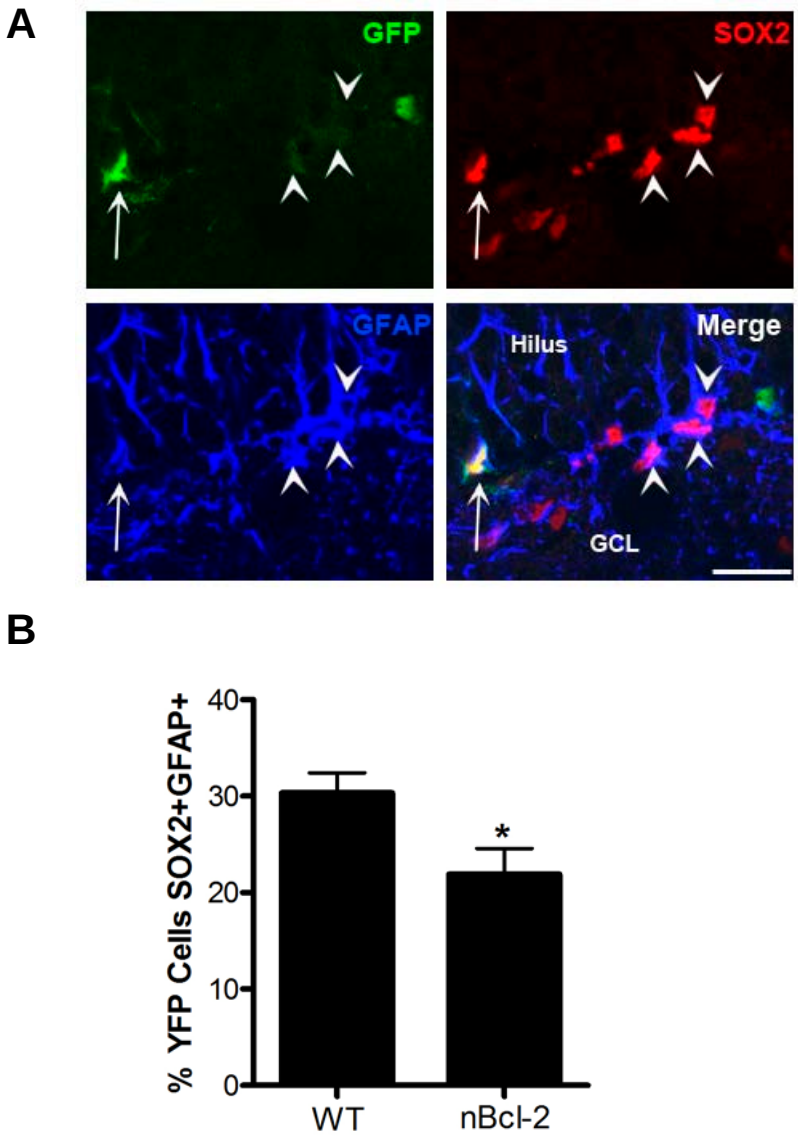

Figure 13. nBcl-2 KO Mice Have Significantly Reduced Number of Stem-like PCs

A) Representative image of GFP, Sox2, GFAP triple positive cells (arrow) and Sox2+GFAP+ (arrowhead) in nBcl-2 KO mice. **B)** nBcl-2 KO mice have a significant reduction in YFP+ NPCs expressing Sox2 and GFAP. (Scale Bar: 20um)

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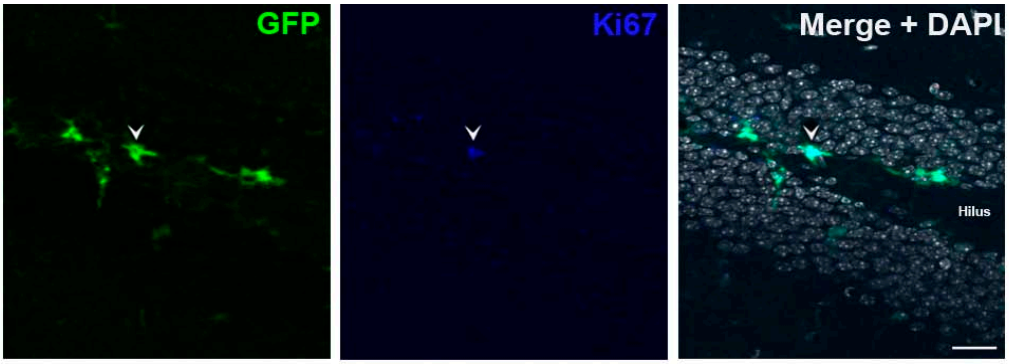

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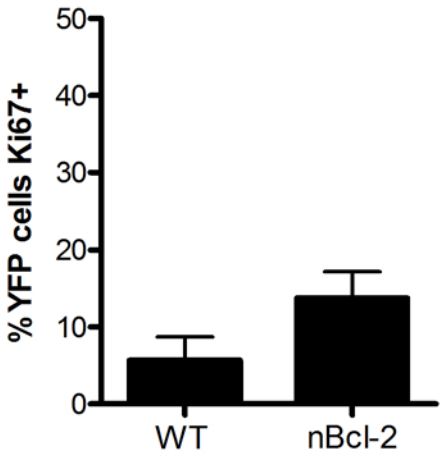


Figure 14. nBcl-2 KO Mice Do Not Have Any Difference in the Proportion of Proliferating Cells (GFP+Ki67+)

A) Representative image of GFP, Ki67, and DAPI staining in the SGZ with GFP+Ki67+ PCs shown with an arrowhead. **B)** No significant difference was found in proportion of PCs dividing (Ki67+), but there is a trend for the nBcl-2 KO mice to have more dividing cells. (Scale Bar: 20um)

null immature neurons in the nBcl-2 KO mice compared to WT mice ($t(8)=2.45$, $P<0.05$) **(Figure 15)**.

Together these findings suggest Bcl-2 has a role in regulating the number of stem-like PCs and immature neurons, but does not alter the proportion of proliferating PCs. It seems paradoxical that there would be a decrease in the proportion of stem-like cells that does not result in a concurrent reduction in proportion of proliferating PCs. One possibility is that there is no change in the proportion of proliferating PCs because there is compensation that involves enhanced proliferation due to the reduction in stem and immature neuronal populations. In support of this hypothesis there was a non-significant trend for increased number of proliferating cells in the fBcl-2 mice compared to WT controls. In order to address this question and examine the longer time effect of removal of Bcl-2 from the nestin-expression PCs, animals were analyzed 1 month after TAM.

3.5 Removal of Bcl-2 in Nestin-expressing Cells and their Progeny Altered the Mature Neuron Population Developing at 30 Days

There was no significant difference in the number of recombined (GFP+) **(Figure 16A-B)** or apoptotic (AC3+)**(Figure 17D)** cells in the nBcl-2 KO and WT mice 30 days after TAM treatment. This was surprising given the significant reduction in number of Bcl-2 null PCs at 12 days following TAM. The recombined GFP+ Bcl-2 null and WT PCs were phenotyped and there was also

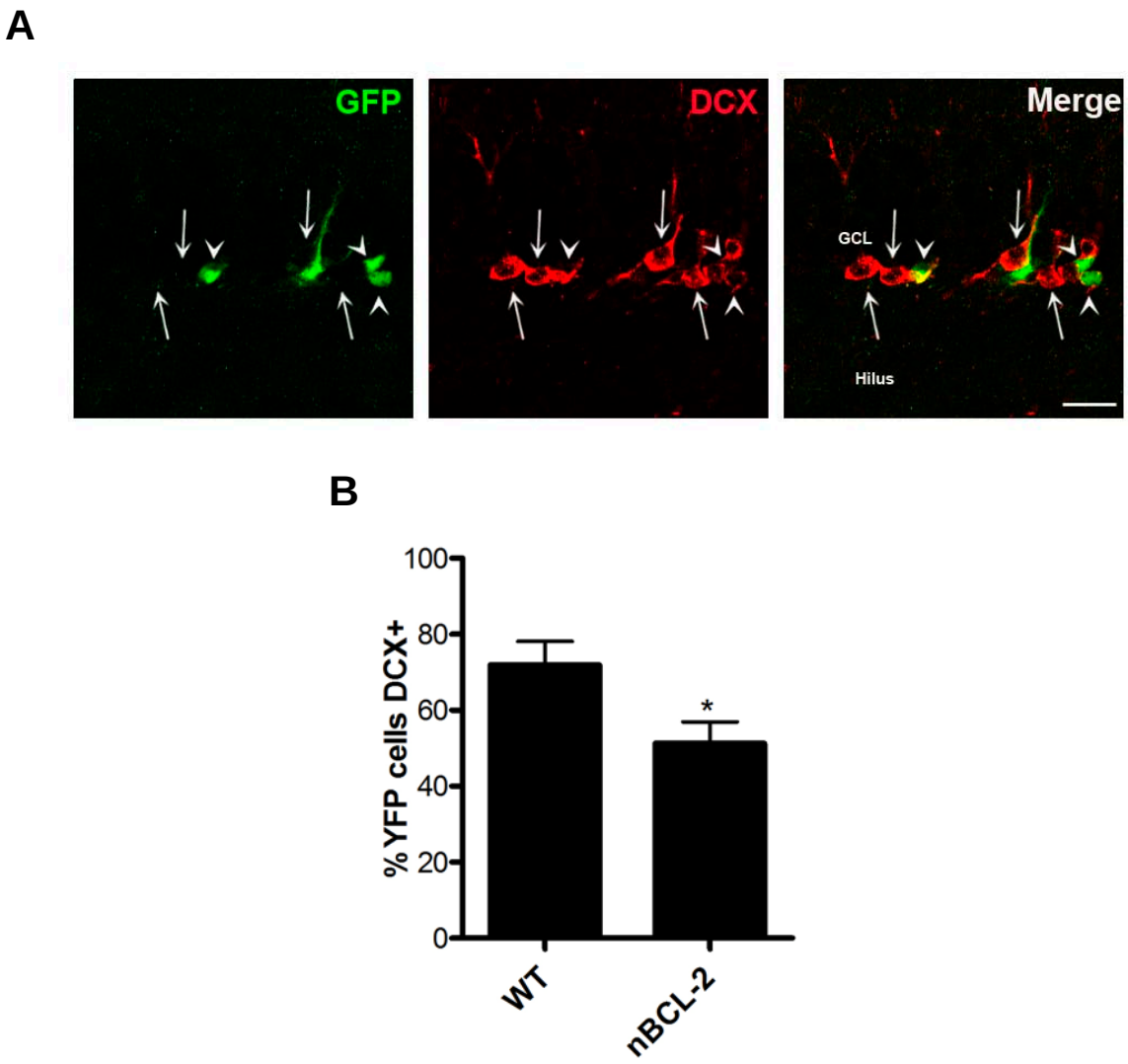


Figure 15. nBcl-2 KO Mice Have Significantly Reduced Proportion of Immature Neurons (GFP+DCX+)

A) Representative images of GFP and DCX staining in the SGZ, with GFP+DCX+ PCs shown by arrowheads and DCX+ only by arrows. **B)** There is a significant reduction in proportion of immature neurons (DCX+) in nBcl-2 KO mice (Scale Bar: 20um)

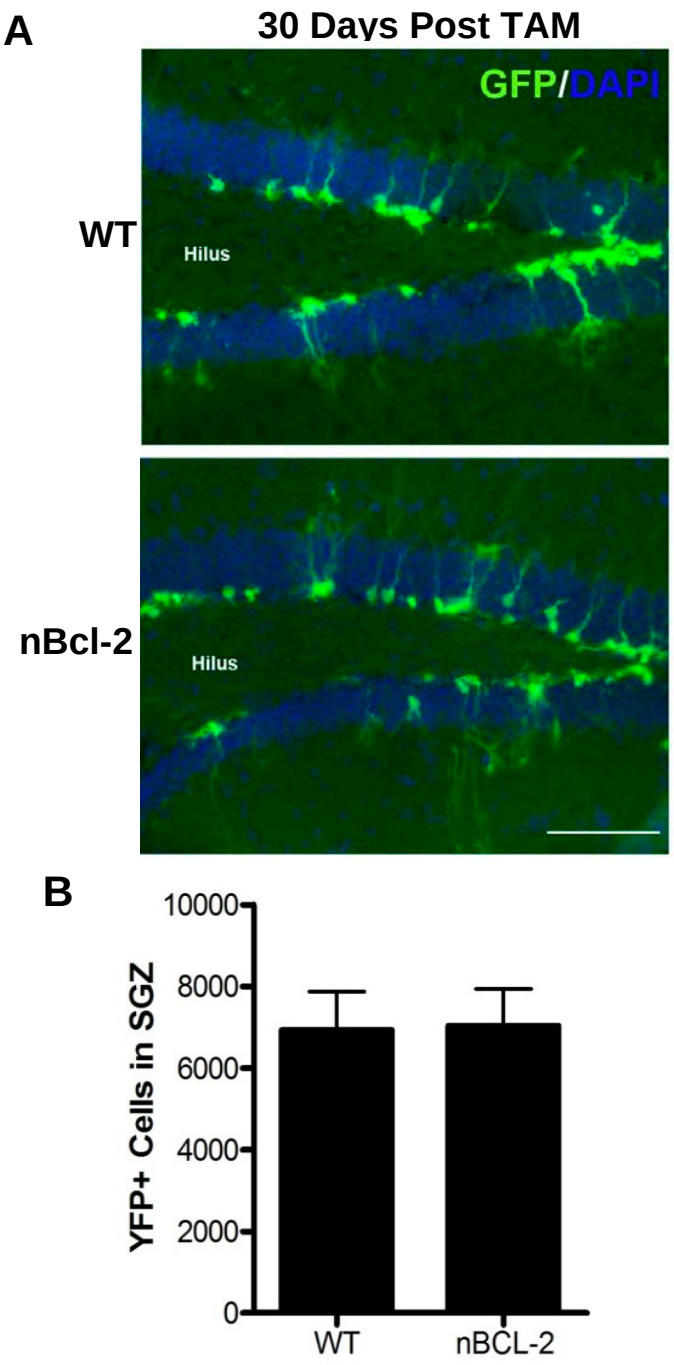


Figure 16. The nBcl-2 Mice at 30 Days After Removal of Bcl-2 Do Not Have Altered Number of Recombined PCs Compared with WT Mice.

A) Representative Image and **B)** quantification of GFP+ cells at 30 days after TAM resulted in no difference in the PCs.

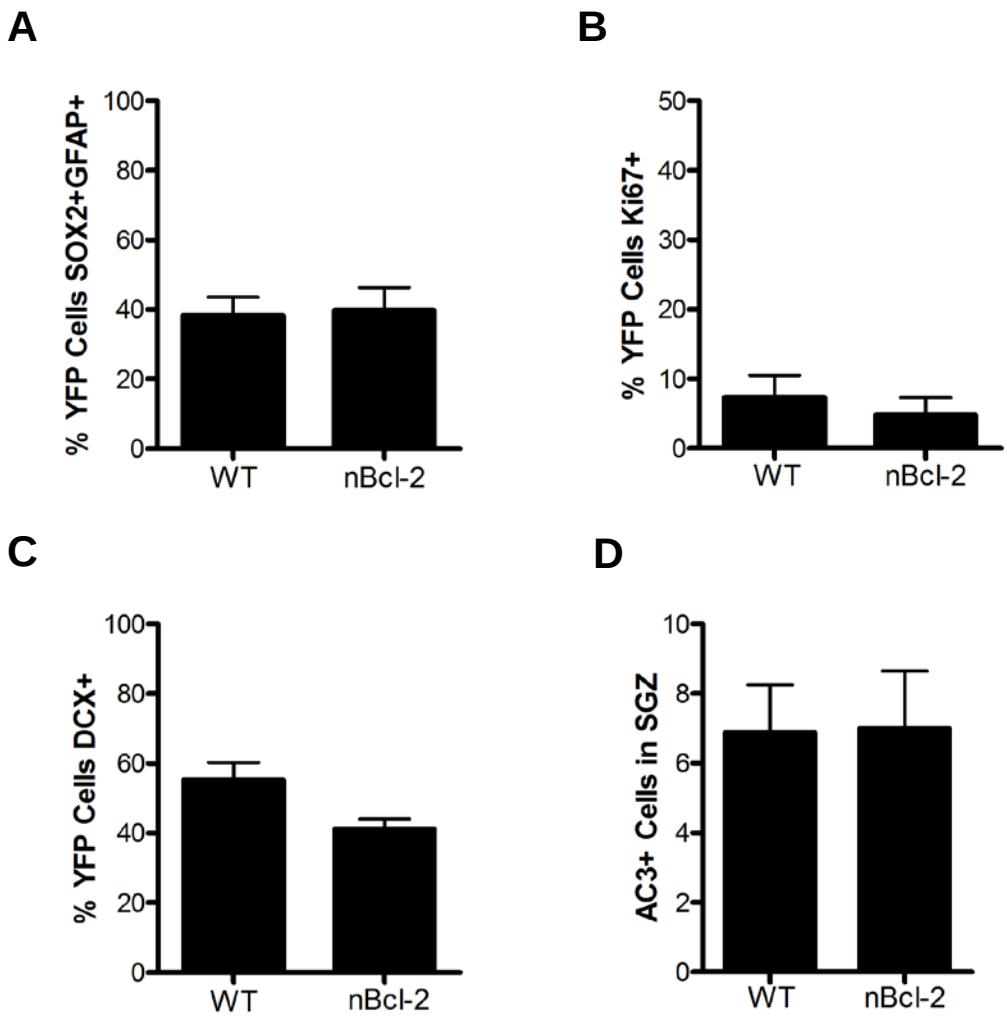
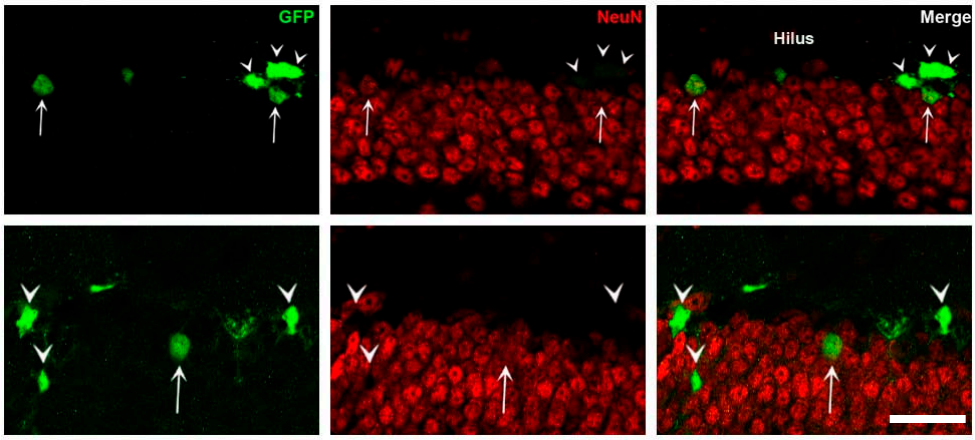


Figure 17. The nBcl-2 Mice at 30 Days After Removal of Bcl-2 Did Not Have Altered Proportion of Phenotyped PCs or Changes in Number of Dying Cells (AC3+).

A) GFP+ cells were phenotyped with SOX2+GFAP+ for stem cells **B)** Ki67+ for proliferating cells **C)** DCX+ for immature neurons showed no significant difference in the proportion of nBCL-2 KO animals compared with the WT littermates. **D)** No difference was seen in the number of AC3+ cells in the SGZ at 30 days post TAM.

A



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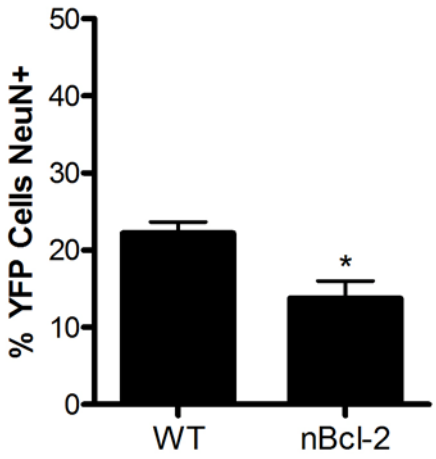

Figure 18. The nBcl-2 Mice at 30 Days After Removal of Bcl-2 Have Fewer Recombined Neurons Expressing NeuN.

A) Representative Image and **B)** quantification shows a significant difference in the proportion of mature neurons, NeuN+ (Arrowheads indicate GFP+ only cells; Arrows are GFP+NeuN+).

no significant difference in the proportion of recombined stem-like (GFAP+Sox2+), proliferating (Ki67+) or immature neuron (DCX+) population between the nBcl-2 and WT mice **(Figure 17A-C)**. The proportion of recombined PCs that expressed the mature neuronal marker, NeuN (Ming and Song, 2011) was also determined since others have previously shown in this model that recombined NeuN+ cells can be detected at 30 days post TAM (Lagace et al., 2007b). There was a significant decrease in proportion of recombined mature neurons that expressed NeuN in the nBcl-2 KO compared to WT mice ($t(8)=3.2$, $p<0.05$) (**Figure 18)**. These findings are interesting as they support our hypothesis that compensation may occur, as there is a loss of PCs at 12 days but no change in number of PCs and progeny labeled at 30 days post TAM.

4. DISCUSSION

During the process of adult neurogenesis relatively few new neurons are created despite the large number of dividing cells. This apparent inefficiency arises due to apoptosis occurring through the developmental stages of neurogenesis (Biebl et al., 2000; Tashiro et al., 2006b; Sierra et al., 2010). The present study addresses the need to identify regulators of apoptosis in adult neurogenesis by specifically examining the role of the anti-apoptotic protein, Bcl-2. Experiments using both viral-mediated gene transfer and an inducible transgenic mouse approach demonstrate that Bcl-2 has an essential cell-autonomous role during adult neurogenesis. Removal of Bcl-2 specifically from adult-generated cells reduced the stem-cell population, immature neuron population and newly formed mature neurons. Additionally the effects of Bcl-2 on adult neurogenesis could be rescued by the removal of BAX which further supports Bcl-2 effects on adult neurogenesis are BAX dependent. Together with the findings from other recent papers (Sierra et al., 2010; Sahay et al., 2011), these data demonstrate that Bcl-2/BAX mediates apoptosis during neurogenesis in the adult hippocampus.

4.1 Bcl-2 has a Cell-Autonomous Essential Role in Adult Neurogenesis

Retroviral-mediated removal of Bcl-2 completely prevented new neurons from developing within the dentate at 30 dpi. This effect appeared to be mediated

by Bcl-2 actions within the immature neurons (type-2b/3) since there was a significant reduction in the proportion of Bcl-2 null cells that expressed DCX. Similarly, in the nBcl-2 KO mice there was a significant reduction in proportion of Bcl-2 null cells that expressed DCX at 12 days after TAM. These findings suggest that the DCX+ population is especially susceptible to apoptosis, which supports previous findings (Kuhn et al., 2005; Tashiro et al., 2006b; Tashiro et al., 2006a; Sierra et al., 2010). For example, Kuhn et al., (2005) demonstrated that in the adult SGZ the TUNEL-positive PCs are most frequently colabeled with DCX. More recently, Sahay et al. (2011) identified an increase in the number of DCX+ PCs after the removal of BAX. In combination with our data, this work strongly suggests that PCs are susceptible to apoptosis at the immature neuron stage of development.

It is hypothesized that PCs expressing DCX are most susceptible to cell death is because at this time period the PCs begin to express NMDA-type glutamate receptors and their survival is dependent on synaptic activity (Nacher and McEwen, 2006; Abdipranoto et al., 2008). Evidence to support this claim illustrated viral-mediated removal of NR1 significantly enhanced the death of PCs and this occurred most frequently in PCs expressing DCX (Tashiro et al 2006). Moreover the death of PCs occurred after 14 days, and was highest at 21 days after removal of NR1. Interestingly, in comparison, we find that the death of PCs occurred after 7 days, and resulted in complete ablation of PCs at 30 days after removal of Bcl-2. The differences in these time courses suggest that the Bcl-2

mediated apoptosis observed in our study is likely not only due to apoptosis that is induced through the NR1 subunit since we have effects that occur earlier than NR1 expression. This then raises the possibility that the death of PCs during adult neurogenesis by apoptosis may not always be mediated by an NR1 dependent mechanism.

The complete ablation of new neurons following removal of Bcl-2 by a retroviral expression of Cre can raise the concern that our results are due to the potentially confounding toxic effects of overexpressing Cre (Forni et al., 2006). However, WT mice transduced with Cre had no difference in survival compared to non-Cre (RFP+) infected cells. Moreover, unpublished data from our lab and the published work of others has also reported that viral-mediated expression of Cre does not prevent adult neurogenesis (Tashiro et al., 2006b; Jagasia et al., 2009). In fact, others have shown that not all PCs are removed after viral expression of NR1 (Tashiro et al., 2006b) or CREB (Jagasia et al., 2009). In contrast our findings demonstrate a complete ablation of PCs following removal of Bcl-2. This result suggests that Bcl-2 is essential for adult neurogenesis and there are no other mechanisms that can compensate for the removal of Bcl-2 to sustain PC survival.

4.2 Bcl-2-Mediated Effects on the Stem-cell Like Population

Removal of Bcl-2 from the stem-cell like population in the nBcl-2 KO mice provided insight into the role of Bcl-2 in this slowly dividing population. Our findings are consistent with previous studies that have suggested stem-like PCs

are susceptible to apoptosis mediated cell-death (Sleeper et al., 2002; Sierra et al., 2010). More specifically, Sleeper et al., (2002) identified the expression of stem-like cell marker, nestin, to be colabeled with the apoptotic marker, TUNEL. Additionally, Sierra et al., (2010) identified nestin-positive cells to be phagocytized by microglia. Both studies used nestin as a marker to identify these PCs, however, only a subset of the nestin population are identified to be stem-like. In comparison, we used both GFAP and Sox2 to identify stem-like cells and discovered specifically in our nBcl-2 KO mice at 12 days following removal of Bcl-2 from PCs there was a significant reduction in this population.

Since there is a debate about how best to quantify and phenotype the stem-like cells in the SGZ (Palmer et al., 1997; Seaberg and van der Kooy, 2002a), ongoing work is examining stem cells in the SVZ to further assess whether Bcl-2 induces apoptosis in adult stem cells. Since cells migrate from the SVZ to the RMS and OB as they mature analysis of the stem cells in the SVZ will provide more insight into whether Bcl-2 is essential for adult neuronal stem cells. Ongoing work will also determine if treating neurospheres from fBcl-2 mice with virally expressed Cre alters the survival of spheres. One caveat of this approach is that since this is an *in vitro* assay, the results may not be representative of the *in vivo* approaches. As an alternative, future experiments could ablate Bcl-2 in only the stem-like PCs using the newly developed Hes5-CreERT2 (Lugert et al., 2012) mouse that specifically allows for inducible Cre expression in only the

stem-like cells instead of the nestin-CreERT2 mouse which targets both stem- and dividing PCs.

4.3 Differences in Neurogenesis between Floxed Bcl-2 Mice Infected with Retroviral Cre and nBcl-2 KO Mice

One of the most surprising findings of this study was that the nBcl-2 KO mice had no decline in Bcl-2 null PCs at 30 days post tamoxifen. This was an unexpected finding since retroviral-mediated removal of Bcl-2 in PCs completely ablated the Bcl-2 null PCs. One possible explanation for this finding could be that in the nBcl-2 KO mice the loss of Bcl-2 was able to be compensated, whereas there was no compensation in fBcl-2 mice injected with retroviral Cre. This ability to compensate may be due to the difference between a retroviral-mediated versus nestin-Cre inducible transgenic gene ablation approach in the adult neurogenic cells. Although both the retroviral and inducible nestin KO mouse model specifically target the neurogenic PCs, our data and the work of others show that the retrovirus infects mostly rapidly dividing cells (Tashiro et al., 2006b; Jessberger et al., 2008; Jagasia et al., 2009), whereas the nestin-Cre targets stem-like and some dividing PCs (type 1/2a) (Lagace et al., 2007a). Furthermore the viral approach targets approximately 1000x fewer cells than the inducible transgenic approach. Thus, it may be that due to targeting a larger population of PCs at an early stage of development the Bcl-2-null PCs are able to be compensated through feedback mechanisms to restore levels of neurogenesis to levels that are the same as that observed in wild-type mice.

4.4 The Expression of Bcl-2 During Adult Neurogenesis

Overall our results suggest that Bcl-2 has a role in type-1 stem-like cell and immature neurons in the SGZ. These results imply also that the Bcl-2 protein is expressed and active in both of these PC populations. In support of this, early work by Merry et al., (1994) identified Bcl-2 expression in postnatal and adult hippocampus and suggested Bcl-2 expression was high in the neurogenic regions. Bcl-2 mRNA and protein expression within the hippocampus has also been demonstrated to be regulated by conditions (such as stress or antidepressant treatment) that can alter the level of neurogenesis (Perera et al., 2007; Shishkina et al., 2010). Identification of what exact stages during PC development in the hippocampus Bcl-2 is expressed remains unknown. We tried using 3 different antibodies and various immunohistological techniques to identify Bcl-2 (unpublished data). Similar to others, we have found that this is a difficult question to answer with the antibodies that are available due to their nonspecific effects. Therefore ongoing work is attempting to identify the expression of Bcl-2 in different populations using fluorescence activated cell sorting (FACS). Using offspring from Nestin-GFP and DCXDsred mice, we are specifically aiming to sort out the GFP+, Dsred+, and combined GFP and Dsred+ population which represent the stem-like, immature neurons and combination of both stem-like and immature neurons, respectively. Following isolation of the cells, future work

would then perform either RNA and/or protein analysis to identify the expression of Bcl-2 in these distinct populations.

4.5 The Role of Bcl-2 in Regulating Apoptosis in Adult Neurogenesis

The role of Bcl-2 in adult neurogenesis is dependent on BAX as demonstrated by Bcl-2 null PCs being rescued when BAX was also removed. This suggests that Bcl-2-BAX are involved in adult neurogenesis and raises questions about the mechanism by which Bcl-2 regulates cell death during adult neurogenesis. For example, would the BH3 only members of the Bcl-2 family which have been identified to interact with Bcl-2 and regulate its function in the apoptotic pathway (Esposti, 2002; Martinou and Youle, 2011) also contribute to the role of Bcl-2 in apoptosis in adult neurogenesis? Secondly, would the phosphorylation status of Bcl-2 be an important regulator of adult neurogenesis? It has been previously shown that phosphorylation of Bcl-2 by Cdk5 in post mitotic retinoic ganglionic cells regulates apoptosis and support neuronal survival. It has also been shown that Cdk5 has an essential role in neuronal maturation and survival during adult neurogenesis (Jessberger et al., 2008; Lagace, 2008). Thus it is possible to hypothesize that Cdk5 is essential for adult neurogenesis due to its ability to phosphorylate Bcl-2. To address this hypothesis future studies could be completed that utilize a recently developed non-phosphorylatable Bcl-2 knock-in transgenic mouse (He et al., 2012).

Lastly, it is interesting to speculate that Bcl-2 may not only regulate apoptosis to cause cell death, but may also be regulating autophagy-mediated cell death

during adult neurogenesis. Autophagy is a conserved pathway in which proteins and organelles are sequestered to the lysosome where they are degraded and recycled. The autophagic process functions to promote survival by removing mis-folded proteins or damaged organelles through the digestion of essential constituents of the cell(Mizushima, 2007). Within the autophagy pathway, Bcl-2 is identified to bind to Beclin-1, a key autophagic regulator, to inhibit autophagy (Pattingre et al., 2005). Specially, it has been shown that pBcl-2 allows for the dissociation of the Bcl-2/Beclin-1 complex and subsequent activation of the autophagy pathway. Unpublished data from our lab has recently identified autophagy to play a role in the survival of PCs in the adult hippocampus. Since autophagy provides for another mechanism through which PCs could die, it will be very interesting to further explore if the observed effects of Bcl-2 are due to autophagy-mediated cell death, in addition to apoptosis-mediate cell death.

Conclusion

In conclusion, two different *in vivo* knockout strategies have identified the anti-apoptotic protein Bcl-2 is essential for the survival of PCs in adult hippocampal neurogenesis. Moreover this work begins to reveal the mechanism(s) by which Bcl-2 functions during adult neurogenesis by demonstrating its dependence on BAX. Uncovering the basic molecular mechanisms regulating this process in the adult brain is important since it is hoped that this will help develop novel therapeutics that could enhance the survival of PCs.

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