Mcl-1 IS A CRITICAL SURVIVAL FACTOR IN NEURAL PRECURSOR CELLS OF THE ADULT MAMMALIAN BRAIN

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Mcl-1 is a Critical Survival Factor in Neural Precursor Cells of the Adult Mammalian Brain

By

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Abstract

Although the aetiology of many neurodegenerative diseases differ, they all share a common feature in that they result in neural cell death. Neural precursor cells (NPCs) of the adult brain are thought to be capable of regenerating the injured brain by replacing cells that are injured or have died. NPCs of the adult brain are maintained in a balance of proliferation and death; however, little is known about the molecular mechanisms that regulate these processes. As Mcl-1 is a critical survival factor for NPCs in the embryonic brain, I questioned whether Mcl-1 regulates the survival of adult NPCs. To determine whether Mcl-1 is expressed in adult NPCs, I used BrdU to identify different subpopulations of NPCs based on their proliferation kinetics. In doing so, I demonstrated that Mcl-1 is expressed in both the neural stem and neural progenitor populations of the adult subventricular zone (SVZ). To determine the role of Mcl-1 in adult NPC's, I used the Cre/*lox* conditional knockout system to conditionally knock out Mcl-1 in adult NPCs. To assess Mcl-1 loss-of-function in NPCs *in vitro*, I first drove Cre recombinase off the chicken β-actin promoter and stained the cultures with Nestin. Secondly, I drove Cre recombinase off the Nestin promoter (NesCre), which resulted in Cre expression specifically in NPCs. In both experiments, Mcl-1 loss-of-function resulted in a 2-fold increase in apoptosis in Cre transfected Mcl-$1^{f/f}$ NPCs. When Mcl-1 loss-of-function was characterized *in vivo*, there was a 2-fold increase in apoptosis in Cre transfected Mcl-$1^{f/f}$ NPCs. Finally, Mcl-1 gain-of-function was assessed, *in vitro*, which resulted in a 2-fold reduction of apoptosis in $Nestin^{+}$ NPCs. Collectively, these data demonstrate that Mcl-1 is a prosurvival factor in adult NPCs. Therefore, Mcl-1 gain-of-function may lead to an expansion of the adult NPCs, which could offer a putative therapy for neurodegenerative conditions and ultimately facilitate neural regeneration.

Abbreviations

1X PBS = 1X phosphate buffer solution

Apaf-1 = apoptotic protease activation factor-1

Bcl-2 = b cell lymphoma 2

BrdU = 5-bromo-2-deoxyuridine

CARD = caspase recruitment domain

CNS= central nervous system

DNA = deoxyribonucleic acid

Dlx2 = Distal-less2

EGF = epidermal growth factor

FGF-2 = fibroblast growth factor 2

GFAP = glial fibrillary acidic protein

GFP = green fluorescent protein

Mcl-1 = myeloid cell leukemia 1

Mcl-$1^{f/f}$ = myeloid cell leukemia 1 homozygous floxed alleles

NesCre = Cre recombinase driven off of the Nestin enhancer promoter

NesGFP = green fluorescent protein off of the Nestin enhancer promoter

NSCs = neural stem cells

OMM = outer mitochondrial membrane

PCR = polymerase chain reaction

PFA = para-formaldehyde

SGZ = subgranule zone

SVZ = subventricular zone

TACs = transient amplifying cells

TPBS = tween20 phosphate buffer solution

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Chapter 1

Introduction

1.1 Adult Neural Stem Cells and Neurodegenerative Disease

Although the etiology of many central nervous system (CNS) diseases such as stroke, Alzheimer's disease and Parkinson's disease differ, they all share a common feature in that they result in neural cell death, which leads to functional impairment. Various cell replacement therapies have been attempted including transplant therapies; however, cell death remains the biggest drawback to exogenous cell therapies. With the discovery of endogenous neural stem cells (NSCs) in the adult mammalian brain (Reynolds & Weiss, 1992), there has been a lot of excitement surrounding the possibility of therapeutically manipulating these populations in a variety of neurodegenerative conditions. For instance, several studies have shown a transient increase in cellular proliferation in the subventricular zone (SVZ) after ischemic stroke (Arvidsson *et al.,* 2002; Jin *et al.,* 2001). However, this response from the SVZ seems to be insufficient as only a small number of cells differentiate into neurons. By identifying the molecular mechanisms regulating proliferation, survival, migration, differentiation and integration, it may be possible to optimize neurogenesis and ultimately regeneration after neural degeneration or injury. This thesis focuses on the survival of neural precursor cells (NPCs) and puts forward the hypothesis that the expression levels of a specific protein, myeloid cell leukemia 1 (Mcl-1), dictates the sensitivity of NPCs to apoptosis within the adult mammalian brain.

1.2 Neural Precursor Cells of the Adult Mammalian Brain

In 1992, Reynolds and Weiss (1992) used the two defining properties of all stem cells, self-renewal and potentiality, to show that cells found within the SVZ of 3- to 18-month old adult mice are mitotic and have the capacity to differentiate into neural lineages *in vitro*, thus providing empirical evidence for the existence of adult NSCs. Subsequent studies have found that adult neurogenesis occurs within the SVZ and the hippocampal subgranular zone (SGZ). Within the SVZ there are several subpopulations, which are collectively known as NPCs (Figure 1). The most primitive cell types are the neural stem cells (type B cells), which are defined as having long-term, self-renewing capacity and multipotentiality, meaning that they can differentiate into all three neural lineages, which include neurons, astrocytes and oligodendrocytes (Morshead et. al, 1998). The more restricted type C transit amplifying cells (TACs) have lost their self-renewal capacity and are restricted to a single neural cell lineage (Morshead et. al, 1994). These cells give rise to the tangentially migrating neuroblasts (type A cells) that migrate to the olfactory bulb via the rostral migratory stream and differentiate into both granule and periglomerular inhibitory neurons (Doetsch, 2003). Table 1 provides a list of phenotypic markers that will be used to identify these cell types in our work.

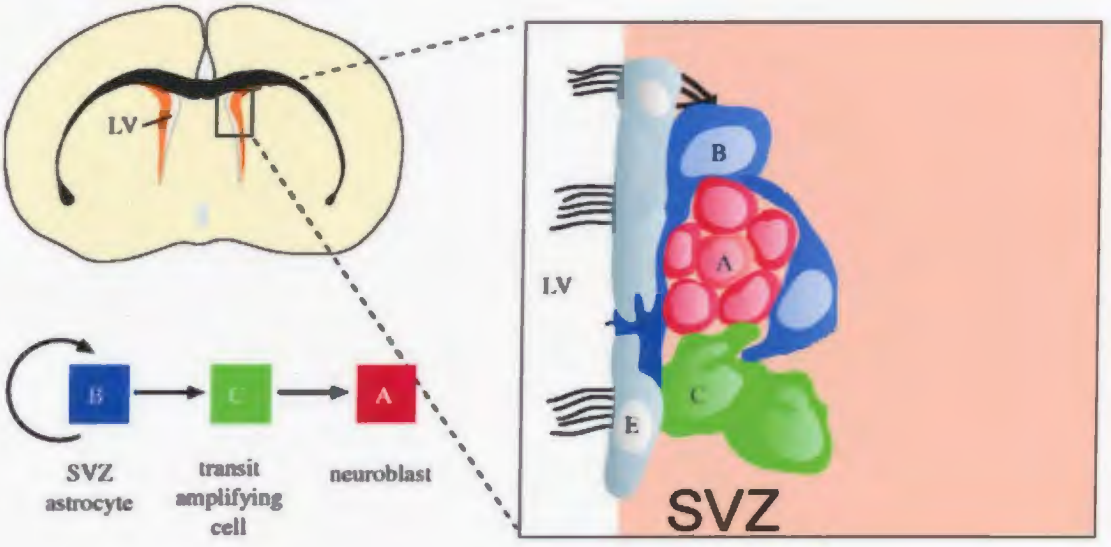
The type B, NSCs persist in the adult mammalian brain largely due to their specialized microenvironments or niches that support their lifelong self-renewal and multipotential capabilities. As stated above, one of the main neurogenic niches includes the SVZ (Figure 1). This area consists of a thin layer of dividing cells that is separated from the cerebrospinal fluid by a layer of multi-ciliated ependymal cells. This dividing layer contains the type B cells, which

have been identified as astrocytes based on their ultrastructural features, phenotypic markers as well as electrophysiological properties (Doetsch *et al.,* 1999). Although these SVZ astrocytes have the characteristics of multipotent stem cells both *in vitro* and *in vivo*, there is some debate as to whether the SGZ neurogenic niche contains neural stem cells or only committed neurogenic precursors (Seaberg & van der Kooy, 2002). Therefore, this thesis will focus on the SVZ NPC's from this point unless otherwise stated.

	Neural Precursor Cells			
		Neural Progenitor Cells		
	Type B Neural Stem Cells	**Type C Transit Amplifying cells**	**Type A Neuroblasts**	**Neurons**
GFAP	+			
SOX2	+			
Nestin	+	+	+	
Dlx2		+	+	
PSA-NCAM			+	
Dcx			+	
Tuj-1			+	
NeuN				+

Table 1: Marker expression for the different stages of neural cell differentiation in the adult brain. Modified from Okano *et al.*, 2007

Figure 1. Cell Types of the Adult Subventricular Zone. The SVZ (orange) is adjacent to the lateral ventricles and is lined with multi-ciliated ependymal cells (grey). Type B SVZ astrocytes (blue) are stem cells that give rise to migrating neuroblasts (A, red) destined for the olfactory bulb via the transit-amplifying type C cells (green). (Adapted from Riquelme *et al.*, 2008).


LV
B
C
A
SVZ
astrocyte
transit
amplifying
cell
neuroblast
LV
B
A
C
E
SVZ

1.2.1 Survival and Proliferation of Adult Neural Precursor Cells

The NPCs found within the adult SVZ exist in equilibrium, such that new cells are constantly being produced while cell death is also occurring. In a defining study Morshead and van der Kooy (1992) used the thymidine analog 5-Bromo-2-deoxyuridine (BrdU) to demonstrate that the majority of mitotically active cells of the adult subependyma are the type C and type A neural progenitors, which collectively have a cell cycle time of approximately 12.7 hours. Furthermore, this study suggests that for every neural progenitor cell division, 1 progeny dies. In a subsequent study, Morshead *et al.,* (1994) demonstrated that when the neural progenitors of the subependyma are depleted using high doses of tritiated thymidine (^{3}H-thy) the much smaller, more quiescent NSCs will repopulate the neural progenitor population by temporarily shortening their cell cycle time. Normally, the quiescent NSC population has a cell cycle time of roughly 28 days and constitutes less than 1% of the proliferating subependyma population, *in vivo* (Craig *et al.,* 1994; Morshead *et al.,* 1994). Whether there is an endogenous level of cell death occurring in the NSC population has yet to be determined. However, for every type C division, one progeny undergoes cell death (Morshead and van der Kooy, 1992).

The NPCs of the adult SVZ can also be isolated and grown to form clonal neurospheres *in vitro* when epidermal growth factor (EGF) is added to serum free media (Reynolds and Weiss, 1992; Morshead *et al.*, 1994). Cells that are derived from neurospheres are multipotent and self-renew, thus meeting the criteria of type B neural stem cells. On this basis, it had been assumed that only the quiescent neural stem cells were capable of producing neurospheres (Morshead *et al.*, 1994) and as a result, many used the neurosphere assay to assess how various manipulations to NSCs affected these cardinal properties of neural stem cells. However, Doetsch *et al.*, (2002)

seriously challenged the assumption that type B NSCs were the primary neurosphere forming cells when they showed that the majority of EGF-responsive cells appear to be $Dlx2^+$ neural progenitors. The expression of $Dlx2^+$ is indicative that the neural progenitor cells have begun the differentiation process toward inhibitory neurons destined for the olfactory bulb (Panganiban and Rubenstein, 2002), therefore the demonstration that $Dlx2^+$ cells are capable of returning to a proliferating, sphere forming stem cell underscores the plasticity within the NPC population.

Although we have known since the early 1990's that roughly half of the progeny of type C cell divisions die in the adult brain, little is known about the molecular mechanisms that result in such a high rate of cell death. As the expansion of adult SVZ NPCs is hypothesized to be a putative therapy for a variety of neurodegenerative diseases, understanding how endogenous cell death of adult NPCs occurs is of huge significance. As apoptosis regulates the size of the embryonic NPC population (Kuida, *et al.,* 1998; Woo, *et al.,* 1998), this thesis focussed on identifying a potent anti-apoptotic factor that is expressed in adult NPCs, which could be used to expand the NPC population and provide the injured brain with an enhanced regenerative capacity.

1.3 Regulation of Adult Neural Precursor Cells

1.3.1 Apoptosis

Apoptosis was a term first used by Kerr *et al.*, (1972) to describe an energy dependent form of cell death that is characterized by DNA fragmentation, nuclear condensation and

membrane changes without harming neighboring cells. The two major protein families that regulate apoptosis are the caspase family of proteases and the B cell lymphoma (Bcl-2) family.

1.3.1.1 Caspase Family of Proteases

There have been fourteen caspases identified and all share common characteristics including being aspartate-specific cysteine proteases, having the conserved pentapeptide active site "QACXG" (X can be R, Q or D) (Fan *et al.*, 2005). Furthermore, the caspase family is divided into 3 subfamilies based on amino acid homology: the initiator caspases, which include caspase 9, the apoptosis executioners including caspase 3 and 7, and the inflammatory mediators. With the exception of caspase 14, all of the caspases are translated as inactive zymogens and are regulated at the posttranslational level. The initiator caspases contain domains such as the caspase recruitment domain (CARD) that enable these proteins to interact with other molecules such as apoptotic protease activation factor-1 (Apaf-1), which then cleaves the executioner proteins (Fan *et al.*, 2005). Once activated, the executioner proteins begin cleaving cellular proteins resulting in the morphological changes that are characteristic of apoptosis such as plasma membrane blebbing and nuclear condensation (Fan *et al.*, 2005).

There are a variety of caspase family members involved in both developmental apoptosis and apoptosis that occurs after acute injury or in CNS degeneration. For instance, embryonic NPCs from either caspase 9 or caspase 3 null mice fail to undergo normal developmental apoptosis (Kuida, *et al.*, 1998; Woo, *et al.*, 1998). This results in an increased number of surviving NPCs and differentiated cells causing an expanded ventricular zone and cortex, which ultimately leads to lethality at the perinatal period (Kuida, *et al.*, 1998; Woo, *et al.*, 1998). Furthermore, a variety of injury models of the adult CNS including spinal cord injury, ischemia and traumatic brain injury, have demonstrated that significant cell loss occurs through the

activation of active caspase-3 and ultimately apoptosis (Springer, 2002). In these models, neurons, glia and newly born cells that have migrated from the NSC niches are vulnerable to apoptosis (Springer, 2002). Finally, caspase 3, 6 and 8 activity has been demonstrated in both neurons and glia of Alzheimer's disease models (Zhang *et al.,* 2000). In all models of Alzheimer's disease that identified apoptosis as a contributing factor to degeneration, the apoptotic cascade converged on the activation of caspase 3 (Gervais *et al.,* 1999). Collectively, these data demonstrate that active caspase 3 is the major executioner caspase for CNS apoptosis.

1.3.1.2 Bcl-2 Family

The Bcl-2 family is composed of 12 core proteins that contain at least one of the Bcl-2 homology domains (BH domains) (Chipuk *et al.*, 2010). As described in Figure 2, proteins within the Bcl-2 family are functionally divided into three groups based on the presence of the four BH domains. The anti-apoptotic proteins contain all four BH domains and function to inhibit the oligomerization of the Bax and Bak. The pro-apoptotic proteins are functionally subdivided into two groups: the multidomain effector proteins and the BH3-only proteins. The multidomain, pro-apoptotic proteins, Bax and Bak, contain BH1-3 domains and activate the mitochondrion-mediated pathway of apoptosis after an apoptotic signal has been given. The second major class of pro-apoptotic proteins is the BH-3 only proteins and this group is further subdivided based on their ability to interact with the anti-apoptotic Bcl-2 proteins, or both the anti-apoptotic and multidomain effector proteins. The sensitizer BH-3 only proteins only interact with the anti-apoptotic proteins and the current theory suggests that they displace the activator proteins, which are then free to facilitate the oligomerization of Bak and Bax, which leads to mitochondrial permeablization and ultimately apoptosis (Chipuk *et al.*, 2010).

Within the CNS, Bcl-2 expression peaks from E11-15 in the mouse brain and then begins to decline with little staining occurring at birth (Krajewska *et al.,* 2002). Expression of Bcl-x_L, another Bcl-2 anti-apoptotic protein, peaks in the neural population during the formation of the neural tube and continues to stay high through birth only to decline about 1 week after birth (Krajewska *et al.,* 2002). With regard to the multidomain pro-apoptotic Bcl-2 proteins, it is known that Bax and Bak are both expressed in the proliferative areas of the brain, as concomitant knock out (KO) of these proteins results in hypercellularity (Lindsten *et al.,* 2005). Although there has been some evidence of the BH-3 only protein Bid in the developing CNS (Krajewska *et al.,* 2002), little is known about the expression and/or function of other BH-3 proteins within either the proliferative zones of either embryonic or adult brain. Furthermore, the molecular mechanisms that regulate cell death in the adult NPC population have yet to be identified, despite the fact that this could optimize the therapeutic potential of this population.

1.3.1.3 The Mitochondrial–Mediated Apoptotic Pathway

Figure 3 provides a summary of the molecular mechanisms involved in mitochondrial-mediated apoptosis. Multiple apoptotic signals such as endoplasmic reticulum stress (ER stress), trophic factor withdrawal and UV radiation have the capacity to induce apoptosis by causing BH-3 only proteins to catalyze Bax and Bak homo-oligomerization, which ultimately leads to the permeablization of the outer mitochondrial membrane (OMM). While it is known that Bax and Bak are the proteins that homo-oligomerize to create the pores within the OMM, there have been several key questions surrounding the mechanism by which homo-oligomerization of these proteins occurs. In the healthy state, both Bax and Bak exist as monomers with Bax being unbound in the cytosol and Bak existing as an OMM transmembrane protein. The Bak monomer is inserted into the OMM because its C-terminal α9 helix does not interact with the N-terminal

α1 helix, which is the case for the Bax monomer (Suzuki *et al.*, 2000). It is known that certain BH-3 only molecules interact with Bak and Bax to induce homo-oligomerization (Cheng et al., 2001) but the precise mechanism by which this occurs was not established until recently. In a landmark study, Kim *et al.,* (2009) showed that under ER stress, the BH-3 only activator proteins tBid, Bim and PUMA bind to the N-terminal α1 helix of Bax, which releases its C-terminal α9 helix enabling the Bax monomer to insert into the OMM. Once inserted into the OMM, the activator proteins can continue to interact with N-terminal of both Bax and Bak to induce the higher-ordered tetramers that create the transmembrane channel known as the mitochondrial pore that allows cytochrome c to be released from the mitochondria (Saito *et al.,* 2000).

Once cytochrome c is released into the cytosol, it binds to Apaf-1 in an energy dependent manner (Li *et al.,* 1997). Other proteins such as second mitochondria-derived activator of caspase (SMAC) are released through mitochondrial pore, which interfere with the ability of proteins such as X chromosome-linked inhibitor of apoptosis protein (XIAP) that block the activation of caspase 3, 7 and 9. Once activated, caspase 9 can interact with cytochrome c and Apaf-1 to form a complex known as an apoptosome (Rodriguez & Lazebnik, 1999), which is capable of cleaving the effectors caspase 3 and caspase 7. Once activated, these proteins begin cleaving cellular proteins ultimately resulting in morphological changes that are characteristic of apoptosis such as plasma membrane blebbing and nuclear condensation (Fan *et al.,* 2005).

The importance of cytochrome c in the developing central nervous system was underscored when Hao *et al.,* (2005) created a knock-in mouse that expressed a mutant cytochrome c protein capable of functioning in oxidative phosphorylation but defunct in its apoptotic role. This resulted in an overexpansion in the proliferating ventricular zone as well as an overexpansion of the cortex and midbrain by E14.5. Similar results were found in Apaf-1

knockouts (Hao *et al.,* 2005), which collectively underscores the importance of mitochondrial-mediated apoptosis in regulating the size of the NPC population during development.

Within the mitochondrial-mediated apoptotic pathway, the initiation of apoptosis can be inhibited if the formation of the mitochondrial pore is prevented (Lindsten *et al.,* 2005). Kim *et* al., (2006) have demonstrated that the key role of the major anti-apoptotic proteins, Bcl-2, Bcl-X_L and Mcl-1 is to sequester and inhibit the BH-3 activator proteins that catalyze the homo-oligomerization of Bak and Bax. This study went on to show that the role of the remaining BH-3 only proteins, Bad, Noxa, Bmf and Bik-Blk is to prevent the anti-apoptotic Bcl-2 proteins from sequestering the activator BH-3 proteins. More specifically, Kim *et al.*, (2006) showed that Bad, Bmf and Bik-Blk were all capable of inducing apoptosis by displacing Bid from both Bcl-2 and Bcl-X_L, while Noxa was the only BH-3 protein capable of inducing apoptosis by displacing Bid from Mcl-1.

When comparing the roles of the three major Bcl-2 anti-apoptotic proteins in the developing CNS, Mcl-1 emerges as a critical survival factor in NPCs during neurogenesis. Loss-of-function studies have shown that Bcl-2 deficiency results in a loss of sympathetic, motor and sensory neurons postnatally (Michaelidis *et al.*, 1996), while Bcl-X_L deficiency causes a reduction in catecholaminergic neurons (Savitt *et al.*, 2005). However, Mcl-1 is the only anti-apoptotic family member required for embryonic NPC survival during neurogenesis (Arbour *et al.,* 2008). As Mcl-1 plays such an important role in the survival of the embryonic NPC population, it is foreseeable that Mcl-1 may play a similar role in the adult NPC population. Furthermore, if Mcl-1 was a survival factor for adult NPCs, it could be an appropriate target when addressing the question of how one could decrease the endogenous rate of apoptosis within the adult NPC population.

Bcl-2 Protein Family			
Anti-Apoptotic Proteins	**Multidomain Proapoptotic Proteins**	**BH3-only Proteins**	
		BH3-only Sensitizer Proteins	**BH3-only Activator Proetins**
Bcl-2 Bcl-x_L Mcl-1	Bax Bak	Bad Noxa Bmf Bik-Blk	tBid Bim Puma

Table 2. Bcl-2 protein family and the various subclasses

Figure 2. Bcl-2 protein family classes. A representation of each of the three major classes of proteins found within the Bcl-2 family. The anti-apoptic proteins contain all four Bcl-2 homology (BH) domains while the multidomain effector proteins contain BH1-3 domains. In the BH-3 only class, both the sensitizer and activator BH3-only proteins contain just the BH-3 domain.

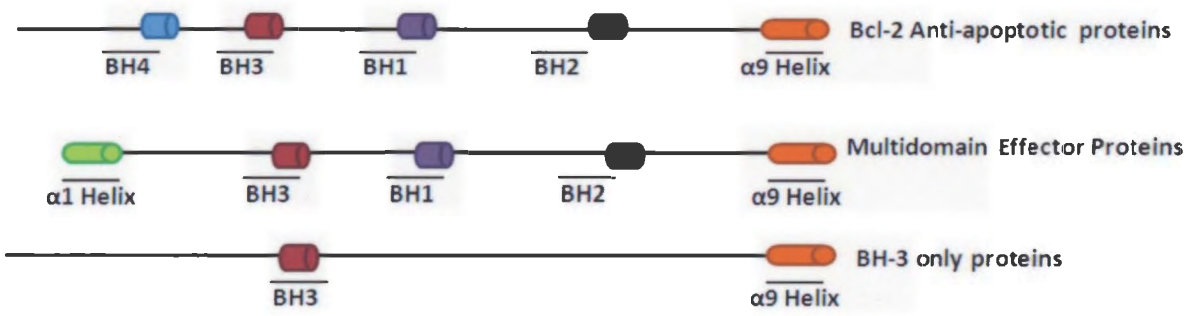
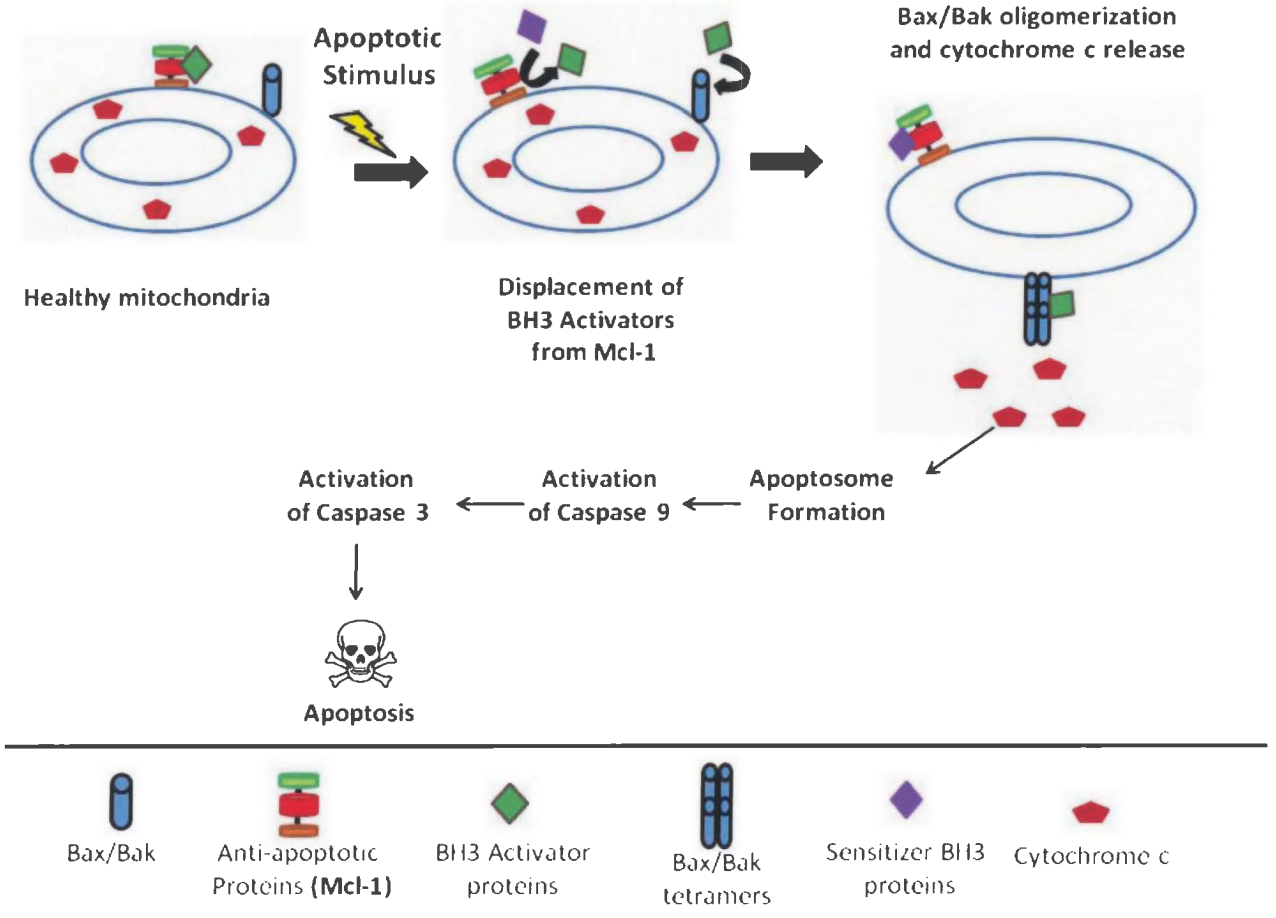

Bcl-2 Anti-apoptotic proteins
BH4
BH3
BH1
BH2
α9 Helix
Multidomain Effector Proteins
α1 Helix
BH3
BH1
BH2
α9 Helix
BH-3 only proteins
BH3
α9 Helix

Figure 3. Mitochondrial mediated apoptosis. In the healthy cell, Bax and Bak exist as monomers. Upon an apoptotic stimulus, the sensitizer BH-3 only proteins displace the activator BH-3 only proteins from the anti-apoptotic Bcl-2 family members. This allows the activator BH-3 only proteins to induce homo-oligomerization of Bax and/or Bak, which results in OMM permeablization releasing cytochrome c into the cytosol where it interacts with Apaf-1 to form the apoptosome. Caspase 9 is activated by the apoptosome, which in turn activates caspase 3. Active caspase 3 cleaves cellular proteins resulting in the morphological changes that are characteristic of apoptosis.


Apoptotic Stimulus
Bax/Bak oligomerization and cytochrome c release
Healthy mitochondria
Displacement of BH3 Activators from Mcl-1
Apoptosome Formation
Activation of Caspase 9
Activation of Caspase 3
Apoptosis
Bax/Bak
Anti-apoptotic Proteins (Mcl-1)
BH3 Activator proteins
Bax/Bak tetramers
Sensitizer BH3 proteins
Cytochrome c

1.3.2 Mcl-1

The Mcl-1 protein was first isolated from the ML-1 human myeloid leukemia cell line during the differentiation of monocytes and macrophages and its gene is located on chromosome 1q21 (Kozopas *et al.,* 1993). The *Mcl*-1 gene contains three exons and alternative splicing produces mRNA transcripts that either contain or lack exon 2 producing the Mcl-1_L and Mcl-1_S isoforms, respectively (Bingle *et al.,* 2000). With respect Mcl-1_L, this isoform contains the C-terminal transmembrane domain, which allows the protein to be inserted into the OMM. It is thought that this is where Mcl-1_L exerts its anti-apoptotic function by inhibiting the activation of Bax/Bak (Bingle *et al.*, 2000). Focussing on Mcl-1_S, when exon 2 is excluded, there is a shift in the reading frame and the resulting Mcl-1_S protein contains only the BH3 domain, thus this form of Mcl-1 is proapoptotic (Bingle *et al.,* 2000). The shift in reading frame occurs when the cell enters into an apoptotic cascade resulting in an upregulation of pro-apoptotoic Mcl-1_S (Marriot *et al.,* 2005). Furthermore, Mcl-1_S isoform does not contain the C-terminal transmembrane domain, which may account for some studies suggesting that Mcl-1 is found in the nucleus of the cell and in turn, has physiological roles that are distinct from its anti-apoptotic role (Jamil, *et al.,* 2005).

The characterization of Mcl-1 has been minimal compared to other Bcl-2 proteins due to the fact the Mcl-1 germline KOs cannot implant *in utero* because of trophectoderm defects, which results in lethality at the peri-implantation stage (Rinkenberger *et al.*, 2000). However, we do know that Mcl-1 has a relatively short half life of approximately 3 hours (Weng *et al.*, 2005) and it is strictly regulated at transcriptional, translational and post-translational levels (Akgul, 2009). For instance, the signal transducers and activators of transcription (STATS) family of

proteins regulate are capable of regulating survival by targeting the Mcl-1 promoter at the transcriptional level while various microRNAs such as mir-29b can directly inhibit Mcl-1 mRNA translation upon an apoptotic stimulus (Isomoto, *et al.,* 2005). This tight regulation also appears to be crucial in the prevention of cancer, as Mcl-1 expression is enhanced in a variety of cancers including multiple myeloma (Wulleme-Toumi *et al.*, 2005) and B-cell non-Hodgkin's lymphomas (Cho-Vega, *et al.,* 2004).

Much of the current understanding of the role of Mcl-1 has come about through conditional knockout systems such as the Cre/lox system. For instance, Opferman et al., (2003) generated a conditional KO of Mcl-1 to specifically assess the function of Mcl-1 within the hematopoietic system. Their results demonstrate that Mcl-1 is essential for the survival of hematopoietic stem cells (Opferman *et al.* 2005) and development and survival of the B and T cell populations (Opferman *et al.* 2003). Furthermore, Mcl-1 expression is a critical survival factor for hepatocytes, which are the proliferating progenitors required for normal liver development and functioning (Hikita *et al.*, 2009).

1.3.2.1 Mcl-1and the Central Nervous System

Most of the data surrounding apoptosis of the proliferating populations in the CNS has been restricted to the developing brain. Recently, Arbour *et al*, (2008) have shown that embryonic neural precursors express Mcl-1 during cortical neurogenesis. When Mcl-1 loss-of-function was induced during this time period, there was an increased level of apoptosis in Nestin-expressing precursor cells, which ultimately lead to severe deficits in cortical neurogenesis (Arbour *et al*, 2008). Moreover, Arbour *et al*, (2008) used a conditional KO system to demonstrate that Mcl-1 depletion at embryonic day 8 results in embryonic death by E16-E17,

which underscores the crucial role that Mcl-1 plays in the developing brain. Other studies have demonstrated that the anti-apoptotic activity of Notch1 in NPCs can be attributed to Mcl-1 upregulation (Oishi *et al.*, 2004). In contrast to embryonic NPCs, Mcl-1 loss-of-function does not directly induce apoptosis in differentiated neurons, but Mcl-1 deficiency potentiates neuronal cell death in an acute DNA damage model of apoptosis (Arbour *et al*, 2008), demonstrating that Mcl-1 does play a pro-survival role in postmitotic cells of the CNS.

Currently there is little known about the regulation of apoptosis within the adult SVZ. There is evidence demonstrating that enhanced trophic factor support (Reynolds & Weiss, 1992; Craig *et al.*, 1996), exercise (van Praag *et al.*, 1999), cell cycle regulators such as Bmi (He *et al.*, 2009) can enhance proliferation within the adult SVZ. However, there has yet to be a potent anti-apoptotic Bcl-2 family member implicated in the regulation of adult NPC apoptosis or survival. However, the function of Mcl-1 in adult NPC's has yet to be characterized, which could be a reflection of the peri-implantation lethality that exists in germline kockouts of this protein.

1.4 Summary and Hypothesis

Many neurodegenerative conditions are most prevalent during adulthood; therefore focus has recently been directed towards regenerative strategies within the adult brain. When compared to the embryonic brain, the endogenous neural precursor populations of the adult brain are quite small and without manipulation offer the damaged brain an insignificant level of functional regeneration. One putative therapeutic approached to regeneration of the adult brain is to expand the endogenous neural precursor population. When looking for potential genetic targets, Mcl-1 has emerged as a viable candidate as the Mcl-1 conditional KO results in a dramatic increase in apoptosis of the embryonic NPCs (Arbour *et al*, 2008). Although this is

promising, there has yet to be any evidence that Mcl-1 is even expressed in the adult neural precursor population, let alone whether Mcl-1 is a prosurvival factor within this population. Therefore, to begin this journey toward improving the therapeutic efficiency of the endogenous neural precursor population of the adult brain, I put forward the following hypothesis:

Hypothesis:

Mcl-1 is a pro-survival factor within the adult neural precursor population of the adult mammalian brain.

1.5 Objectives

The main objectives of this thesis are as follows:

1. To demonstrate that Mcl-1 is expressed in proliferating cells of the adult mammalian brain;

2. To demonstrate that Mcl-1 loss-of-function increases endogenous apoptosis of adult NPCs;

3. To investigate whether Mcl-1 gain-of-function reduces endogenous apoptosis in adult NPC's.

Chapter 2

Materials and Methods

Mice and Genotyping

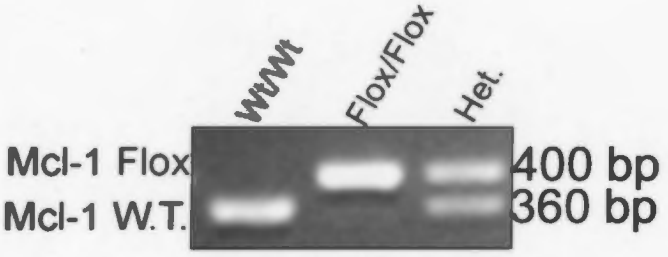
Adult floxed Mcl-1(Mcl-$1^{f/f}$) mice or FVBN wild type control mice ranging from 2-6 months of age were used. Mice were kept on a 12hr light/dark cycle and food/water was administered *ad libitum*. All experiments were approved by the Memorial University of Newfoundland's Internal Animal Care Committee adhering to the Guidelines of the Canadian Council on Animal Care.

Mcl-$1^{f/f}$ mice were previously generated by Opferman *et al.*, (2003). To determine the genotype of each animal, DNA was acquired from tail clippings and isolation was completed using the REDExtract-N-Amp tissue PCR kit (Sigma, 029K6262). Once isolated, the DNA was combined with the polymerase chain reaction (PCR) reaction components as outlined in Table 2. The PCR reaction (94°C for 6 min; 55°C for 1 min; 72°C for 1min) was then carried out for 30 cycles. The products of the PCR reaction were run on a 2% agarose gel containing ethidium bromide (15585-011; Invitrogen), which binds to DNA and fluoresces under ultraviolet light. As the Mcl-$1^{wt/wt}$ allele (360bp) lacks the two 34bp *loxP* sites that flank exon 1 of the Mcl-$1^{f/f}$ allele (400bp) (Operferman *et al.*, 2003), it can be separated from the Mcl-$1^{f/f}$ allele via electrophoresis on an agarose gel (Figure 4). Therefore, the two possible alleles can be distinguished according to band size as shown in Figure 4 and the genotype of each mouse can be determined.

Component	**Volume/Sample (μL)**
10X Reaction Buffer	5.0
Primers (2.5μM): *6Mcl-1 **7Mcl-1	 5.0 5.0
1.25mM dNTP's	8.0
50mM $MgCl_2$	1.5
Taq polymerase	0.5
Water	22.0
DNA	3.0
*6Mcl-1 = 5' GCA GTA CAG GTT CAA GCC GAT G3' **7Mcl-1 = 5'CTG AGA GTT GTA CCG GAC AA3'	

Table 3. Components of Mcl-1 PCR reaction solution

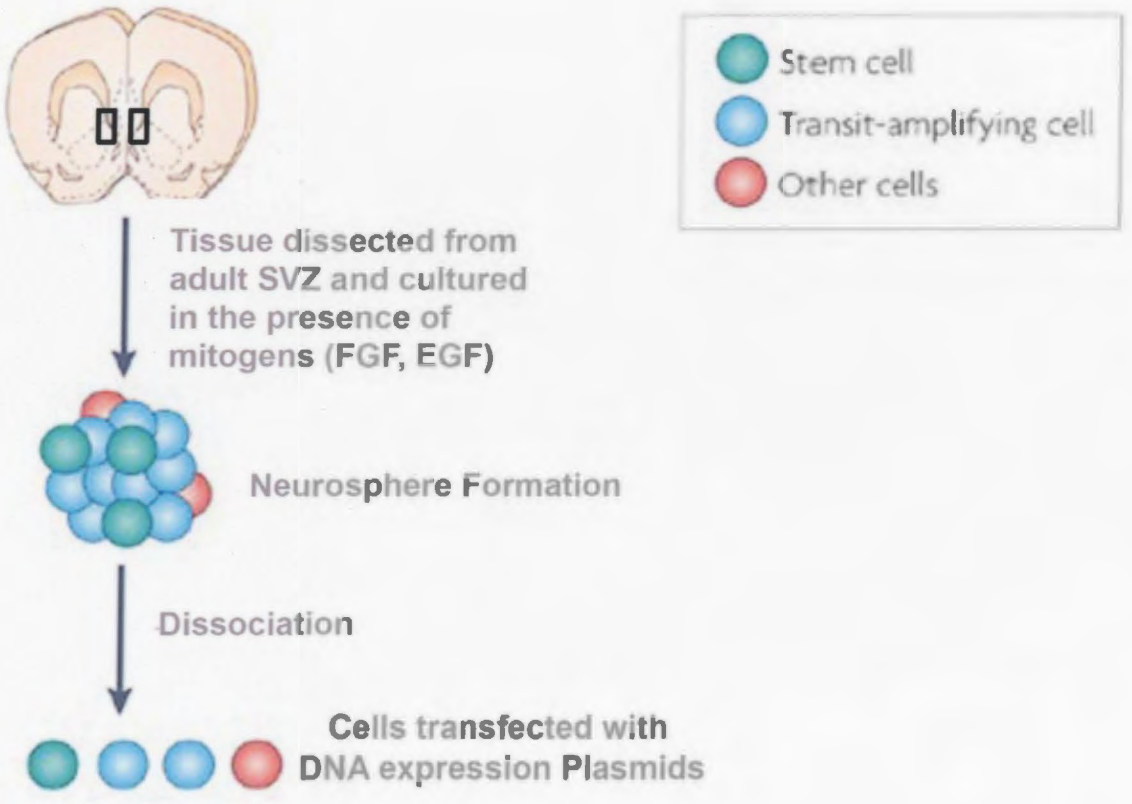
Figure 4. Mcl-1 genotypes can be determined through PCR and gel electrophoresis. The $Mcl^{f/f}$ allele contains two 34-bp *loxP* sites, which makes it larger than the wild type Mcl-1 allele. The PCR products of these two alleles are separated by placing them in a 2% agarose gel and applying electrophoresis. By doing this, the different alleles will separate and the genotype can be determined.


Wt/Wt
Flox/Flox
Het.
Mcl-1 Flox
Mcl-1 W.T.
400 bp
360 bp

Acquisition of Clonally Derived Neural Precursor Cells

NPCs were obtained from adult Mcl-$1^{f/f}$ and wild type control mice (2-6 months old). Mice were euthanized with a 0.3ml intraperitoneal (i.p.) injection of Euthanol (2.5mg/kg). NPCs were acquired as depicted in Figure 5. Access to the SVZ was acquired by separating the hemispheres along the longitudinal fissure and removing the overlying cortex. The exposed SVZ was then dissected out, cut up and dissociated by manually triturating in artificial cerebrospinal fluid [(2M NaCl (S271-3, Sigma), 1M KCl (P333, Sigma), 1M $MgCl_2$ (M2393, Sigma), 155mM $NaHHCO_2$ (Sigma, S576), 1M glucose (G7528, Sigma), 108mM $CaCl_2$ (C7902, Sigma)] and incubated at 37°C for 15 min with trypsin enzyme (T4549, Sigma) to chemically digest the tissue. The solution was then spun at 5000rpm for 5 min and the pellet was resuspended and plated in DMEM/F12 (911330, Gibco) and the following: 0.0033mol/L D-glucose (G7528, Sigma), 0.5% penicillin-streptomycin (15140-122; Invitrogen), 0.5% insulin (I-5500, Sigma), 0.2% apo-transferrin (T4382, Sigma), 0.002% progesterone (P8783, Sigma), 0.12% putrescine (P5780, Sigma), 0.015% selenium (S5290, Sigma), 0.1% fungizone (Gibco; 15290-018) and 0.02% Heparin (H3149, Sigma) supplemented with 1% B27 (Invitrogen; 17504-044), 0.1% FGF-2 (Sigma; F0291) and 0.02% EGF (Invitrogen; 13247-051) (Tropepe et al., 1997). Neural precursor cells (NPCs) were plated at clonal density (10cells/μl) unless otherwise stated and primary neurospheres were grown for plasmid transfection.

Figure 5. Acquisition of Clonally Derived Neural Precursor Cells. The SVZ of Mcl-1$^{f/f}$ and wild type mice were dissected, then expanded in serum-free media containing FGF-2, EGF and heparin. Neurospheres were mechanically dissociated and plated as single cells 12-16hrs before transfection. (Modified from Chojnacki *et al.*, 2009).


Stem cell
Transit-amplifying cell
Other cells
Tissue dissected from adult SVZ and cultured in the presence of mitogens (FGF, EGF)
Neurosphere Formation
Dissociation
Cells transfected with DNA expression Plasmids

Plasmid Constructs

Cre, Mcl-1, NesGFP and NesCre were cloned into the pCIG2 vector (Megason & Mcmahon, 2002) in our laboratory at Memorial University of Newfoundland and their maps are shown in Appendix A. To assess Mcl-1 loss-of-function, the Cre construct (Tronche *et al.*, 1999) was cloned into the pCIG2 expression vector (Megason & Mcmahon, 2002). To selectively target the NPC population, the heat shock protein 68 minimal promoter (Rossant *et al.*, 1991) linked to the 2nd intron Nestin enhancer element (Kawaguchi *et al.*, 2001) was used to drive either GFP or Cre expression in Nestin expressing neural precursors. The Mcl-1 expression construct (Rinkenberger *et al.*, 2000) was cloned into the pCIG2 vector (Megason & Mcmahon, 2002) to assess Mcl-1 gain-of-function. Protein expression of both Cre and Mcl-1 in HEK 293A cells was verified by western analysis (Fig, 6 A,B).

***In Vitro* Transfection and Immunocytochemistry**

Cells were plated at a density of 2 x 10^5 cells/ml in Neurobasal media (Gibco; 21103-049) supplemented with 0.25% L-glutamine (Sigma; 25030-081), 0.1% fungizone (Gibco; 15290-018), 2% B27 (Invitrogen; 17504-044), 1% N2 (Sigma; 17502-048), 0.1%FGF-2 (25µg/ml; Sigma; F0291) 0.02% EGF (Invitrogen; 13247-051). 12-16hrs after plating, the cultures were transfected with GFP, Mcl-1 or Cre plasmids using Lipofectamine Plus reagent (Invitrogen; 11668-027). After 6-hrs, additional media was added such that the cells were at clonal density. Cultures were incubated for 24, 36, 48, or 60 hrs and fixed with cold paraformaldehyde (PFA, pH; Sigma; F8775).

All GFP positive cells were assessed for apoptosis based on nuclear condensation as visualized by Hoechst staining (60µg/ml; Sigma; B1155). To specifically identify NPCs that

were transfected with the plasmids that are driven off of the universal chicken β-actin promoter, cultures were immunostained with Nestin antibody (mouse monoclonal, 1:200; Millipore MAB353).

In vivo Plasmid Injections and Electroporation

Plasmid transfection of NPCs i*n vivo* was accomplished as described previously (Barnabe-Heider *et al.,* 2008). Adult Mcl-$1^{f/f}$ and wild type control mice were anesthetized using isofluorane. The dorsal portion of the head was shaved and the animal was secured in the stereotaxic device. Once secured, the shaved portion of the skin was sterilized with provoiodine solution (Rougier; 00172944) and the skull was exposed. Both Bregma and Lamda were landmarked to ensure that the head was positioned equally in all three axes. Once aligned, the injection site was marked with the following coordinates: Anterior-Posterior: 4.0mm from bregma; Medial-Lateral: 0.6mm from the midline. After drilling through the skull, the dura was pierced to allow the injection needle to go through with ease. The loaded injection needle was then secured to the stereotaxic device and lowered to the surface of the brain and measurement was taken at the cortical surface. The injection needle was then slowly lowered ventrally by 3-4mm and the 2μL plasmid volume (6-10μg/μL) was infused into the lateral ventricle over a 2 minute period. Within 1 minute of completion of the injection, electrode gel (Spectra) was applied to both sides of the dorsolateral aspects of the skull and a 5-pulse current at 200V and a duration of 50ms and an interpulse interval of 950ms was passed through the brain. The injection needle was then slowly removed from the brain and skin was sutured.

BrdU labeling of Proliferating Cells

5-Bromo-2-deoxyuridine (BrdU, Sigma; B5002) was used to label the proliferating cells of the SVZ. Wild type mice were given an intraperitoneal BrdU injection (100μg/g body weight) every two hours for 10 hours. To specifically target the neural progenitor cells, the mice were euthanized 30 minutes after the last BrdU injection. To specifically target the slowly dividing neural stem cells, animals were allowed to live for 28 days after the last injection. In both groups, coronal sections were collected beginning at the formation of the corpus callosum and continuing caudally until the third ventricle. Roughly every fifth section was assessed and quantification was measured by calculating the percentage of BrdU positive cells that were also positive for Mcl-1 staining.

Tissue Processing and Immunohistochemistry

Adult mice were euthanized and perfused with 1X phosphate buffer solution (1X PBS) followed by cold 4% PFA. Brains were removed and postfixed overnight in 4% PFA and cryoprotected incrementally using 12%, 16% and 22% sucrose (Sigma) dissolved in 1XPBS. Brains were then frozen and coronal sections (14-μm) were collected on Superfrost Plus slides (Fisher Scientific) using a freezing cryostat microtome (Microm, HM520). For all subsequent BrdU immunostaining, sections were incubated in 2N HCl at 37°C for 30min followed by a 10-min wash in 0.1M sodium borate (pH 8.0) to denature the DNA. Immunohistochemistry was performed with primary antibodies for Mcl-1 (1:400; Santa Cruz; sc-819), BrdU (1:100; BD Biosciences; 347580) and Hoechst (60μg/ml; Sigma; B1155) was used to stain nuclei.

For the *in vivo* electroporation study, apoptosis of transfected cells (GFP^+) was assessed at 72hrs after electroporation. Mice were perfused and tissue was processed as described above.

Coronal sections were collected beginning at the rostral site of transfection (i.e. as soon as GFP^+ cells were present) and apoptosis was assessed via nuclear condensation as visualized by Hoechst staining. A minimum of 50 GFP^+ cells was assessed per brain in each treatment group (NesGFP range: 58-158; NesCre range: 50-200). The percentage of apoptotic cells was calculated by dividing the number of apoptotic GFP^+ cells by the total number of GFP^+ cells per brain.

Western blot Analysis

As shown in Figure 6, western blot analysis was performed on HEK 293A cells to demonstrate plasmid expression. To extract the protein, complete immunoprecipitation (IP) buffer [25mM Tris-base (Roche, 10708976001); 150mM NaCl (Sigma, C7902); 1mM $CaCl_2$ (Sigma, C7902); 1% Triton X-100 (Sigma, 93426); 100µL phenylmethylsulfonyl Floride (Sigma, P7626); 10µL DTT (Sigma, 43816)] was added to cell pellets and incubated on ice. Samples were mechanically triturated and centrifuged at 2000 rpm for 5 min and the supernatant was transferred to a new pre-chilled tube. Protein concentration was determined using the Bio-Rad protein Assay kit I (500-0001) and a Thermo Scientific Genesys 10UV spectrophotometer reading at 595nm absorbance.

Separating gels and stacking gels were made as shown in Table 4 and 5, respectively. 30µg of protein was added to 5µL of protein loading buffer [250mM Tris-HCl (Fisher, B9153); 0.5 DTT; 10% sodium dodecyl sulphate (SDS, 155-25-017 Invitrogen); 50% Glycerol (Sigma, G5516); 0.5% Bromophenol Blue (Fisher, B392-5)] and topped up to 25µL with complete IP buffer. Protein was denatured by boiling for 5 minutes. After denaturation, 10µL of each protein sample and kaleidoscope prestained protein standards (Bio-Rad, 161-0324) were loaded into the

gel, which was then submerged in running buffer [0.6% Tris-base, 1.125% glycine (Bio-Rad, 161-0724), 0.1% SDS] and run at 110V for approximately two hours. Protein was then transferred to a nitrocellulose membrane (Hybrid-ECL, LRPNK/95/81) by sandwiching the nitrocellulose membrane with the separating gel. The cassette was then submerged in transfer buffer [0.3% Tris-base, 1.125% glycine and 20% methanol (Sigma, 179337)] and run at 190 amps for 1.5hrs. Following protein transfer, the membrane was washed in Tween20 phosphate buffer solution [(TPBS); 1.42% Na_2HPO_4 (Fisher Scientific, S374-500), 1.38% NaH_2PO_4 (Fischer Scientific, S399-500), 1% NaCl (Fisher Scientific, S271), 0.1% Tween20 (Sigma, P7949)], blocked with 5% blotto (milk powder in TPBS) and incubated overnight with primary antibodies. The membranes then underwent 3x15 min washes of 0.5% blotto and were incubated with Horseradish peroxidase conjugated to secondary antibodies (1:2000; BioRad; anti-rabbit IgG: 1706515, anti-mouse IgG: 1706515) for 1 hr. After secondary antibody exposure, membranes underwent 3, 15 min washes of TPBS and the peroxidase reaction was then catalyzed with chemiluminescence reagent (Western lightning (Nel100) for 1 min. Blots were blotted and exposed to Fuji Medical X-Ray film (100NIF) for 1-3min and developed on a Mini Medical Series developer. Primary antibodies used were as follows: Mcl-1 (1:10,000; Rockland Immunochemicals; 600-401-394); Cre recombinase (1:10,000; Chemicon, MAB3120); and anti-β-Actin (1:2000; Sigma; A5316-2) as a loading control.

Stock Solution	Separating gel 10% (20ml Final Volume)
dd water	7.0ml
0.5M Tris, 1.5M glycine	4.0ml
10% SDS (RT)	0.8ml
50% glycerol	2.0ml
40% acrylamide; 0.25% bisacrylamide	5.0 ml **Filtered**
Ammonium persulphate (made fresh)	30mg in 1 ml of dd water
10% TEMED	0.02ml (TEMED)

Table 4. Separating gel.

Stock Solution	Stacking gel 14% (10ml Final Volume)
dd water	4.05ml
0.5 M Tris-HCl pH 6.8	1.4ml
10% SDS (RT)	0.4ml
50% glycerol	1.0ml
40% acrylamide, 0.25% bisacrylamide	1.25ml **FILTERED**
Ammonium persulphate (made fresh)	25mg in 1ml of dd water
10% TEMED	0.01 ml (TEMED)

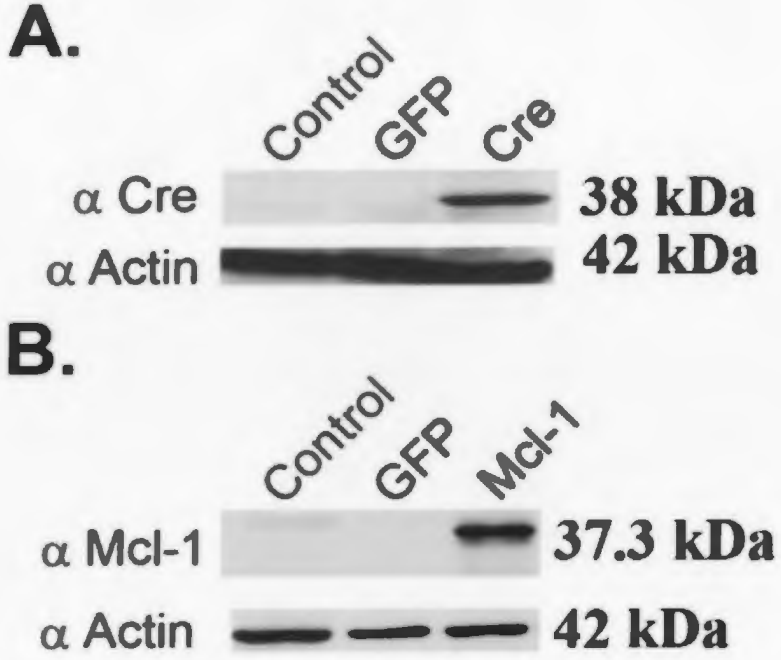
Table 5. Stacking gel.

Microscopy and Statistics

Immunostained cultures were examined on a Zeiss AxioObserver A.1 microscope while immunostained sections were examined on a Zeiss AxioImager Z.1 microscope. All images were taken with a Zeiss AxioCam MRm camera using Zeiss AxioVision 4.8 software. All figures were created using Adobe Photoshop CS2. If required, manipulations of brightness and contrast were made such that all treatment groups received the same adjustments. All statistics were completed on GraphPad Prism 5 software, including unpaired T-test and both one and two way analysis of variance. Tukey's posthoc analysis was used to determine differences between treatment groups.

Figure 6. Western analysis of protein expression. A, Western analysis of protein expression from 293A cells that were transfected with a pCIG2 vector (GFP) or pCIG2 vector containing Cre recombinase (Cre) or untransfected cells (control). 48hrs after transfection, cells were collected for Cre protein analysis. **B,** Western analysis of Mcl-1 protein expression from 293A cells that were transfected with a pCIG2 vector (GFP) or pCIG2 vector containing Mcl-1 (Mcl-1) or untransfected cells (control). 48hrs after transfection, cells were collected for Mcl-1 protein analysis. Blots were reprobed with Actin as a loading control.

\


A.
Control
GFP
Cre
α Cre
38 kDa
α Actin
42 kDa
B.
Control
GFP
Mcl-1
α Mcl-1
37.3 kDa
α Actin
42 kDa

Chapter 3

Results

Mcl-1 is Expressed in NPCs of the Adult SVZ

To demonstrate that Mcl-1 is expressed in NPCs of the adult SVZ, I used a cumulative BrdU labeling assay that labels cells undergoing S-phase of the cell cycle. By administering a BrdU injection every two hours for ten hours and euthanizing the animals 30 minutes after the last injection, it is possible to label the majority of the TACs of the adult SVZ and a very few neural stem cells (Morshead & van der Kooy, 1992). When the proliferating cells of the SVZ were labeled in this manner, approximately 63% of the BrdU cells also expressed Mcl-1 (Fig. 7A, B; an average of 225 per brain BrdU positive cells were assessed; n=3), suggesting that a subpopulation of the transint amplifying population expresses Mcl-1.

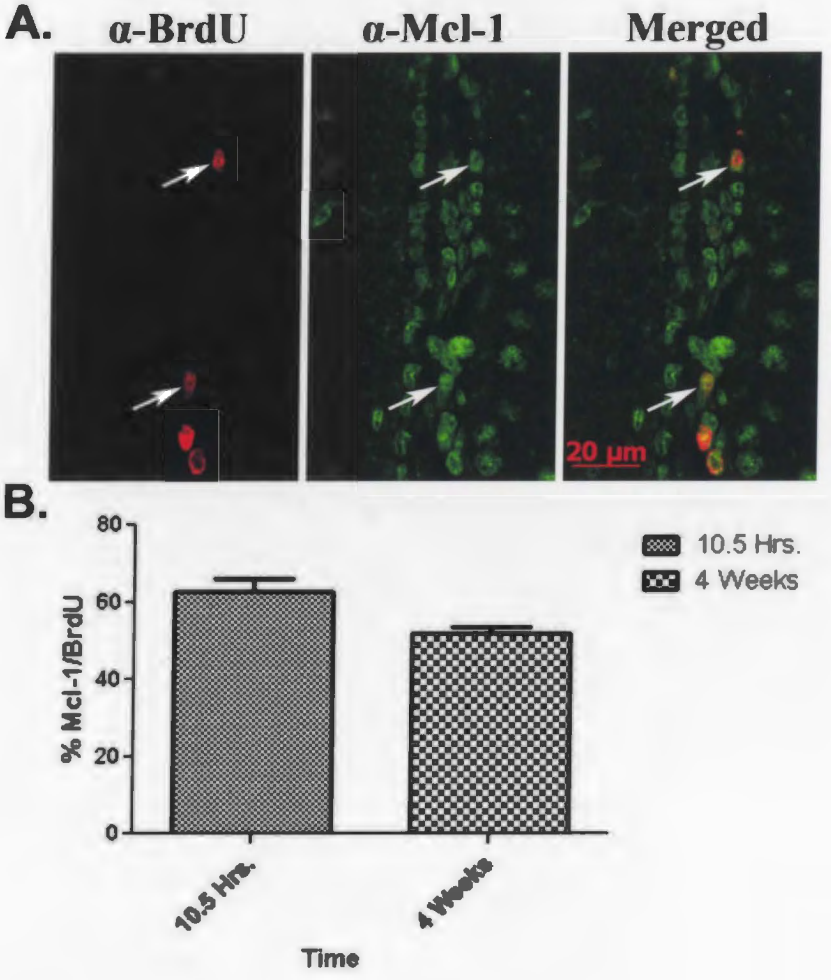
To specifically assess whether Mcl-1 was expressed in the quiescent stem cell population of the adult SVZ, I took advantage of the fact that the cell cycle time of this population is approximately 28 days (Craig *et al.,* 1994; Morshead *et al.,* 1994) while the cell cycle time of the TACs is 12.7 hours (Morshead & van der Kooy, 1992). By using the same BrdU labeling assay as above and allowing the animals to live for 28 days, the few quiescent stem cells that underwent S-phase during the BrdU assay will be the only cells that retain BrdU in the SVZ. One can assume that all the BrdU positive cells remaining in SVZ are the quiescent stem cell population because TACs that were initially labelled will die, migrate away from the SVZ or dilute the BrdU signal out as they continue to divide (Morshead *et al.,* 1994; Craig *et al.,* 1994). When the percentage of BrdU positive cells that were co-labeled with Mcl-1 was quantified,

approximately 52% of the BrdU labeled neural stem cells also expressed Mcl-1 (Fig. 7B; an average of 91 per brain BrdU positive cells assessed; n=4). Taken together, these results demonstrate that Mcl-1 is expressed in both the TACs and the more quiescent NSC population of the adult SVZ.

Mcl-1 Loss-of-Function Increases Apoptosis of Adult NPCs *in vitro*

To characterize the function of Mcl-1 in adult NPCs, I began by using loss-of-function strategies *in vitro*. In the first set of experiments, Mcl-1 loss-of-function was induced by transfecting DNA expression plasmids into SVZ-derived cultures from both wild type and Mcl-$1^{f/f}$ brains. The plasmids used in this initial study directed either Cre recombinase (Cre) or control (GFP) off of the chicken β-actin promoter which induces robust protein expression in all SVZ-derived cell types. Expression of both the GFP and Cre plasmids occurred by 24hrs (Fig. 8C) and I assessed apoptosis on the basis of nuclear condensation because this measure of apoptosis is less transient compared with other measurements of apoptosis, such as active caspase 3. This resulted in a significant degree of chromatin condensation in Mcl-$1^{f/f}$ cultures that were transfected with the Cre plasmid. Indeed, when this measure of apoptosis in Cre transfected Mcl-$1^{f/f}$ cultures was compared to all controls, there was a 2 fold increase in apoptosis (Fig.8C; Wt.GFP vs. Mcl-$1^{f/f}$Cre, Wt.Cre vs. Mcl-$1^{f/f}$Cre, Mcl-$1^{f/f}$GFP vs. Mcl-$1^{f/f}$Cre: $p<0.001$; n=3). This effect was sustained at all time points measured (Fig. 8C) indicating that a higher rate of apoptosis was sustained for at least 60 hrs in Cre transfected Mcl-$1^{f/f}$ cells when compared to controls.

Figure 7. Mcl-1 is expressed in proliferating cells of the adult subventricular zone, *in vivo*. FVBN mice received a BrdU injection (100μg/g) every 2hrs for 10hrs and were euthanized at 30min. or 4 weeks after the last injection. **A,** Representative photomicrographs of proliferating neural precursor cells labelled with antibodies against Mcl-1 (green) and BrdU (red). **B,** Quantification of the percentage of BrdU positive cells that are also Mcl-1 positive.


A.
α-BrdU
α-Mcl-1
Merged
20 μm
B.
10.5 Hrs.
4 Weeks
% Mcl-1/BrdU
80
60
40
20
0
10.5 Hrs.
4 Weeks
Time

As previous studies have demonstrated that embryonic NPCs undergo apoptosis after Mcl-1 is knocked out (Arbour et al., 2008), a Nestin antibody was applied to these cultures to specifically label adult NPCs. When apoptosis was assessed at 36hrs, there was at least a 2 fold increase in apoptosis in Cre transfected Mcl-$1^{f/f}$ cells compared to control groups (Fig. 8A,B,D; Wt.GFP vs. Mcl-$1^{f/f}$Cre: $p<.05$; Wt.Cre vs. Mcl-$1^{f/f}$Cre: $p<.01$; Mcl-$1^{f/f}$GFP vs. Mcl-$1^{f/f}$Cre $p<0.05$; n=3). This effect was also seen at 60 hrs. (Fig. 8D; Wt.GFP vs. Mcl-$1^{f/f}$Cre: $p<0.05$; Wt.Cre vs. Mcl-$1^{f/f}$Cre: $p<.05$; Mcl-$1^{f/f}$GFP vs. Mcl-$1^{f/f}$Cre $p<0.05$; n=3). Overall, this indicates that Mcl-1 is a pro-survival factor in Nestin-positive NPCs, *in vitro*.

To underscore the fact that Mcl-1 loss-of-function increases apoptosis in adult NPCs *in vitro*, I did another set of experiments using DNA expression plasmids that directed either Cre or GFP off of the Nestin enhancer promoter. This promoter is only functional in Nestin expressing NPCs (Lothian *et al.*, 1999). Therefore, only NPCs will express Cre (NesCre) or GFP (NesGFP) proteins. As in our previous experiment, there was a 2 fold increase in apoptosis in Cre transfected Mcl-$1^{f/f}$ NPCs at 36 hrs (Fig.9A,B,C; Wt.NesGFP vs. Mcl-$1^{f/f}$NesCre: $p<0.001$; Wt.NesCre vs. Mcl-$1^{f/f}$NesCre: $p<.01$; Mcl-$1^{f/f}$NesGFP vs. Mcl-$1^{f/f}$NesCre $p<0.05$ n=3). Furthermore, this effect was also observed at 60 hrs (Fig.9C; Wt.NesGFP vs. Mcl-$1^{f/f}$NesCre: $p<0.01$; Wt.NesCre vs. Mcl-$1^{f/f}$NesCre: $p<0.01$; Mcl-$1^{f/f}$NesGFP vs. Mcl-$1^{f/f}$NesCre $p<0.05$; n=3). Overall, these data, combined with the data using the chicken β-actin promoter show that Mcl-1 is a pro-survival factor of adult NPCs, *in vitro*.

Figure 8. Mcl-1 loss-of-function results in increased apoptosis of Nestin-positive adult NPCs, *in vitro*. A, Representative photomicrographs of wild type (top panel) and Mcl-$1^{f/f}$ (bottom panel) Nestin-positive NPCs transfected with expression plasmids containing GFP. **B,** Representative photomicrographs of wild type (top panel) and Mcl-$1^{f/f}$ (bottom panel) Nestin-positive NPCs transfected with expression plasmids containing Cre recombinase. Cells were plated at 2 x10^5cells/mL and transfected with GFP or Cre expression plasmids 12hrs after plating. **C,** Quantification of the percentage of transfected cells that are apoptotic. Apoptosis was assessed by nuclear condensation visualized by Hoechst staining at 24, 36, 48 and 60hrs after transfection. **D,** Percentage of Nestin-positive transfected cells that are apoptotic. Cells were stained with primary antibody against Nestin at 36 and 60hrs after transfection. Results are expressed as mean ± SEM (n=3). *p<0 .05, **p<0.001

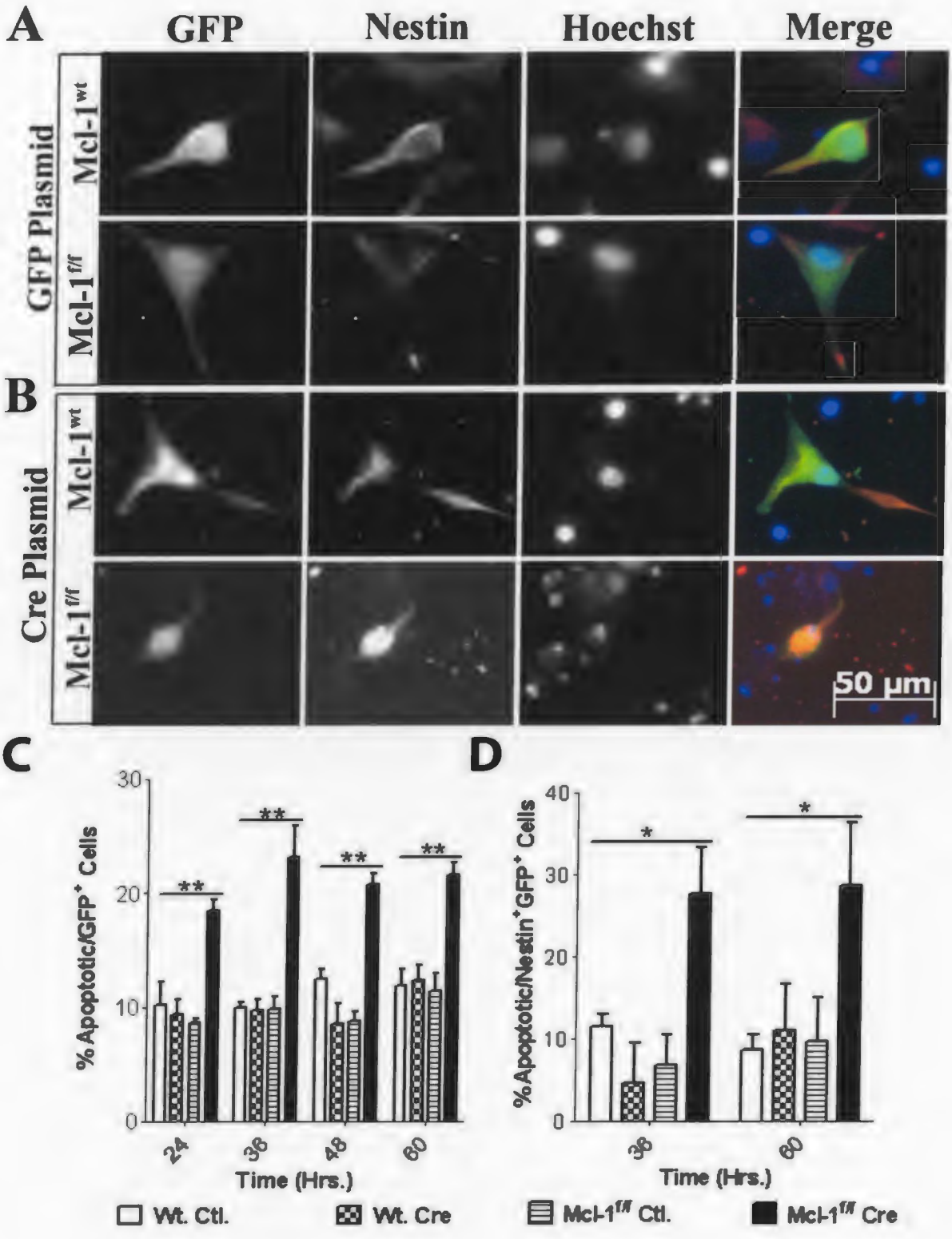
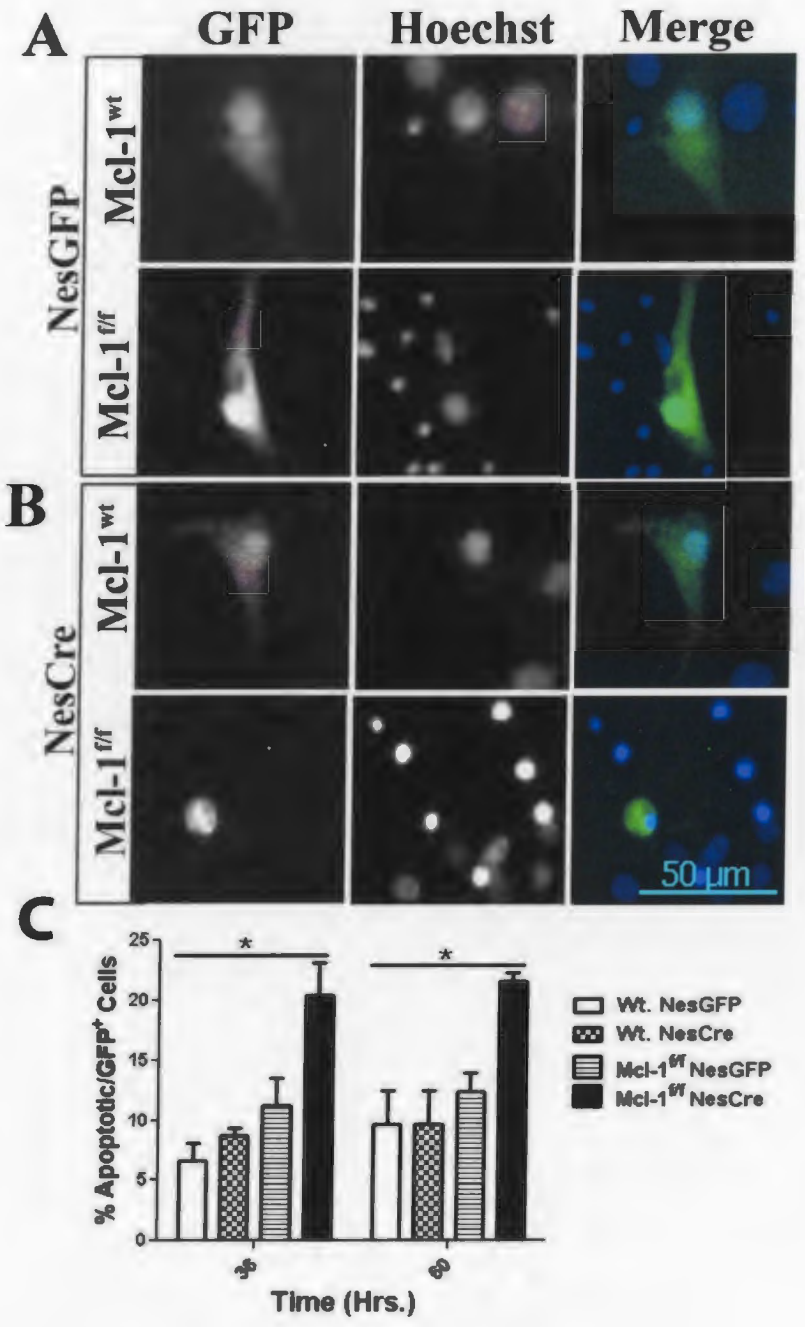

A
GFP
Nestin
Hoechst
Merge
GFP Plasmid
Mcl-1wt
Mcl-1f/f
B
Cre Plasmid
Mcl-1wt
Mcl-1f/f
50 µm
C
% Apoptotic/GFP+ Cells
30
20
10
0
**
**
**
**
24
36
48
60
Time (Hrs.)
Wt. Ctl.
Wt. Cre
D
% Apoptotic/Nestin+GFP+ Cells
40
30
20
10
0
*
*
36
60
Time (Hrs.)
Mcl-1f/f Ctl.
Mcl-1f/f Cre

Figure 9. Mcl-1 loss-of-function results in increased apoptosis of SVZ-derived NPCs, *in vitro*. **A,** Representative photomicrographs of wild type (top panel) and Mcl-$1^{f/f}$ (bottom panel) NPCs transfected with expression plasmids containing NesGFP. **B,** Representative photomicrographs of wild type (top panel) and Mcl-$1^{f/f}$ (bottom panel) NPCs transfected with expression plasmids containing NesCre. Cells were plated at 2 x10^5cells/mL and transfected with NesGFP or NesCre expression plasmids 12hrs after plating. **C,** Quantification of the percentage of transfected cells that are apoptotic. Apoptosis was assessed by nuclear condensation at 24, 36, 48 and 60hrs after transfection. Results are expressed as mean ± SEM (n=3). *p<0 .05


A
GFP
Hoechst
Merge
NesGFP
Mcl-1wt
Mcl-1f/f
B
NesCre
Mcl-1wt
Mcl-1f/f
50 µm
C
% Apoptotic/GFP+ Cells
25
20
15
10
5
0
*
*
Time (Hrs.)
Wt. NesGFP
Wt. NesCre
Mcl-1f/f NesGFP
Mcl-1f/f NesCre

Mcl-1 Loss-of-Function Increases Apoptosis of Adult NPCs *in vivo*

As I have shown that Mcl-1 loss-of-function results in increased apoptosis of adult NPCs *in vitro*, I next addressed the question of whether Mcl-1 loss-of-function also affects the survival of NPCs, *in vivo*. To address this question, I used *in vivo* electroporation (Barnabe-Heider *et al.*, 2008) to transfect either NesGFP or NesCre into NPCs in the SVZ of adult wildtype or Mcl-$1^{f/f}$. By 72 hrs after transfection, there was robust expression of both plasmids in all genotypes (Fig.10A,B). When chromatin condensation was assessed at 72 hrs, Mcl-$1^{f/f}$ NPCs transfected with NesCre had a significantly higher rate of apoptosis when compared to controls (Fig.10C; Wt. NesGFP vs. Mcl-$1^{f/f}$NesCre, Wt. NesCre vs. Mcl-$1^{f/f}$NesCre: $p<0.01$; Mcl-$1^{f/f}$NesGFP vs. Mcl-$1^{f/f}$NesCre $p<0.05$; n=3). These results support the *in vitro* findings demonstrating that Mcl-1 is a potent prosurvival factor for NPCs *in vivo*.

Mcl-1 Gain-of-Function Decreases Apoptosis in Adult NPCs *in vitro*

To assess whether Mcl-1 gain-of-function is sufficient to reduce apoptosis in the adult neural precursor population *in vitro*, I performed gain-of-function experiments by overexpressing Mcl-1 in adult SVZ-derived cultures. As in our loss-of-function studies, expression of both control and Mcl-1 plasmids had occurred by 24 hrs. When apoptosis was assessed by nuclear condensation, Mcl-1 overexpression resulted in roughly a 2-fold decrease in apoptosis at 24 hrs (Wt. GFP vs. Wt. Mcl-1: $p<0.01$; n=3). This was sustained for 60-hrs after transfection (Fig.11A). As I saw a significant increase in apoptosis specifically in neural precursor cells in the Mcl-1 loss-of-function studies (Figs. 8,9,10), I questioned whether overexpressing Mcl-1 in adult SVZ-derived NPCs would decrease the endogenous rate of apoptosis. As in our loss-of-function studies, the cultures were labeled with the NPC marker Nestin and assessed for

apoptosis based on nuclear condensation. When apoptosis was assessed at 36hrs, the level of apoptosis did not differ between Mcl-1 overexpression (Fig. 11B). However, when the level of apoptosis was assessed in Nestin positive NPCs at 60hrs, there was a significant reduction in apoptosis in Mcl-1 transfected cells (Fig.11B; Wt. GFP vs. Wt. Mcl-1: $p<0.01$; $n=3$). These data demonstrate that Mcl-1 gain-of-function in NPCs leads to an overall reduction in endogenous apoptosis, *in vitro*.

Figure 10. Mcl-1 loss-of-function results in increased apoptosis in adult NPCs *in vivo*. NesGFP or NesCre plasmids were electroporated into the rostral lateral ventricles and apoptosis was assessed at 72hrs. **A,** Representative photomicrographs of GFP^+ cells from coronal wild type (top panel) and Mcl-$1^{f/f}$ (bottom panel) brains that were transfected with NesGFP. **B,** Representative photomicrographs of GFP^+ cells from coronal sections of wild type (top panel) and Mcl-$1^{f/f}$ (bottom panel) brains that were transfected with NesCre. **C,** Quantification of the percentage of apoptotic wild type and Mcl-$1^{f/f}$ cells that were transfected with NesGFP or NesCre *in vivo*. Transfected GFP positive apoptotic cells were identified by nuclear condensation as visualized by Hoechst staining. Results are expressed as the mean ± SEM (n=3). *$p<0.05$

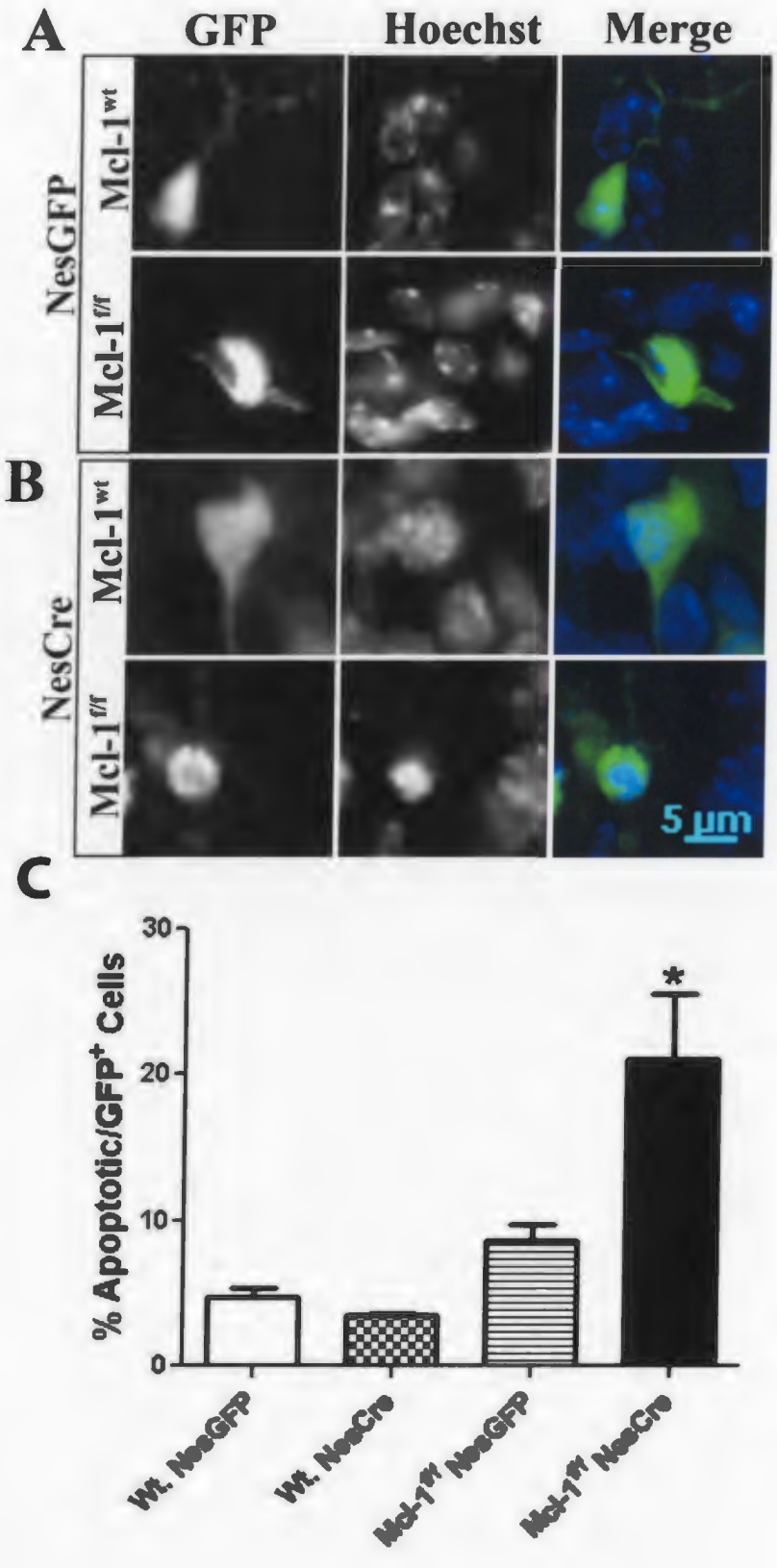
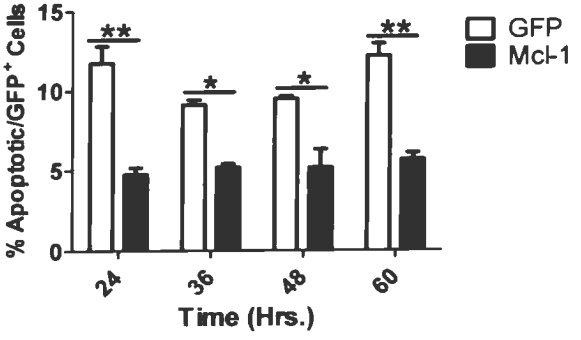
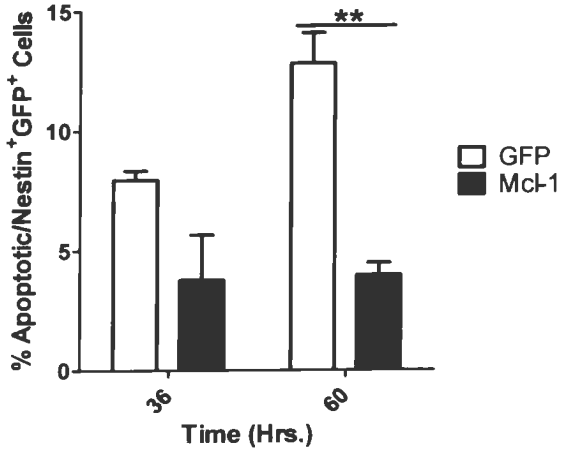

A
GFP
Hoechst
Merge
NesGFP
Mcl-1wt
Mcl-1f/f
B
NesCre
Mcl-1wt
Mcl-1f/f
5 µm
C
% Apoptotic/GFP+ Cells
30
20
10
0
*
Wt. NesGFP
Wt. NesCre
Mcl-1f/f NesGFP
Mcl-1f/f NesCre

Figure 11. Mcl-1 gain-of-function results in decreased apoptosis of SVZ-derived cultures and Nestin-positive adult NPCs *in vitro*. Cells were plated at 2 x10^5cells/mL and transfected with GFP and Mcl-1 expression plasmids 6hrs after plating. **A,** Quantification of the percentage of transfected cells that are apoptotic. Apoptosis was assessed by nuclear condensation as visualized by Hoechst staining at 24, 36, 48 and 60hrs. **B,** Quantification of the percentage of Nestin-positive transfected cells that are apoptotic. Cells were stained with primary antibody against Nestin and apoptosis assessed based on nuclear condensation as visualized by Hoechst staining at 36 and 60hrs. Results are expressed mean ± SEM (n=3). *$p<0.05$, *$p<0.001$


% Apoptotic/GFP+ Cells
15
10
5
0
**
*
*
**
24
36
48
60
Time (Hrs.)
GFP
Mcl-1


% Apoptotic/Nestin+GFP+ Cells
15
10
5
0
**
36
60
Time (Hrs.)
GFP
Mcl-1

Chapter 4

Discussion

Mcl-1 is Expressed in NPCs of the Adult SVZ

Our initial Mcl-1 co-labeling studies demonstrate that Mcl-1 is expressed in both the neural stem cell and neural progenitor populations, *in vivo*. This is the first time that a Bcl-2 prosurvival protein has been found to be expressed within the adult neural precursor population. The other major Bcl-2 prosurvival proteins, Bcl-2 and Bcl-X_L, are expressed at specific times during CNS development but the expression of both of these proteins significantly declines by the first week of postnatal life (Lindsten *et al.*, 2005). Moreover, the fact that Mcl-1 is the only Bcl-2 prosurvival factor expressed in NPCs from the early stages of CNS development (Arbour *et al.,* 2008) into adulthood provides a huge opportunity to manipulate NPC apoptosis in both developmental conditions as well as the neurodegenerative diseases that are seen in adulthood.

In addressing whether Mcl-1 is expressed in adult NSCs, the animals were euthanized at 0.5hrs and 28 days after BrdU labeling, which specifically labels the neural progenitor and neural stem cell populations, respectively (Morshead & van der Kooy, 1992; Craig *et al.*, 1994). Roughly half of BrdU positive cells seen in both populations were co-labeled with Mcl-1, which could suggest that the NPC population is heterogenous with some cells having a requirement for Mcl-1 while others do not. This is supported by the fact that not all embryonic NPCs require Mcl-1 for survival (Arbour *et al.,* 2008). Furthermore, Barraud *et al.,* (2005) also suggested that

the NPC population is heterogenous by creating a Sox1-GFP transgenic mouse and using fluorescence-activated cell sorting (FACS) to demonstrate that there are region specific morphological and potentiation differences within the Sox1-GFP population.

Another explanation as to why only half of the NPC population are Mcl-1^{+} may relate to the level of cell death that occurs endogenously within this population. Since 1 progeny will die for every neural progenitor division (Morshead & van der Kooy, 1992) the proportion of NPCs that do not express Mcl-1 may be those cells destined to die. As there is a lack of cell specific markers for the different NPC subpopulations, one could FACS sort NPCs based on Mcl-1 expression. If this technique did separate the NPC subpopulations, then it would support that there is a heterogenous population. Furthermore, one could then carry out characterization studies for self-renewal, proliferation kinetics and survival to assess potential differences between the subpopulations. These studies may provide a significant contribution to our understanding of the adult population. Investigations into Mcl-1's function in systems that have the ability to isolate stem and progenitor cells have demonstrated distinct roles for Mcl-1 in the different subpopulations. For instance, Opferman *et al.*, (2005) demonstrated that conditional deletion of Mcl-1 within the Hematopoietic system specifically kills the hematopoietic stem cells. If a similar type of specificity was found within the adult NPC population, it could have dramatically different impacts and as a result, the strategies that would be developed to use Mcl-1 in a regenerative capacity would also differ.

Mcl-1 Loss-of-Function Increases Apoptosis of Adult NPCs both *in vitro* and *in vivo*

Collectively the *in vitro* and *in vivo* Mcl-1 loss-of-function experiments demonstrate that Mcl-1 is a survival factor in adult NPCs of the SVZ. There have been several studies demonstrating increased levels of apoptosis in NPCs cell lines (Walls *et al.*, 2009) or primary NPCs *in vitro* (Hung & Porter, 2009); however, all of these studies use exogenous initiators of apoptosis such as nitric oxide (Hung & Porter, 2009) and hypoxia (Walls *et al.*, 2009). As these initiators of apoptosis are present in a variety of disease states, these studies were completed in order to characterize the pathophysiology of NPCs during CNS injury. However, compounds such as nitric oxide or a hypoxic state are not present in the healthy brain and therefore, these studies do not provide any insight into how adult NPC apoptosis occurs within the healthy brain. Having an understanding of the molecular mechanisms that regulate adult NPC apoptosis in the healthy brain may provide strategies that facilitate regeneration. This thesis demonstrates that Mcl-1 is not only expressed within the adult NPC population but that Mcl-1 loss-of-function increases NPC apoptosis without any exogenous apoptotic initiator. This demonstrates that Mcl-1 is key regulator of adult NPC apoptosis in the healthy brain.

The Mcl-1 loss-of-function studies presented here are the first experiments to demonstrate that an anti-apoptotic protein of the Bcl-2 family has a pro-survival effect on the adult NPC population. Since Mcl-1 loss-of-function increases NPC apoptosis both *in vitro* and i*n vivo* and Mcl-1 gain-of-function inhibits the endogenous apoptosis in adult NPCs it can be suggested that Mcl-1 gain-of-function could possibly provide a protective strategy against NPC apoptosis and ultimately facilitate regeneration.

Mcl-1 Gain-of-Function Decreases Apoptosis in Adult NPCs *in vitro*

The Mcl-1 gain-of-function experiment uses the wild type form of Mcl-1 and demonstrates that enhancing Mcl-1 expression in adult NPCs *in vitro* decreases the endogenous rate of apoptosis that is seen within this population. Although this suggests that Mcl-1 gain-of-function could potentially lead to an expansion of the adult NPC population, unpublished data from the Vanderluit laboratory suggest that Mcl-1 gain-of-function affects the proliferation kinetics of embryonic NPC by causing the cells to exit the cell cycle. In spite of this, Mcl-1 gain-of-function may have a different effect of the adult NPC population, therefore the risk of tumorigenesis is still present. As such, studies of how Mcl-1 gain-of-function affects the proliferation kinetics of adult NPCs are currently being conducted within the Vanderluit laboratory. However, independent of its affect on cell proliferation, the ability of Mcl-1 to reduce apoptosis in adult NPCs has major implications for adult regeneration. For instance, one of the current challenges in stroke regeneration is the fact the NPCs that migrate to the site of injury and differentiate ultimately undergo apoptosis (Kernie & Parent, 2009). Therefore, by using a Mcl-1 gain-of-function strategy in conjunction with other regenerative strategies such as trophic factor administration (Reynolds & Weiss, 1992; Craig *et al.*, 1996), it may be possible to inhibit apoptosis in this situation while also circumventing the issue of tumorigenesis that exist with many prosurvival proteins.

A variety of mechanisms have been shown to expand the NPC population both *in vitro* and *in vivo*. Studies have demonstrated that enhanced trophic factor support such as EGF can expand the NPC population both *in vitro* and *in vivo* (Reynolds & Weiss, 1992; Craig *et al.*, 1996). Fittingly, exogenous stimuli such as exercise, which are capable of enhancing trophic factor production in the stem cell niches have been shown to increase proliferation of the NPC

population (van Praag *et al.*, 1999). Other studies have shown that particular cell cycle regulators such as PCNA, the Cip/Kip family and p53 (Bertand & Hermanson, 2010) have the potential to alter proliferation within adult NPCs (He *et al.*, 2009), while inactivation of cell cycle inhibitors such as the $p16^{Ink4a}$ and $p19^{Arf}$ tumor suppressor proteins via Bmi-1 enhances stem cell self-renewal but has little effect on neural progenitor proliferation (Molofsky *et al.*, 2003).

There has been some evidence suggesting that Mcl-1 expression levels are influenced by trophic factors including insulin-like growth factor 1 (Zhang & D'Ercole, 2004). As trophic factor administration has been identified as a mechanism to expand the adult NPC population (Reynolds & Weiss, 1992; Craig *et al.*, 1996), Mcl-1 gain-of-function strategies may offer a potential additive effect to trophic factor expansion by reducing the endogenous rate of apoptosis that occurs within this population. One advantage of this approach lies with current techniques such as retroviral gene delivery that can be used in the experimental setting to allow Mcl-1 gain-of-function to be maintained during proliferation, migration and differentiation, which could dramatically enhance the regenerative capacity of adult NPCs.

One of the major drawbacks that our Mcl-1 gain-of-function study has, is that it does not address whether Mcl-1 gain-of-function selectively rescues a particular subpopulation of the neural precursor population. There was an overall decrease in apoptosis in the Mcl-1 gain-of-function study, but whether Mcl-1 gain-of-function selectively rescues NSCs over neural progenitor cells, or vice versa could have different effects. For instance, if Mcl-1 gain-of-function selectively rescues NSCs, there would presumably be more NSCs proliferating, thus the entire NPC population would expand. However, if Mcl-1 gain-of-function selectively rescues the neural progenitor population that are already committed to a neuronal fate, then Mcl-1 gain-of-

function could result in an increase in neurogenesis without altering the NPC population. To specifically assess how Mcl-1 gain-of-function affects the adult neural stem cell population, a self-renewal assay (Reynolds & Weiss, 1992) could be performed on SVZ-derived NPCs that are infected with a Mcl-1 expressing lentiviral construct that integrates into the host genome. By using a construct that stably integrates into the host genome, Mcl-1 gain-of-function would be maintained in all progeny and would thus avoid the dilution effect that would be seen using DNA plasmid constructs. If Mcl-1 gain-of-function enhanced NSC survival without altering cell cycle kinetics, then the self-renewal assay should yield a higher number of secondary neurospheres demonstrating an expansion of the neural stem cell population.

To specifically assess how Mcl-1 gain-of-function affects the neural progenitor population, one could use both *in vitro and in vivo* strategies. More specifically, if the spheres formed in an *in vitro* neurosphere assay were much larger as compared to controls, this would also suggest that more progenitors are surviving demonstrating that Mcl-1 gain-of-function affects the neural progenitors *in vitro.* Furthermore, to assess how Mcl-1 gain-of-function affects neural progenitors *in vivo,* one could overexpress Mcl-1 through *in vivo* electroporation, as over 99% of the SVZ proliferating population are neural progenitors (Craig *et al.*, 1994; Morshead *et al.,* 1994). To specifically address whether Mcl-1 gain-of-function enhances the survival of NPCs *in vivo* is very challenging, as many of the techniques used to measure survival also measure proliferation. For instance, the 10.5hrs BrdU labelling protocol could be used to address whether Mcl-1 gain-of-function leads to an expansion of the neural progenitor population. However, if there was an increased number of $BrdU^+$ cells in the Mcl-1 gain-of-function group, an equally strong argument could be made for either survival or proliferation. Although both arguments are legitimate, increasing either survival and/or proliferation would lead to an

expansion of the neural precursor population, which could have a putative therapeutic role. However, one way to differentiate between proliferation and survival in a potential NPC expansion would be to do Mcl-1 gain-of-function transfection experiments in a Bax knockout. By eliminating the pro-apoptotic affects of Bax, there should be no difference in the level of apoptosis in Mcl-1 gain-of-function group and controls. Therefore, any difference in the number of transfected cells would be the result of altered proliferation and not survival. Conversely, if there was no difference in the proliferation of Mcl-1 vs. control transfected cells, this would suggest that the NPC expansion that is seen is due to survival and not proliferation.

Future Directions

Collectively, the results of this thesis demonstrate that Mcl-1 is a critical survival factor for adult NPCs. This has a number of implications for both the healthy aging brain as well as the injured brain. Firstly, Tropepe *et al.,* (1997) have shown that there is a decline in neural progenitors in the SVZ of aging animals and that this is a result of a lengthening of the cell cycle within this population. This presents a major problem when targeting adult NPCs for the treatment of different neurodegenerative diseases, as many of these are more prevalent in the aging brain. One way to remedy this situation would be to enhance the expression of Mcl-1 in TACs, which would presumably decrease the endogenous rate of apoptosis in the TAC population. Moreover, by enhancing the survival of NPCs *in vivo,* the cells that were rescued could also contribute to the SVZ niche, which would further support the maintenance of the neural precursor population throughout the life time (Riquelme, *et al.,* 2008). In summary, Mcl-1 gain-of-function in NPCs of the aging healthy brain may offer several key benefits that would

allow the NPC population to be poised to offer an enhanced regenerative response when a neurodegenerative insult occurs.

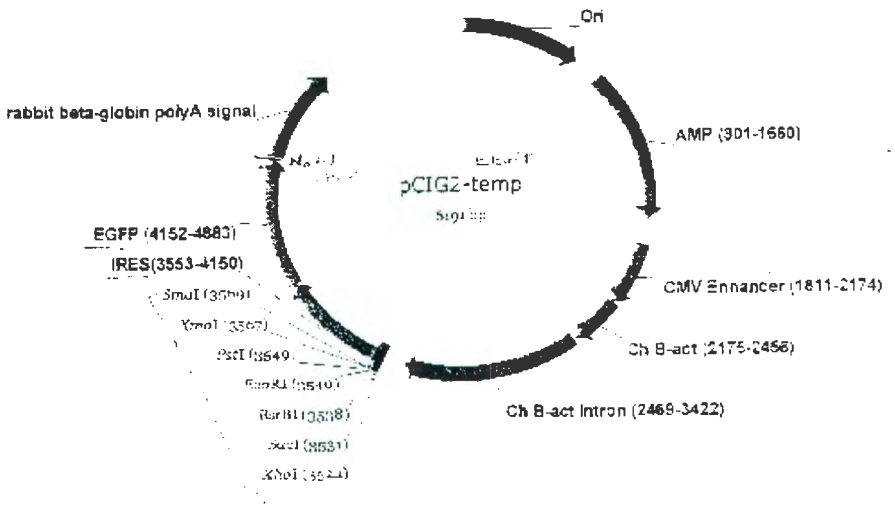
There have been many studies demonstrating that endogenous NPCs respond to stroke in a potentially therapeutic manner (for review see Lindvall & Kokaia, 2010). Furthermore, this therapeutic response can be enhanced by applying trophic factors that promote proliferation and subsequently applying factors that direct differentiation toward a neuronal fate (Kolb *et al.*, 2007). However, one of the greatest challenges that exists within this area of research is the high rate of cell death that occurs in these newly born cells (Lindvall & Kokaia, 2010). To combat this problem, one approach could involve genetic manipulation of the NPC population such that they are resistant to apoptosis after migrating away from the SVZ niche. The Mcl-1 gain-of-function experiment completed in this thesis suggests that Mcl-1 would be a good target to enhance the survival of endogenous NPCs that migrate away from the SVZ when responding to an injury such as a stroke.

DNA expression plasmids were used to alter the genetic makeup of adult NPCs in all of the experiments carried out in this thesis. This type of genetic manipulation is beneficial for short-term experiments that do not involve multiple cell divisions. To fully assess how Mcl-1 gain-of-function affects both the aging healthy brain as well as the injured brain, one would need to stably insert a copy of the Mcl-1 gene driven off a constitutively active promoter into the NPC genome so that Mcl-1 gain-of-function would be carried forward during proliferation. This could be accomplished by using a retrovirus and such a strategy would be sufficient for proof-of-principle Mcl-1 gain-of-function studies. Obviously, the ultimate goal when applying Mcl-1 gain-of-function to any disease model would be to achieve recovery of function. However, because any study that employed a retrovirus could only enhance Mcl-1 expression in a

proportion of the NPC population, it is reasonable to suggest that this type of approach would not provide the full benefit Mcl-1 gain-of-function has to offer. To achieve the optimal benefit that Mcl-1 gain-of-function offers would require an inducible transgenic mouse that drives Mcl-1 off of the Nestin promoter. Once induced, Mcl-1 gain-of-function would be present within the entire neural precursor population as well as all of the subsequent NPC progeny. This would provide the optimal setting to assess whether Mcl-1 gain-of-function leads to functional recovery in a variety of neurodegenerative diseases.

Appendix

pCIG2-GFP



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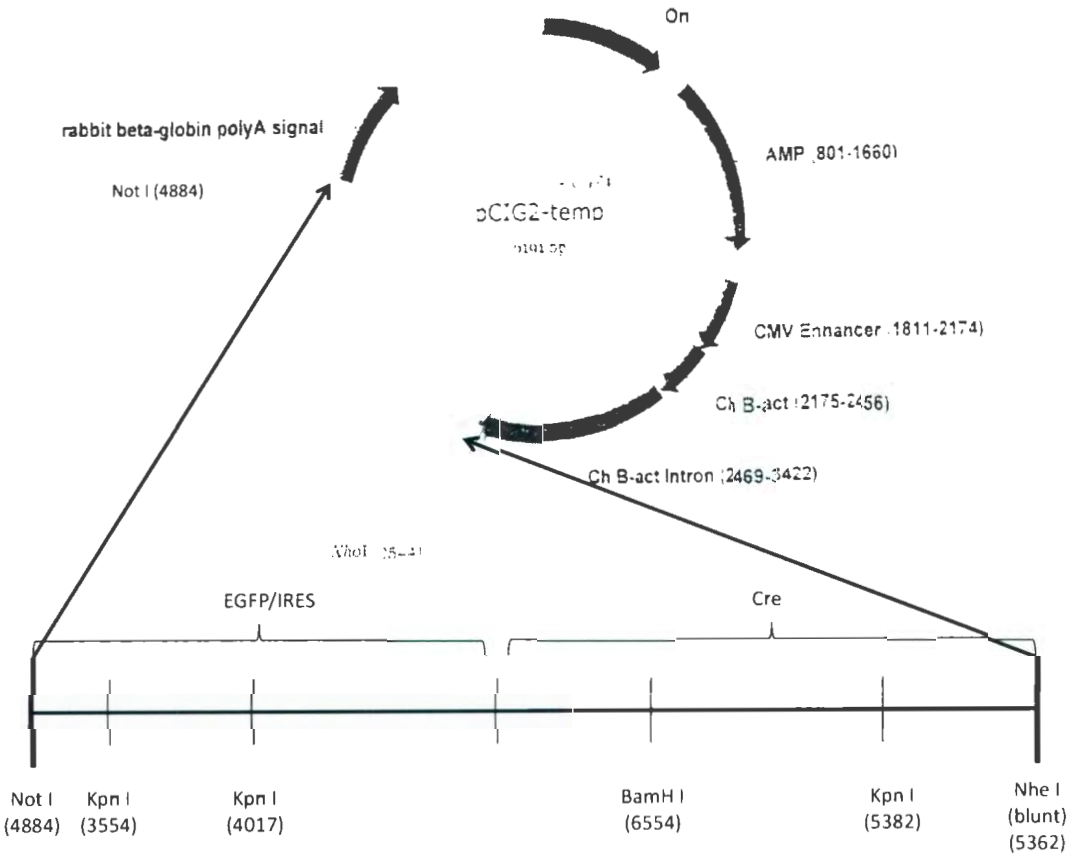
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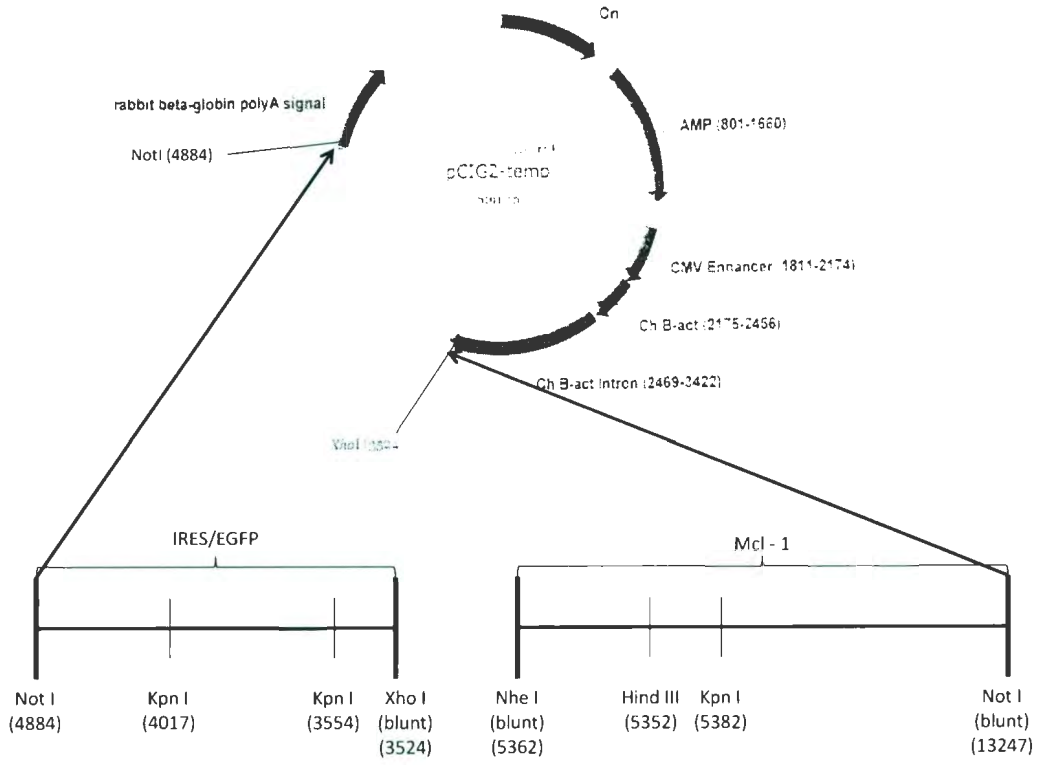
AMP (801-1660)
pCIG2-temp
CMV Enhancer (1811-2174)
Ch B-act (2175-2456)
Ch B-act Intron (2469-3422)
EGFP/IRES
Cre
Not I (4884)
Kpn I (3554)
Kpn I (4017)
BamH I (6554)
Kpn I (5382)
Nhe I (blunt) (5362)

pCIG2-Mcl-1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IRES/EGFP
Mcl - 1
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Kpn I (4017)
Kpn I (3554)
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Kpn I (5382)
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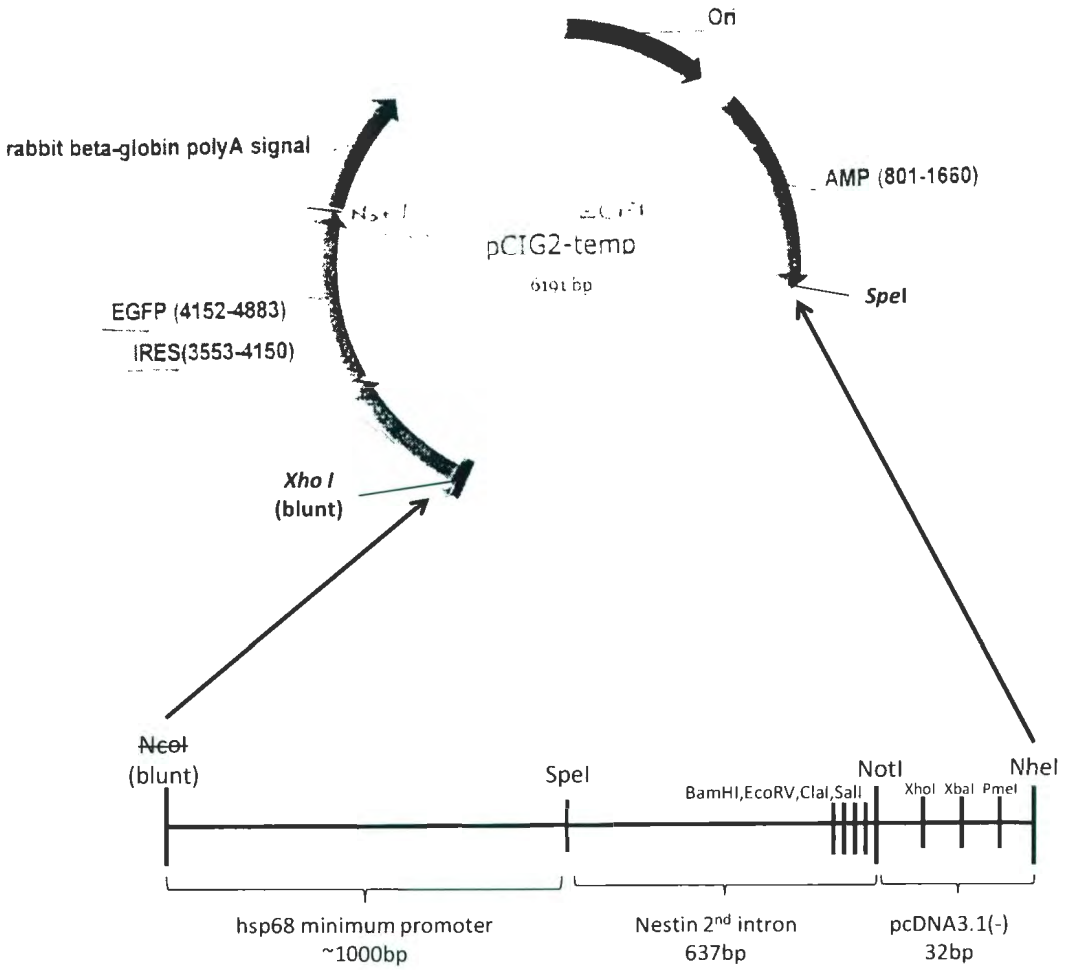
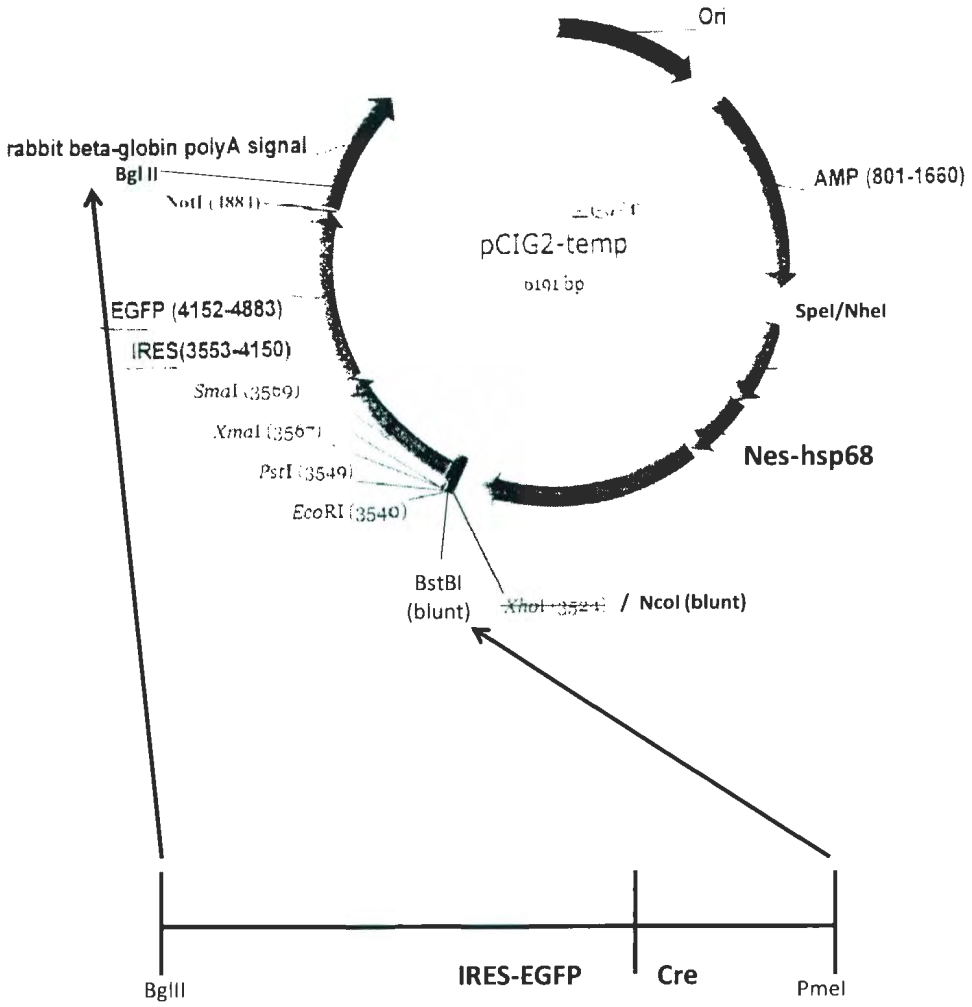
NesGFP



Total Size: ~1670bp

NesCre



From pCIG2-Cre

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