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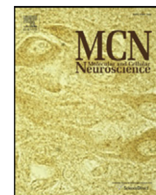

Mcl-1 regulates the survival of adult neural precursor cells

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A B S T R A C T

Since the discovery of neural precursor cells (NPCs) in the adult mammalian brain, there has been a lot of excitement surrounding the potential for regeneration in the adult brain. For instance, many studies have shown that a significant number of NPCs will migrate to a site of injury and differentiate into all of the neural lineages. However, one of the main challenges affecting endogenous neural regeneration is that many of the NPCs that migrate to the injury site ultimately undergo apoptosis. Therefore, we sought to determine whether myeloid cell leukemia-1 (Mcl-1), an anti-apoptotic Bcl-2 protein, would promote the survival of adult NPCs by impeding apoptosis. To do this, we first confirmed that Mcl-1 is endogenously expressed within the adult NPC population using BrdU labeling assays. Next, we conditionally deleted Mcl-1 in adult NPCs using cre/lox technology and expressed Cre from the NPC-specific promoter Nestin. *In vitro*, cells that had Mcl-1 conditionally deleted had a 2-fold increase in apoptosis when compared to controls. *In vivo*, we used electroporation to conditionally delete Mcl-1 in adult NPCs and assessed apoptosis at 72 h. after electroporation. As in our *in vitro* results, there was a 2-fold increase in apoptosis when Mcl-1 was conditionally deleted. Finally, we found that Mcl-1 over-expression reduced the endogenous rate of adult NPC apoptosis 2-fold *in vitro*. Collectively, these results demonstrate that Mcl-1 is crucial for the survival of adult NPCs and may be a promising target for future neural regeneration therapies.

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Introduction

The discovery of stem cells in the adult mammalian brain has created excitement surrounding the idea of regeneration in the adult central nervous system. Studies in rodents show that endogenous neural stem cells respond to an injury by proliferating and producing new neurons and glia in an attempt to replace cells that have been lost or damaged (Arvidsson et al., 2002; Liu et al., 1998; Magavi et al., 2000; Parent et al., 2002; Wang et al., 2007). Perhaps most exciting is the demonstration that regenerative neurogenesis also occurs in the human brain following stroke (Jin et al., 2006). However, for successful regeneration to occur NPCs must overcome a variety of challenges including switching from their normal quiescent state to a proliferative state to expand NPC numbers (Bouab et al., 2010; Morshead et al., 1994; Tropepe et al., 1997), successfully migrating to the site of injury, differentiating into specific neural lineages (specific neuronal subtypes and glial cells) and integrating into existing neural networks (Kernie and Parent, 2010; Okano et al., 2007). Although NPCs proliferate and migrate to the site of injury and some differentiate into neurons (Arvidsson et al., 2002; Parent et al., 2002), the vast majority of cells undergo apoptosis within a month of arriving at the injury site (Arvidsson et al., 2002; Haas et al., 2005; Kim and Szele, 2008; Okano et al., 2007; Parent, 2003). Therefore, strategies aimed at promoting the survival of NPCs and their progeny may enhance regeneration of the injury site and allow NPCs to differentiate and integrate into neural networks to affect functional recovery.

One of the main neural stem cell niches in the adult brain is found adjacent to the lateral ventricles in an area known as the subventricular zone (SVZ) (for review see Duan et al. (2008)). This highly vascular niche contains several NPC subpopulations, which include the slowly cycling neural stem cells (B cells), and the more rapidly dividing progenitor cell populations, which are composed of the transit amplifying progenitor cells (C cells) and neuroblasts (A cells) (Doetsch et al., 1997). Based on their distinct cell cycle kinetics, neural stem cells can be distinguished from the more rapidly dividing progenitor cells through proliferation assays with ^{3}H-thymidine or BrdU labeling (Morshead and van der Kooy, 1992; Morshead et al., 1994; Vanderluit et al., 2004). The NPC subpopulations can also be differentiated by labeling with cell specific markers, including GFAP, which labels neural stem

Abbreviations: Bcl-2, B cell lymphoma 2; Bcl-xL, B cell lymphoma X large fragment; CKO, conditional knockout; BrdU, Bromodeoxyuridine; Cre, Cre recombinase; CMV, cytomegalovirus; Dcx, Doublecortin; DIV, days *in vitro*; FGF-2, fibroblast growth factor 2; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; hrs, hours; Mcl-1, myeloid cell leukemia-1; Nes, Nestin; NPC, neural precursor cell; PSA-NCAM, polysialated n-cell adhesion molecule; SVZ, subventricular zone; TAT, trans-activator of transcription.

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cells, or the transit amplifying cell marker Dlx2 and the migratory neuroblast cell markers Dcx and PSA-NCAM (Ming and Song, 2005; Okano et al., 2007). Furthermore, the entire NPC population also expresses the intermediate filament Nestin, which can be used to distinguish NPCs from differentiated cells (Lendahl et al., 1990; Okano et al., 2007).

The size of the NPC population in the adult brain is tightly regulated, such that each cell division is balanced by apoptotic elimination resulting in an apoptotic rate of ~60% (Morshead et al., 1994). As a result, apoptosis serves a physiological role within the healthy brain; however, when the brain suffers an injury, apoptosis can impede the ability for NPCs to regenerate the injured areas. Therefore, limiting the degree of NPC apoptosis could be one strategy to enhance brain regeneration.

One mechanism that could be used to inhibit apoptosis in adult NPCs, would be to identify and manipulate anti-apoptotic proteins that are endogenously expressed within the adult NPC population. To date, few anti-apoptotic proteins have been identified as adult NPC survival factors. For instance, the anti-apoptotic proteins Bcl-w and BFl1 (A1) are not expressed in the nervous system (Lin et al., 1993; Orlofsky et al., 1991; Print et al., 1998) and germ line deletion of either Bcl-2 or Bcl-xL does not affect NPC survival demonstrating that these proteins do not have an endogenous role in promoting NPC survival (Michaelidis et al., 1996; Motoyama et al., 1995).

One Bcl-2 anti-apoptotic protein whose function in adult NPCs has yet to be assessed is Mcl-1. Unlike anti-apoptotic proteins, Bcl-2 and Bcl-xL, Mcl-1 is expressed during the cell cycle with expression peaking in M-phase (Harley et al., 2010). As such, Mcl-1 is highly expressed in many proliferating precursor cells and the conditional deletion of Mcl-1 has lead to widespread apoptosis within these various precursor populations (Arbour et al., 2008; Opferman et al., 2003, 2005; Sitailo et al., 2009; Vick et al., 2009). For instance, Opferman et al. (2003) used a tissue-specific Mcl-1 conditional knockout model to demonstrate that Mcl-1 depletion in the hematopoietic system leads to an almost complete ablation of hematopoietic stem cells and their progenitor populations demonstrating that Mcl-1 is a survival factor for the hematopoietic precursor population (Opferman et al., 2003, 2005). Furthermore, conditional deletion of Mcl-1 within the embryonic nervous system revealed that Mcl-1 is required for the survival of embryonic NPCs (Arbour et al., 2008). Loss of Mcl-1 in the Nestin-expressing NPC population results in widespread apoptosis of embryonic NPCs and severely impairs neurogenesis (Arbour et al., 2008). Mcl-1, therefore is the only identified anti-apoptotic Bcl-2 protein that regulates embryonic NPC survival. However, the embryonic lethality of the nervous system-specific conditional knockout mice has hampered investigations into the role that Mcl-1 plays within the adult NPC population.

In this study, we used a different approach to examine the role of Mcl-1 in adult NPCs. We first confirmed that Mcl-1 is endogenously expressed within the adult NPC population by using BrdU labeling assays. To manipulate Mcl-1 expression in adult NPCs, we used loss- and gain-of-function strategies by combining cre/lox technology and standard transfection techniques *in vitro*, whereas *in vivo* we used electroporation of plasmids into NPCs in the SVZ of the brain. Our results demonstrate that Mcl-1 loss-of-function in adult NPCs both *in vitro* and *in vivo* causes NPC apoptosis while Mcl-1 gain-of-function decreases apoptosis. Collectively, these findings are the first to demonstrate that the anti-apoptotic protein, Mcl-1, is a crucial survival factor for adult NPCs. Furthermore, this finding also suggests that Mcl-1 may be a promising target to promote NPC survival and neural regeneration in the injured brain.

Results and discussion

Mcl-1 is expressed in proliferating neural precursors within the adult SVZ

Embryonic NPCs were previously shown to be dependent on Mcl-1 for survival (Arbour et al., 2008). Numerous changes occur within NPCs from early embryonic development to the adult brain such that adult NPCs are distinctly different from embryonic NPCs with respect to their differentiation potential, cell cycle kinetics and trophic factor responsiveness (reviewed in (Molofsky et al., 2004; Vaccarino et al., 2001)). Therefore, we questioned whether Mcl-1 was also required for adult NPC survival.

To determine whether adult NPCs require Mcl-1 for survival, we first examined whether Mcl-1 is expressed in adult NPCs within the SVZ. To address this question, we used a BrdU labeling protocol to distinguish slowly cycling neural stem cells from the rapidly cycling progenitor cells based on their distinct cell cycle kinetics (Morshead and van der Kooy, 1992; Morshead et al., 1994, 1998; Reynolds and Weiss, 1992). Mice received i.p. BrdU injections every 2 h over a 10 h period to label the majority of proliferating cells in the adult SVZ and were euthanized 30 min (10.5 h time point) or 4 weeks after the last injection. Since the majority of proliferating cells in the SVZ are progenitor cells, the 10.5 h BrdU labeling protocol predominantly labels this population (Morshead and van der Kooy, 1992). Within this BrdU$^+$ population approximately 60% of the cells co-labeled with Mcl-1, indicating that neural progenitor cells within the adult SVZ express Mcl-1 (Figs. 1A, B). The 4-week survival group allowed us to examine the slowly dividing neural stem cell population which retains the BrdU label due to a protracted cell cycle time of ~15 days (Morshead et al., 1994). Immunohistochemistry revealed that ~50% of the BrdU$^+$ cells in the SVZ at 4 weeks also co-labeled with Mcl-1 (Fig. 1B). Taken together, these results demonstrate that Mcl-1 is expressed in >50% of proliferating cells within the NPC population of the SVZ.

The neural progenitor population in the adult brain contains a heterogeneous mix of cells including the rapidly proliferating transit amplifying cells and committed neuroblasts. Since the majority of BrdU$^+$ cells in the 10.5 h time point are predominantly the rapidly proliferating transit amplifying cells, we questioned whether Mcl-1 is also expressed in the neuroblast population. Immunohistochemistry for Mcl-1 and the committed neuroblasts markers doublecortin (Dcx) or PSA-NCAM (not shown), revealed that neuroblasts in the adult SVZ also express Mcl-1 (Fig. 1C). Furthermore, the Mcl-1 antibody was tested for specificity on tissue sections from embryonic day 12 wild type and Mcl-1 conditional knockout embryos, which demonstrated that Mcl-1 was not expressed in Mcl-1 conditional knockouts (Fig. 1D). Collectively, our results show that Mcl-1 is expressed in the different NPC populations within the adult SVZ from the slowly cycling neural stem cells to the rapidly proliferating progenitors and committed neuroblasts.

Mcl-1 loss-of-function causes apoptosis of adult neural precursors

Having demonstrated that Mcl-1 is expressed by proliferating NPCs in the adult brain, we next questioned whether Mcl-1 functioned in a survival capacity in this population. Since the embryonic lethality of Mcl-1 CKO mice (Arbour et al., 2008) precluded our analysis of Mcl-1 loss-of-function in adult mice, we used a plasmid transfection protocol to deliver Cre recombinase into adult NPCs *in vitro*. Cre protein expression was confirmed following transfection of HEK 293A cells (Fig. 2A). NPCs were cultured from adult wild type and Mcl-1$^{f/f}$ mice and transfected with the pCIG2 plasmid expressing Cre recombinase (Cre) or the pCIG2 vector alone, control (Ctl), and apoptosis was assessed at different time points following transfection (Fig. 2B). Cre expression alone did not induce apoptosis as no increase in cell death was observed in Cre-transfected wild type NPCs. There was an apoptotic baseline rate of 8 - 12% in control cultures; however, this apoptotic rate doubled to 18 - 22% in Cre-transfected Mcl-1$^{f/f}$ cells at each time point (Fig. 2B). Since SVZ-derived neurospheres contain a heterogeneous population of cells, it was unclear which cells were dying as a result of a loss of Mcl-1 expression and specifically whether NPCs required Mcl-1 for survival. To identify NPCs in the population

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