

Regulators of Adult Hippocampal Neurogenesis

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ABSTRACT

One mechanism of plasticity within the adult mammalian brain is the dynamic process of adult neurogenesis that is functionally important in physiological and pathological conditions. During this process, neurons develop from adult neural stem cells (NSCs) via intermediate neural progenitors (NPCs) through several processes including proliferation, survival, differentiation, migration and integration. Despite neurogenesis during development sharing these same processes, there is growing evidence highlighting unique mechanisms that regulate adult versus embryonic neurogenesis.

The studies in this thesis test the cell-intrinsic function of genes that have defined roles in embryonic neurogenesis and undefined roles in adult hippocampal neurogenesis using a combination of transgenic inducible mice and *in vivo* retroviral techniques. The first study examines the microtubule associated protein Doublecortin (DCX), which is transiently expressed by NPCs and is critical for neuronal migration. Our results show that, in the context of adult hippocampal neurogenesis, DCX is not required for the survival or differentiation of the NPCs within the subgranular zone (SGZ). The second study examines the functional role of the autophagy-associated gene 5 (*Atg5*) which is critical for embryonic neurogenesis and survival. Our findings demonstrate that the intracellular recycling process of autophagy is active throughout maturation of adult hippocampal NPCs and that ablation of *Atg5* produces a drastic reduction in NPC survival, without altering the neuronal fate of these cells. The third study examines the requirement of the familial-Alzheimer's disease associated genes, *presenilin 1* and *presenilin 2* (*PS1* & *PS2*), which are critical for embryonic NSC maintenance and differentiation. Similar to the findings with DCX, our results demonstrate that presenilins are dispensable for adult neurogenesis. Altogether, these studies add to the growing evidence suggesting differences

in the regulation of adult versus embryonic neurogenesis, and highlight autophagy as a novel regulator of survival for adult generated granule neurons in the hippocampus.

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LIST OF ABBREVIATIONS

BrdU	Bromodeoxyuridine
NSCs	Neural Stem cells
SVZ	Subventricular Zone
RMS	Rostral Migratory Stream
SGZ	Subgranular Zone
GFAP	Glial fibrillary acidic protein
RGL	Radial glia-like cell (also known as Type-1 NSCs)
DG	Dentate gyrus
BLBP	Brain lipid-binding protein
GLAST	Glutamate aspartate transporter
Sox2	SRY-related HMG-box gene 2
NPCs	Neural Progenitor Cells
Tbr2	Eomesodermin (T-box brain protein 2)
DCX	Doublecortin
GABA	<i>gamma</i> -Aminobutyric acid
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
NMDA	<i>N</i> -methyl-D-aspartate receptor
FAD	Familial autosomal dominant form of Alzheimer's disease
PS1	Presenilin-1
PS2	Presenilin-2
MWM	Morris water maze
OB	Olfactory bulb
Dpi	Days post infection
Bax	Bcl-2-associated X protein

FIP200	Focal adhesion kinase (FAK) family interacting protein of M_r 200K
Atg5	Autophagy-related gene 5
LC3	Microtubule-associated protein 1 light chain 3
mTLE	Mesial temporal lobe epilepsy
HCN	Hippocampal neural stem cells
ACD	Autophagic cell death
mTOR	Mammalian target of rapamycin
PtdIns3K	Phosphatidylinositol 3-kinase complex
PI3P	Phosphatidylinositol-3-phosphate

CHAPTER 1: INTRODUCTION

1. Adult Neurogenesis: The beginnings

Up until the late 20th century, the central dogma in neuroscience had been that the adult brain was static and the generation of new neurons occurred almost exclusively in the embryo during the process of neurogenesis. This view was challenged in the 1960s by the pioneering work of Altman and Das who presented the first anatomical evidence of dividing cells labeled with H³-Thymidine in the adult rat hippocampus (Altman and Das, 1965). These cells were later shown to have ultrastructural characteristics of neurons when examined 30 days after H³-Thymidine labeling (Kaplan and Hinds, 1977). In the 1980s evidence of adult neurogenesis began to emerge in other species including songbirds (Paton and Nottebohm, 1984) and non-human primates (Kaplan, 1983). Adult neurogenesis gained momentum when neural stem cells were isolated from the adult mammalian brain and were shown *in vitro* to be multipotent (Reynolds and Weiss, 1992). There were also advancements in labeling techniques that corroborated these findings including the use of thymidine analogue bromodeoxyuridine (BrdU) to birth-date proliferating cells in the adult brain (Kuhn et al., 1996). Soon after, the seminal discovery of neurogenesis in the adult human brain (Eriksson et al., 1998), marked the beginning of a shift towards an accepted view of neurogenesis occurring in the adult mammalian brain (Ming and Song, 2011).

2. The adult neurogenic niches

Adult neurogenesis has been shown in multiple species to occur within two neurogenic niches including the subventricular zone (SVZ) lining the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG). Both of these complex microenvironments support many different cell populations that include NSCs, NPCs, ependymal cells, vascular cells, astrocytes, microglia and interneurons. Within these niches, NSCs progress through distinct stages of

development to generate mature functional cells. For example in the SVZ, dividing radial glia-like (Type B) cells generate the transient amplifying (Type C) cells which give rise to neuroblasts (Type A). These neuroblasts migrate in chains along the rostral migratory stream (RMS) to reach the olfactory bulb (OB) where they differentiate into granule cells and periglomerular neurons of the OB (Ming and Song, 2011). In the second neurogenic niche, the SGZ, the relatively slowly dividing radial-glia like (Type-1) cells generate rapidly proliferating intermediate progenitors (Type-2 cells) which in turn differentiate into neuroblasts (Type 3 cells) and finally into granule neurons (Ming and Song, 2011; Bonaguidi et al., 2012). Although the SVZ and SGZ are the most characterized neurogenic niches, growing evidence also supports that the median eminence ependymal layer of the third ventricle in the hypothalamus retains the capacity for neurogenesis in the adult brain (Kokoeva et al., 2005; Lee et al., 2012; Haan et al., 2013; Robins et al., 2013; Maggi et al., 2015). In this region, radial glia-like cells termed tanycytes have been shown to proliferate and generate new hypothalamic neurons.

As the work presented in this thesis focuses on adult hippocampal neurogenesis, the remainder of this thesis will primarily describe the identity of NSCs, their transition into NPCs, their survival and maturation within the SGZ neurogenic niche.

3. Neural Stem cells (NSCs) in the adult brain

Determining the source and identity of NSCs that are capable of sustaining lifelong neurogenesis within the neurogenic niches has been a topic of extensive research utilizing both *in vitro* and *in vivo* systems. The existence of multipotent adult NSCs with the ability to self renew and differentiate into neurons, astrocytes and oligodendrocytes was originally described *in vitro* by expanding and differentiating the cells as neurospheres or adherent monolayer cultures (Reynolds and Weiss, 1992; Palmer et al., 1997; Palmer et al., 1999). This technique remains

widely used since primary cultures can be propagated as secondary cultures as an assessment of self-renewal or they can be cultured in the absence of growth factors to assess multipotent nature of adult NSCs through their ability to differentiate into neurons, astrocytes or oligodendrocytes. However, the interpretations derived from long-term expansion of NSCs, isolated from the neurogenic niche, have also raised some concerns. For instance, 1) the neurosphere assay may not give a read-out of *in vivo* NSC frequency since multiple populations including the transient amplifying cells can generate neurospheres (Mich et al., 2014), 2) cell density might affect the clonality of neurospheres and 3) the isolated NSCs may respond differently to exogenous growth factors within an environment of a population of cells (Pastrana et al., 2011). This raises the question of how to effectively identify NSCs *in vivo*.

Newly developed approaches using viral methods and transgenic mice permit visualization, manipulation and determination of cell lineage of adult NSCs *in vivo* (Dhaliwal and Lagace, 2011). The viral approach makes use of lentiviruses to deliver Cre recombinase into NSCs. This is achieved for example, by using a promoter such as glial fibrillary acidic protein (GFAP), that is highly active in NSCs to drive the expression of Cre (Enikolopov et al., 2015). Since viral approaches often only target a small cohort of the total population of cells *in vivo*, a more common approach to genetically manipulate the NSCs and their progeny has been the use of inducible transgenic mouse models that generally consist of three transgenes (**Figure 1**). The first transgene consists of Cre recombinase and a modified estrogen receptor (CreER^{T2}) under control of a promoter that is highly active in NSCs in the neurogenic niche, such as the intermediate filament protein Nestin, GFAP or the Glutamate aspartate transporter (Glast). The second transgene consists of a reporter protein, such as yellow florescent protein (YFP) that is preceded by a stop sequence between two loxP sites. Finally, the third transgene consists of a

floxed gene of interest. In this model, the administration of estrogen receptor ligand tamoxifen, induces CreER^{T2} to translocate to the nucleus, which allows recombination and permanent excision of the gene of interest and expression of the fluorescent protein from NSCs and their progeny. Tamoxifen can be administered to a transgenic animal at any age, thus allowing for temporal control of genetic ablation throughout an animal's lifespan and avoiding early developmental effects of gene ablation as seen in conditional knockout mice.

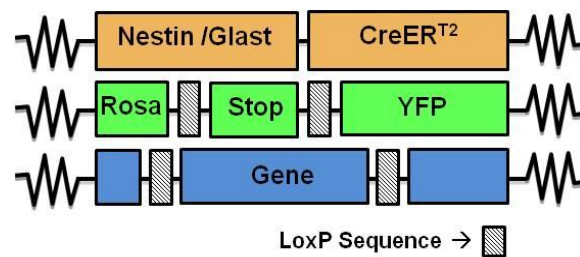
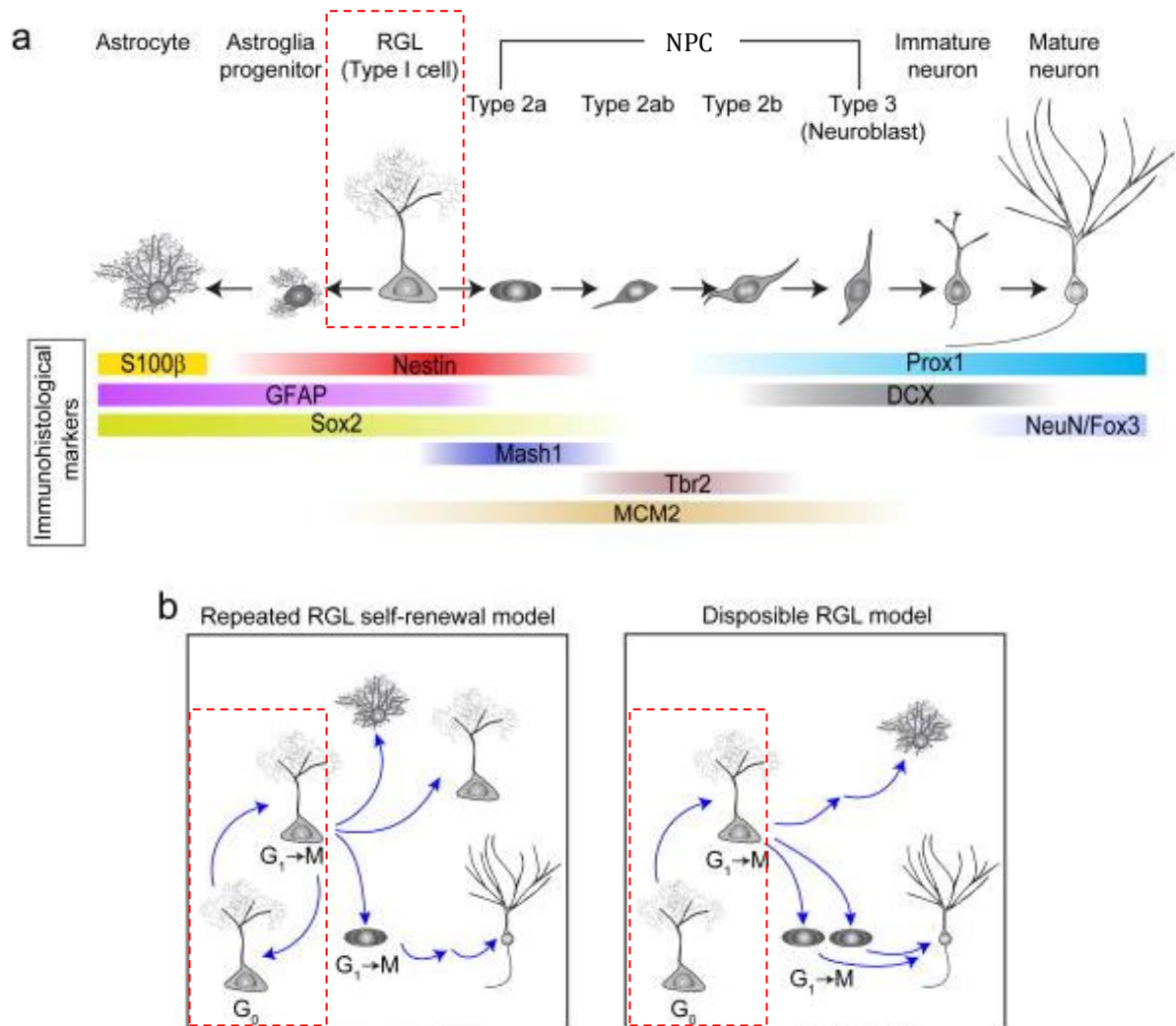


FIGURE 1: A schematic diagram of the three transgenes that are often used to make inducible transgenic mice to study adult neurogenesis. Nestin or Glial are expressed by NSCs and thus drive the expression of CreER^{T2}.

Through the use of these models, in the SGZ, the radial glia-like (RGL, Type-1) cells have been shown to demonstrate characteristics of NSCs and thus, throughout this thesis RGL/Type-1 cells are hereafter referred to as adult NSCs. *In vivo*, the adult NSCs are morphologically identified as having a short radial process terminating in an extensive, bushy arbor (Moss et al., 2016). These cells express Nestin, brain lipid-binding protein (BLBP), GFAP, GLAST and SRY-related HMG-box gene 2 (Sox2) and thus a combination of these immunohistological markers is often used to identify adult NSCs *in vivo* (**Figure 2a**). Upon activation, the NSCs have the potential to generate new NSCs (self-renewal), astroglia and neural progenitor cells (NPCs) and there are two opposing models for how this may occur (**Figure 2b**). *In vivo* clonal analysis led to the “repeated self-renewal model” that proposes that the NSCs undergo multiple rounds of self-renewal and differentiation to generate both neurons and astrocytes in the dentate gyrus.



Modified from (Bonaguidi et al., 2012)

FIGURE 2: Neurogenesis in the adult hippocampus. a) Within the SGZ of the dentate gyrus there is lineage progression of the adult NCSs (RGLs) to generate mature neurons and astrocytes. Often immunohistological markers are used to classify the cells based on their expression within the different cell populations. b) RGLs are considered to have characteristics of NSCs in the SGZ. Two of the proposed models of RGL fate choices: the “Repeated self-renewal” and the “Disposable RGL” are shown.

(Bonaguidi et al., 2011). Alternatively, by performing population analysis, Encinas et al., (2011) proposed a “disposable stem cell model” in which NSCs undergo multiple rounds of cell division, which is coupled with their depletion by terminal differentiation into astrocytes without any long-term maintenance (**Figure 2b**). The differences in these models could be a reflection of labeling of different NSC populations or could reflect labeling of different states (active versus quiescent) of the same NSC population (Bond et al., 2015). Indeed the heterogeneity in the state of NSCs and in the generation of progeny decisions can be considered a unifying principle for adult NSCs (Bonaguidi et al., 2012). Overall these findings therefore highlight the importance of using different transgenic models to target and determine the long-term contribution of these cells towards adult hippocampal neurogenesis.

4. Transitioning from NSCs to NPCs in the SGZ

The NPCs that are generated by the NSCs are often classified into Type 2a, 2b and 3 cells that can be identified based on their associated immunohistological markers (**Figure 2a**) as first described by Kronenberg et al., (2003). Many of these protein markers have also been implicated in regulating the transition from NSCs to NPCs. For example, the proneural transcription factor mammalian achaete scute homolog-1 (Mash1) expressed in activated NSCs and Type 2a NPCs regulates the exit of NSCs from a quiescent state and promotes their proliferation by directly targeting cell-cycle genes (Andersen et al., 2014). The T-box transcription factor Tbr2, expressed by Type 2b NPCs, promotes progression from NSCs to NPCs by repressing the NSC identity marker Sox2, while the loss of Tbr2 is associated with depletion of NPCs and deficits in neuronal differentiation (Hodge et al., 2012). The neuronal committed dividing Type 2b NPCs express prospero-related homeodomain transcription factor (Prox1) and microtubule associated

protein doublecortin (DCX). The expression of Prox1, which is sustained in the Type 3 NPCs, as well as in the immature and mature granule neurons, has a role in promoting neuronal differentiation, independent of affecting survival of adult generated neurons (Karalay et al., 2011). In contrast to Prox1, the expression of DCX is limited to the Type 2b and 3 NPCs, as well as the immature neurons, and is notably absent in the mature granule neurons. Due to this reason, DCX expression is often considered the gold standard for immature neurons and is often used as a proxy of adult hippocampal neurogenesis (Couillard-Despres et al., 2005). The functional role of DCX in neurogenesis is the focus of Chapter 2 of this thesis and is comprised of my published work examining the functional role of DCX during hippocampal neurogenesis (Dhaliwal et al., 2016)

5. Survival of NPCs into mature granule neurons

Of the thousands of new NPCs generated in the adult SGZ the majority die due to apoptosis, with only a small percentage surviving to generate granule cells in the DG. As the rate of survival is one of the biggest regulators of neurogenesis (Ryu et al., 2016), many laboratories, including ours, have focused on understanding when and why the cells die. With one of the goals of regenerative medicine strategies being to enhance neurogenesis, it is important to determine the cell intrinsic factors that mediate survival of new NPCs. Early studies suggested cell death occurred within 2-3 weeks after birth of new NPCs, a period that coincided with the expression of DCX (Dayer et al., 2003; Plumpe et al., 2006). In contrast, more recent work by Sierra et al., (2010) used a cumulative BrdU injection paradigm, and suggested that there are two waves of cell loss. The first wave of apoptosis is suggested to occur between 1-4 days of cell life with ensuing 56% of NPC loss, and the second occurs from 4-8 days resulting in 25% loss, altogether an overall rate of ~81% cell death of NPCs.

To determine the molecular regulators of NPC survival, a number of studies have used retrovirus strategies to label and genetically manipulate the NPCs. One of the seminal papers in the field showed that survival of new neurons is regulated by the NR1 subunit of the *N*-methyl-D-aspartate (NMDA) receptor (Tashiro et al., 2006). This paper developed a retrovirus-mediated, single-cell gene knockout technique that allows for survival of the NPC to be measured independent of any effects within the NSC population. In order to accomplish this task, the retrovirus only infects the dividing NPCs since insertion and integration of the retroviral genome into host DNA requires nuclear envelope breakdown during mitosis. Once infected the retroviral genetic material can be replicated by the host reverse transcriptase to produce DNA from viral RNA (Enikolopov et al., 2015). Thus one of the advantages of using retroviruses is the specific labeling of dividing NPCs and lack of infection of the quiescent NSCs and post-mitotic neurons. By labeling a cohort of dividing NPCs, one can follow the progression of labeled cells at different time points post-viral labeling. Here I describe a retroviral labeling strategy employed in my studies to knockout gene expression.

A mixture of two retroviruses: GFPCre and RFP is injected into floxed mice to knockout the gene of interest specifically from a cohort of dividing cells (**Figure 3**). The GFPCre construct carries a nuclear localization signal, thus GFPCre infected NPCs have nuclear GFP only. The RFP virus serves as an internal injection control. Three possible virally labeled cell populations are obtained post injection: the GFPCre only infected cells (Green), the RFP only infected cells (Red) and both GFPCre+RFP infected cells (Yellow). When injected into floxed mice, Green and yellow NPCs have the gene KO, whereas the red NPCs are WT. The survival of infected NPCs can be measured across two or more time points post infection by generating a survival ratio = (Yellow) / (Red+Yellow).

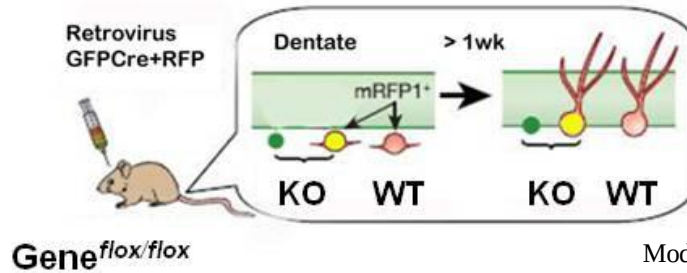


FIGURE 3: Dual GFP-Cre + RFP retrovirus injections into floxed mice results three fluorescently labeled NPC populations with the green and yellow NPCs being KO for the gene of interest and the red NPCs being Wt.

In addition to the apoptotic pathway regulating cell death or survival of adult-generated neurons, my published work (Xi et al., 2016), and the recent work of others (Wang et al., 2013) have suggested a role for autophagy in promoting the survival of adult-generated neurons. Autophagy is an evolutionarily conserved catabolic process by which intracellular components reach the lysosomes for degradation (Mizushima and Komatsu, 2011). In comparison to apoptosis, which regulates the turnover of entire cells, autophagy is responsible for turnover of intracellular, cytoplasmic constituents.

The basic autophagy pathway consists of 1) an isolation membrane that forms upon autophagy induction, 2) the enclosing of the isolation membrane to form a double-membraned autophagosome and finally 3) fusion of the autophagosome with lysosome (autolysosome) for degradation of enclosed contents (Mizushima and Komatsu, 2011). During each of these phases of autophagosome formation key autophagy-associated proteins have been identified to be required, as previously reviewed (Nixon, 2013; Fullgrabe et al., 2014; Yamamoto and Yue, 2014). Briefly, the initiation of autophagy is regulated through the phosphorylation status of the unc-51-like kinase 1 (ULK1) complex which occurs mainly through inhibition of the mammalian target of rapamycin (mTOR) but may also be activated by AMP-activated protein kinase. Changes in the phosphorylation status of individual components the ULK1 complex that includes

ULK1, ULK2, Atg13, Atg101, and the FAK family kinase-interacting protein of 200 kDa (FIP200) allows for stimulation of ULK1 activity and induction of autophagy. During nucleation of isolation membrane the class III phosphatidylinositol 3-kinase complex (PtdIns3K) is then activated to form phosphatidylinositol-3-phosphate (PI3P). The PtdIns3K complex is comprised of the vacuolar protein sorting 34 (VPS34), whose activation depends on the formation of a complex that includes proteins VPS15, Ambra1, ATG14 and Bax-interacting factor 1 (BIF1), and Beclin1. The availability of Beclin1 is important in regulating the induction of the phagophore since the anti-apoptotic BCL-2 protein inhibits Beclin1 through direct binding. Thus loss of the interaction between Beclin1 and BCL-2 is required for activation of VPS34. The elongation of the isolation membrane proceeds through a concerted action of two ubiquitin-like conjugation pathways that results in the formation of autophagosome. In the first pathway, a covalent conjugation forms between ATG12 and ATG5 through the actions of ATG7 and ATG10. ATG12-ATG5 then interact with ATG16-like 1 (ATG16L1) and the tri-protein complex in turn can exhibit an E3 ligase-like activity to promote the lipidation of the light chain 3 (LC3) protein. In the second ubiquitin-like pathway there is the conversion of LC3 I to LC3 II due to conjugation of phosphatidylethanolamine (PE) to LC3 I, via sequential action of ATG4, ATG7 and ATG3. The conjugation of PE to LC3 facilitates the closure of the isolation membrane. ATG4 removes LC3-II from the outer surface of the autophagosome while LC3 on the inner surface is degraded when the autophagosome fuses with the lysosome to form an autolysosome. This overall process combined with shared endocytic pathways allows for control of the elimination of cellular components and the important recycling of metabolites.

During embryonic development autophagy is essential for proliferation and differentiation of different stem and progenitor cells (Phadwal et al., 2013; Rodolfo et al., 2016).

Many of the germline knockout mice with ablation of autophagy genes show early developmental lethality, impeding our understanding of the role of autophagy in the adult brain (Mizushima and Levine, 2010). To circumvent this limitation, my published work as presented in Chapter 3 utilized the retrovirus strategy to determine the cell-autonomous requirement for the autophagy related gene 5 (*Atg5*) in the survival of adult NPCs (Xi et al., 2016). Previous to our study, in the adult brain, autophagy was demonstrated to counteract and limit the cumulative deleterious effects of damaged cellular organelles and proteins since neural-cell-specific conditional knockout models had age-related neurodegeneration and accumulation of ubiquitinated proteins (Hara et al., 2006; Komatsu et al., 2007). By using retroviral labeling in Chapter 3, I determine the requirement of *Atg5* for the survival and maturation of NPCs.

6. Maturation and integration of adult-generated granule neurons

NPCs follow a stereotypic process to generate post-mitotic immature neurons that mature and integrate as granule cells of the DG and form part of the hippocampal tri-synaptic network (Ming and Song, 2011). Maturation of immature neurons into granule cells consists of several morphological changes including growth and extensive branching of the dendrites, increase in the number of dendritic spines and outgoing axonal growth (Zhao et al., 2006). In addition to morphological changes, newborn neurons undergo a dynamic shift in their electrophysiological properties. As reviewed by Ge et al., (2008), shortly after birth, NPCs receive tonic activation by the neurotransmitter gamma-aminobutyric acid (GABA) that is released from local interneurons in the niche. After about one week NPCs forming immature neurons extend dendrites into the molecular layer and receive GABAergic dendritic synaptic inputs. During the maturation process, there is a gradual change in the expression of chloride transporters and as a result, GABA, which is initially depolarizing, gradually becomes hyperpolarizing. Excitatory

neurotransmission is primarily mediated by glutamate via the ionotropic glutamate receptors AMPA and NMDA, as newborn neurons receive abundant glutamatergic inputs around four weeks after birth. Newborn neurons show increased excitability compared to mature granule neurons, however after a period of approximately eight weeks adult born granule cells become almost indistinguishable from developmentally-generated granule cells (Laplagne et al., 2006).

7. Function of adult neurogenesis and implication in disease

Function in naïve adult brain

The hippocampus is essential for the encoding of new information and for the formation of episodic, spatial and emotional memory (Deng et al., 2010). This fact raises the question about how does the addition of new neurons via neurogenesis affect hippocampal learning and memory? Within the neurogenesis field a common strategy to address this question has mainly involved ablating neurogenesis through genetic, pharmacological or by X-ray irradiation methods and then examining the effect on cognitive function (Deng et al., 2010) through the use of hippocampal-dependant behavioural tasks such as the Morris Water Maze (MWM) and/or contextual fear conditioning. These studies have demonstrated the requirement of adult neurogenesis for encoding of new information, for memory retention or for both learning and memory (Snyder et al., 2005; Farioli-Vecchioli et al., 2008; Imayoshi et al., 2008; Zhang et al., 2008; Jessberger et al., 2009). There however also exists a variety of evidence to support no requirement for neurogenesis in learning and memory tasks (Shors et al., 2002; Meshi et al., 2006). This discrepancy is likely due to multiple factors such as behavior protocol, species and strain tested, ablation method used, as well as the sensitivity of the behavioral outcome measure (Deng et al., 2010).

Since not all work has supported a requirement for neurogenesis in learning and memory, others have proposed that adult neurogenesis contributes to only certain forms of hippocampal-dependent learning (Clelland et al., 2009). For example, neurogenesis is often proposed to be required for the ability to distinguish two similar contexts, also known as pattern separation (Yassa and Stark, 2011). In support of this hypothesis, increasing neurogenesis through inhibition of apoptotic death of adult-generated neurons in the adult, is sufficient to improve pattern separation abilities (Sahay et al., 2011). Additionally, recent studies monitoring the activity of adult-generated cells in awake, behaving mice using two-photon calcium imaging has confirmed the direct involvement of adult-generated neurons in context encoding and discrimination, consistent with their proposed role in pattern separation (Danielson et al., 2016). Computational models and experimental evidence have also suggested that neurogenesis should result in destabilization (or forgetting) of previously acquired memories (Weisz and Argibay, 2012). According to this model, as new neurons are added, they compete with other neurons in the network leading to extensive remodeling of the existing hippocampal circuit. In experimental support of this, Akers et al., (2014) discovered that enhancing neurogenesis after memory formation induced forgetting of hippocampal-dependent memories in adult mice. This neurogenesis-mediated forgetting of old memories was later showed to facilitate encoding of new, conflicting information (Epp et al., 2016). Thus, overall there is a growing amount of evidence to support the role for adult-generated granule neurons to be functionally important in particular aspects of learning and memory in naïve animals.

Role for adult neurogenesis in Alzheimer's disease

Since one of the hallmark symptoms of neurodegenerative diseases such as Alzheimer's disease (AD) is profound memory deficits, there is a variety of work that has examined if

alterations in adult neurogenesis are part of the etiology or the outcome of AD. Support for a role of adult neurogenesis in AD comes from reciprocal and correlative evidence that has identified alterations in neurogenesis in different disease models of AD, as reviewed by Mu and Gage, (2011). For example, transgenic mice carrying mutations in amyloid precursor protein (APP) show decreased NPC proliferation, differentiation and survival. This is also true in the case of mice models that express mutations in the *Presenilin 1 (PS1)* gene, where a reduction in neurogenesis has been observed using different BrdU injection paradigms.

Ninety percent of identified mutations in the early-onset familial autosomal dominant form of AD (FAD), occur in the *Presenilin* genes (*PS1* and *PS2*) (Shen and Kelleher, 2007). Presenilins are transmembrane proteins that constitute the catalytic subunit of the proteolytic gamma-secretase complex which is responsible for cleavage of many substrates including the amyloid precursor protein (APP) and Notch1 (van Tijn et al., 2011). Early developmental loss of *PS1* produces severe CNS deficits owing to the reduction in the NPC populations caused by premature cell differentiation and reduced Notch signaling (Yang et al., 2000). In Chapter 4, I test the function of the presenilins in the context of adult hippocampal neurogenesis. In the adult brain presenilins are expressed in the adult hippocampus (Lee et al., 1996), including in the NPCs of the dentate gyrus (Wen et al., 2002), yet their cell-intrinsic roles remain undefined. This work also tests the role of presenilins in modifying neurogenesis during exercise, the rationale being that positive regulators of adult neurogenesis such as physical exercise or environmental enrichment can improve neurogenesis and correlatively increase cognitive performance in AD mice (Costa et al., 2007; Nichol et al., 2007). In support of this, *APP/PS1* double mutant mice exposed to a complex environment showed reduced amyloid plaque deposition and in *APP* single mutants exposure to both running and environment enrichment

improved performance in the Morris water maze (Lazarov et al., 2005; Wolf et al., 2006; Mirochnic et al., 2009; Hu et al., 2010). Together this correlative evidence highlights how increasing neurogenesis can improve cognitive function in AD disease models. Thus, in addition to examining cell-intrinsic roles of presenilins in the naïve condition, our work also examines presenilins as potential mediators of exercise-induced neurogenesis.

8. Hypothesis & Objective

The primary objective of this thesis is to determine cell-intrinsic regulators of adult hippocampal neurogenesis. The main body of this thesis comprises of three manuscripts (2 published & 1 in submission).

1. Chapter 2 is a published manuscript (Dhaliwal and Lagace, 2011) that tested the hypothesis that microtubule protein DCX is essential for the survival and differentiation of adult NPCs.
2. Chapter 3 tested the hypothesis that autophagy-related gene 5 (*Atg5*) is an important regulator of NPC survival and the lack of *Atg5* would produce deficits in neurogenesis due to disruption in autophagy. This chapter is a published manuscript (Xi et al., 2016).
3. Chapter 4 tested the hypothesis that the Familial Alzheimer's Disease (FAD) linked *PS1* and *PS2* genes are required in all stages of adult neurogenesis from the NSCs to integrated adult generated hippocampal neurons. This manuscript is currently in submission.

CHAPTER 2:

Doublecortin (DCX) is not essential for survival and differentiation of newborn neurons in the
adult mouse dentate gyrus

Published Manuscript

Frontiers in Neuroscience

Contribution of co-authors

Jagroop Dhaliwal: Made viruses, performed all *in vivo* virus experiments and data analysis, wrote and revised the manuscript.

Yanwei Xi: Contributed to virus generation.

Elodie Bruel-Jungerman and Johanne Germain: Performed and contributed inducible mice data.

Fiona Francis: Collaborator. Provided the floxed DCX mice and data on the GlastCreER^{T2} inducible mice.

Diane C. Lagace: Contributed to the design, analysis, interpretation of results and writing the manuscript

Doublecortin (DCX) is not essential for survival and differentiation of newborn neurons in the adult mouse dentate gyrus

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Running Title

Doublecortin and adult hippocampal neurogenesis

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Doublecortin, adult neurogenesis, DCX knockdown, DCX knockout, survival, differentiation, retrovirus labeling

Conflict of Interest

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Abstract

In the adult brain, expression of the microtubule-associated protein Doublecortin (DCX) is associated with neural progenitor cells (NPCs) that give rise to new neurons in the dentate gyrus. Many studies quantify the number of DCX-expressing cells as a proxy for the level of adult neurogenesis, yet no study has determined the effect of removing DCX from adult hippocampal NPCs. Here, we use a retroviral and inducible mouse transgenic approach to either knockdown or knockout DCX from adult NPCs in the dentate gyrus and examine how this affects cell survival and neuronal maturation. Our results demonstrate that shRNA-mediated knockdown of DCX or Cre-mediated recombination in floxed DCX mice does not alter hippocampal neurogenesis and does not change the neuronal fate of the NPCs. Together these findings show that the survival and maturation of adult-generated hippocampal neurons does not require DCX.

Introduction

The microtubule-associated protein Doublecortin (DCX) is widely expressed in developing neurons during embryonic and early postnatal development throughout the central and peripheral nervous system (Francis et al., 1999; Gleeson et al., 1999; Belvindrah et al., 2011). In the adult brain the expression of DCX is restricted to the neurogenic regions, as well as specific non-neurogenic regions such as the piriform cortex (Bonfanti and Nacher, 2012). The changes in the expression profile of DCX throughout the lifespan, within both neurogenic and non-neurogenic cells, suggest that DCX may subserve multiple, different functions during development and in the adult, as well as within different cellular contexts during physiological and pathological states (Bonfanti and Nacher, 2012).

Within the two main adult neurogenic regions DCX is transiently expressed in the dividing neuronal precursor cells (NPCs) until the cells become mature neurons after approximately 30 days (Brown et al., 2003). Within the neurogenic region of the adult rostral migratory stream (RMS), where NPCs migrate from the subventricular zone (SVZ) to the olfactory bulb (OB), the function of DCX has been explored. In young adult DCX mutant mice, DCX was first shown to be important for the maintenance of their bipolar shape, migration and fate (Koizumi et al., 2006b). Work in early postnatal mice has also highlighted a novel role for DCX in determining the fate of the neurogenic cells in the RMS (Belvindrah et al., 2011). Yet in direct contrast to these findings, Merz and Lie (2013) recently found no migration or fate phenotype when using a microRNA mediated retroviral approach to knockdown DCX from the RMS in adult mice. These conflicting findings suggest that DCX may have regional and age-dependent roles within the SVZ-RMS-OB.

Within the adult neurogenic region of the dentate gyrus only one study has tested the cell-intrinsic role of DCX. Merz and Lie (2013) found no hippocampal neurogenic deficits following

their microRNA mediated retroviral approach to knockdown DCX. However, as highlighted by Merz and Lie (2013), their conclusion may have been confounded by using a knockdown approach that preserved some DCX expression, as well as examining exercising adult mice. Thus the effect of a complete removal of DCX specifically within adult NPCs that develop into granule neurons within the adult brain remains unknown. This study sought to elucidate the requirement of DCX in the formation of adult-generated granule neurons under normal physiological conditions. In the adult, we used retroviruses to target DCX-expressing NPCs to either 1) knockdown DCX using shRNA in C57Bl6 wild type mice, or 2) knockout DCX through expression of Cre in floxed DCX (DCX^{flox}) mice. Additionally we examined DCX-null NPCs in a novel inducible transgenic mouse that allowed for the removal of DCX from the GLAST-expressing stem cells prior to expression of DCX. The data obtained from these multiple approaches provide convincing evidence that a reduction or removal of DCX is associated with no visible hippocampal neurogenic deficits in the adult.

Materials and Methods

Animals

C57BL/6J mice were purchased from Charles River (female, 6-8 weeks). All procedures associated with the retroviral experiments were performed within the guidelines of the Canadian Council on Animal Care and were approved by the University of Ottawa Animal Care Committee. Generation of the floxed DCX mice has been previously described from a tri-loxP allele (Kappeler et al., 2006) and Cre recombined mice were produced, maintained and manipulated with authorization from the French Ministry of Research (00984.02). DCX is localized to the X-chromosome, thus males (DCX^{flox/Y}) were compared to WT littermate controls. Glast-CreERT2 knockin mice, where the CreERT2 expression is driven by the sodium-

dependent glutamate/aspartate transporter (Glast/Slc1a3), have been described previously (Mori et al., 2006). Mice were housed in a 12-hour light/dark cycle with free access to food and water. Adult GlastCreERT2 heterozygote x DCX flox hemizygote male mice on the 129 Sv/Pas genetic background were treated with tamoxifen (Sigma, France) dissolved in corn oil (Sigma, France), 10 % ethanol. Two x 2.5 mg/day intraperitoneal injections were performed during 5 days, or corn oil (Vehicle) was injected alone (in 10 % ethanol), as described previously (Mori et al., 2006). Four weeks after the last injection of tamoxifen, bromodeoxyuridine (BrdU, Fluka Analytical, Sigma-Aldrich Chemie GmbH, Switzerland # 16880) was injected at 100 mg/kg (4X, each injection spaced by two hours) in order to label dividing NPCs. Mice were sacrificed 10 days after BrdU injection.

Retrovirus Production and Injection

The shDCX sequence (mature sense: CCCTATAGCTGTAGTTAGA) and scrambled control sequence (ATCTCGCTTGGGCGAGAGTAAG) were obtained from Dharmacon (#V2LHS_229318 and #RHS4346). The shDCX was verified to target both human and mouse DCX (nBLAST). The shDCX was further validated in the neuroblastoma cell line SHSY5Y (ATCC # CRL-2266) *in vitro* where it induced a significant 2.2 fold decrease in endogenous DCX protein vs. scrambled control. (0.552 ± 0.008 shDCX vs. 1.214 ± 0.164 control, normalized to tubulin, $p < 0.05$, $N=3$).

For *in vivo* DCX knockdown, the shDCX or control sequences were cloned into a retroviral vector pUEG that co-express shDCX or scrambled control sequence under the U6 promoter and GFP under the EF1 α promoter. The vector was generously provided by Dr. Hongjun Song (Johns Hopkins University).

For *in vivo* DCX knockout, the retroviral vectors CAG-GFP-Cre and the control CAG-RFP and the corresponding packing and envelope constructs were generously provided by Dr.

Fred Gage (The Salk Institute). The retroviruses were generated as previously described and mixed in a 1:1 ratio prior to injection (Tashiro et al., 2006). The titers of viruses were determined by live titer using 293T cells and ranged between ~ 2 and 4×10^8 infectious units (IU) per ml.

The retroviruses were bilaterally injected into the dentate gyrus of 6-8 week old mice. The shDCX or scrambled virus was injected into C57BL/6J mice. The mixture of CAG-GFP-Cre and CAG-RFP was injected into floxed DCX mice and littermate WT controls. The injections were performed using a Hamilton microsyringe and a 33 gauge needle into the dentate gyrus (-1.7mm from Bregma, ± 1.2 mediolateral, -2.4mm dorsoventral; final volume: 1.5 μ l per injection site, injection rate: 0.2 μ l/min). Mice were kept under anesthetic via isoflurane during the surgery procedure and were allowed to recover in a warm chamber after surgery. The mice also received 3 subcutaneous injections of analgesic buprenorphine (0.05mg/kg) one hour prior to, as well as 5-6 hours and 10-12 hours after surgery.

Tissue processing, immunohistochemistry and quantification

Mice were anesthetized and transcardially perfused with cold phosphate buffered saline (1X PBS, pH 7.4) followed by 4% paraformaldehyde in 1x PBS (pH= 7.4). Brains were post-fixed for 1 or 24 hours in 4% paraformaldehyde. All brains that were used for the retroviral studies following post-fix were transferred to 30% sucrose with 0.1% sodium azide in 1X PBS. Brains were cut coronally on a freezing microtome at 30 μ m and stored in 1X PBS with 0.1% sodium azide. The GlastCreER^{T2} x floxDCX inducible mice brains were sectioned into 40 μ m vibratome slices.

Immunohistochemistry was performed on free-floating sections. Sections were washed in 1X PBS and incubated in 0.1% Tween-20 and 0.1% Triton X-100 in 1X PBS with corresponding

primary antibodies at 4°C overnight. The primary antibodies used were: chicken anti-GFP (GFP-1020, Aves, 1:5000), rabbit anti-dsRed (632496, Clontech, 1:5000), goat anti-DCX (sc-8066, Santa Cruz, 1:500), mouse anti-NeuN (MAB377, Millipore, 1:500), rat anti-BrdU (AbCys, France AbC117-7513, 1:1000) and goat anti-NeuroD1 (sc-1084, Santa Cruz, 1:500). Sections were washed in 1X PBS, incubated with corresponding Cy2, Cy3 and Cy5-conjugated IgG secondary antibodies (Jackson Immuno, 1:500) for one hour at room temperature, stained with DAPI (Roche, 1:10000) and washed in 1X PBS prior to mounting on slides and coverslipping. Retroviral labeled cells in the SGZ were counted using an Olympus BX51 fluorescent microscope. The quantification of co-labeled cells was performed using a Zeiss LSM510 confocal microscope or Zeiss AxioObserver.Z1 microscope. For the GlastCreER^{T2} x floxDCX inducible mouse studies the BrdU and DCX labeled cells were counted manually using an apotome microscope (Zeiss, Germany). All counts and phenotype analysis were performed at 400x magnification by an experimenter blind to the experimental conditions and genotypes.

Statistical analysis

All data are reported as mean $\pm$ standard error of the mean (SEM) and statistical analysis was performed using GraphPad Prism (v6.0) software. An outlier test was performed on all retroviral counts and 3/86 counts from individual hippocampi were excluded. Experiments with two groups were analyzed by the two-tailed *Student t-test*. Analyses of two factors were performed using a 2-way ANOVA test followed by a Bonferroni *post hoc*. Statistical significance was defined as $p < 0.05$.

Results

Knockdown of DCX does not affect survival of adult-generated hippocampal neurons

To fate map the NPCs following either a knockdown or knockout of DCX *in vivo*, we utilized a retrovirus approach to infect and track the maturation of the dividing NPCs that develop into granule neurons, expressing NeuN by 30 days post infection (dpi). We and others have shown that this approach is effective at targeting the NPCs and immature neurons expressing DCX, with peak DCX expression found 3-14 dpi (Ge et al., 2006; Tashiro et al., 2006; Jessberger et al., 2008; Jagasia et al., 2009; Schnell et al., 2014; Ceizar et al., 2016).

To knockdown DCX in the NPCs and their progeny, retroviruses expressing either shDCX-GFP or scrambled control (Ctrl-GFP) were created and stereotactically injected into the dentate gyrus in C57BL/6 mice. We generated a time-course to examine and compare survival of Ctrl-GFP+ and ShDCX-GFP+ cells. There were significantly fewer shDCX-GFP+ cells at 3 and 12 dpi compared to Ctrl-GFP+ cells (Supplemental Fig 1). The reduction in number of shDCX-GFP cells at 3dpi was surprising and could be attributed to differences in *in vivo* viral transduction efficiency. In order to control for this variability and determine the survival rate ShDCX-GFP+ and Ctrl-GFP+ cells, the absolute cell numbers at 12 and 30 dpi were normalized to the average number of cells labeled at 3dpi in each group, as previously published also by others (Schnell et al., 2014). Analysis of the infected cells between 3 to 30 dpi demonstrated that relative to the number of cells present at 3 dpi both shDCX-GFP and Ctrl-GFP expressing cells, as expected, showed a decline in cell survival over time (**Fig 1a**). However, there was no significant difference in the relative survival of the cells between the shDCX-GFP+ and Ctrl-GFP+ at 12 and 30 dpi (**Fig 1a**).

To confirm that a knockdown of DCX had occurred *in vivo*, the percentage of shDCX-GFP and Ctrl-GFP expressing cells also expressing DCX was determined. There was a significant reduction in the percentage of the shDCX-GFP cells that expressed DCX compared to

the control at 12 dpi (**Fig 1b**). Expression of DCX was observed in over 90% of control cells compared to only ~50% of shDCX-GFP infected cells. As shown in **Fig 1b**, the majority of the shDCX-GFP+ cells that showed DCX expression also had notably less bright DCX staining relative to the intensity of DCX observed in the Ctrl-GFP cells, suggestive of lower levels of DCX within these cells. In contrast to the significant reduction in percentage of cells expressing DCX, the proportion of surviving NPCs expressing the proneural basic helix-loop-helix transcription factor NeuroD1 was similar between shDCX-GFP and Ctrl-GFP infected cells at 12dpi (**Fig 1c**). Furthermore, on average over 90% of surviving shDCX-GFP and Ctrl-GFP expressing cells at 30 dpi expressed the mature neuronal marker, NeuN (**Fig 1d**) suggesting DCX expression does not impact the neuronal fate of the dividing NPCs. Together these results show that reducing the amount of DCX in the NPCs does not affect the survival and neuronal fate of the cells during their development in the adult dentate gyrus.

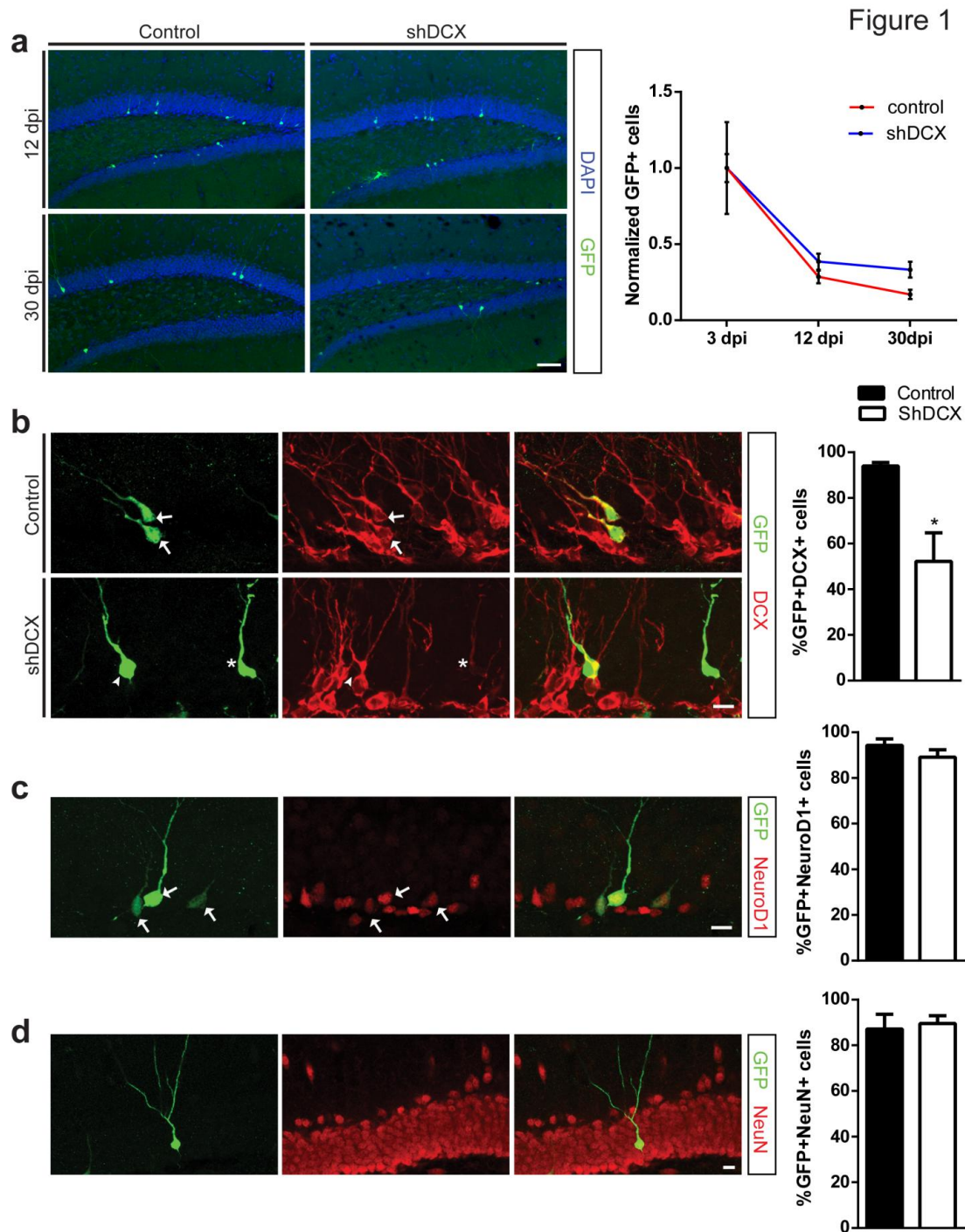


Figure 1. Retroviral knockdown of DCX does not affect NPC survival and differentiation a) Representative images and quantification of NPCs showing no difference in normalized counts between shDCX-GFP cells compared to Ctrl-GFP cells at 12 dpi and 30 dpi; b) Proportion of GFP+ NPCs expressing DCX was significantly reduced in shDCX-GFP compared to Ctrl-GFP, with some shDCX cells having fainter DCX staining (arrows = GFP+ DCX+; arrowhead = GFP+ DCX-; star = GFP+DCX+ faint). c) Expression of the immature neuronal marker NeuroD1 is unaffected between control and shDCX expressing cells (arrows = GFP+NeuroD1+ colabeled cells). d) Neuronal fate was not affected as there was no difference in proportion of GFP+ cells expressing the mature neuronal marker NeuN between control and shDCX at 30dpi. N=3-6 mice per group with the total number of cells analyzed in b) 105 Ctrl and 127 shDCX cells; c) 151 Ctrl and 201 shDCX cells; d) 130 Ctrl and 84 shDCX cells. *p<0.05. Scale bar = 20 μ m for a) and 10 μ m for b-d.

Knockout of DCX does not affect adult-generated hippocampal neurons

In order to completely remove DCX and track the development of the DCX-null NPCs we next used a retroviral method to express Cre in a floxed DCX mouse. A mixture of CAG-GFP-Cre and CAG-RFP was used to infect NPCs in both floxed DCX and WT mice. The CAG-RFP was used as a control virus to allow the survival ratio of the infected dividing cells to be calculated by comparing the ratio of double labeled (GFP+RFP+) to all control RFP+ NPCs, as we and others have previously published (Tashiro et al., 2006; Jessberger et al., 2008; Jagasia et al., 2009; Schnell et al., 2014; Ceizar et al., 2016).

In agreement with the shDCX results, the survival of DCX-null vs. DCX-expressing infected cells was comparable at 7 dpi (**Fig 2a**). This result was not attributable to low efficiency of the GFP-Cre, as all GFP-Cre cells in the floxed DCX mice were absent of DCX expression (**Fig 2b**), yet similar to the shDCX-GFP cells, DCX-null cells expressed NeuroD1 (**Fig 2c**). Similarly, at 30 dpi there was no change in survival of DCX-null cells (**Fig 3a**). DCX also appeared not to be essential for neuronal maturation and fate as nearly all DCX-null and DCX-expressing cells expressed NeuN (**Fig 3b**).

Figure 2

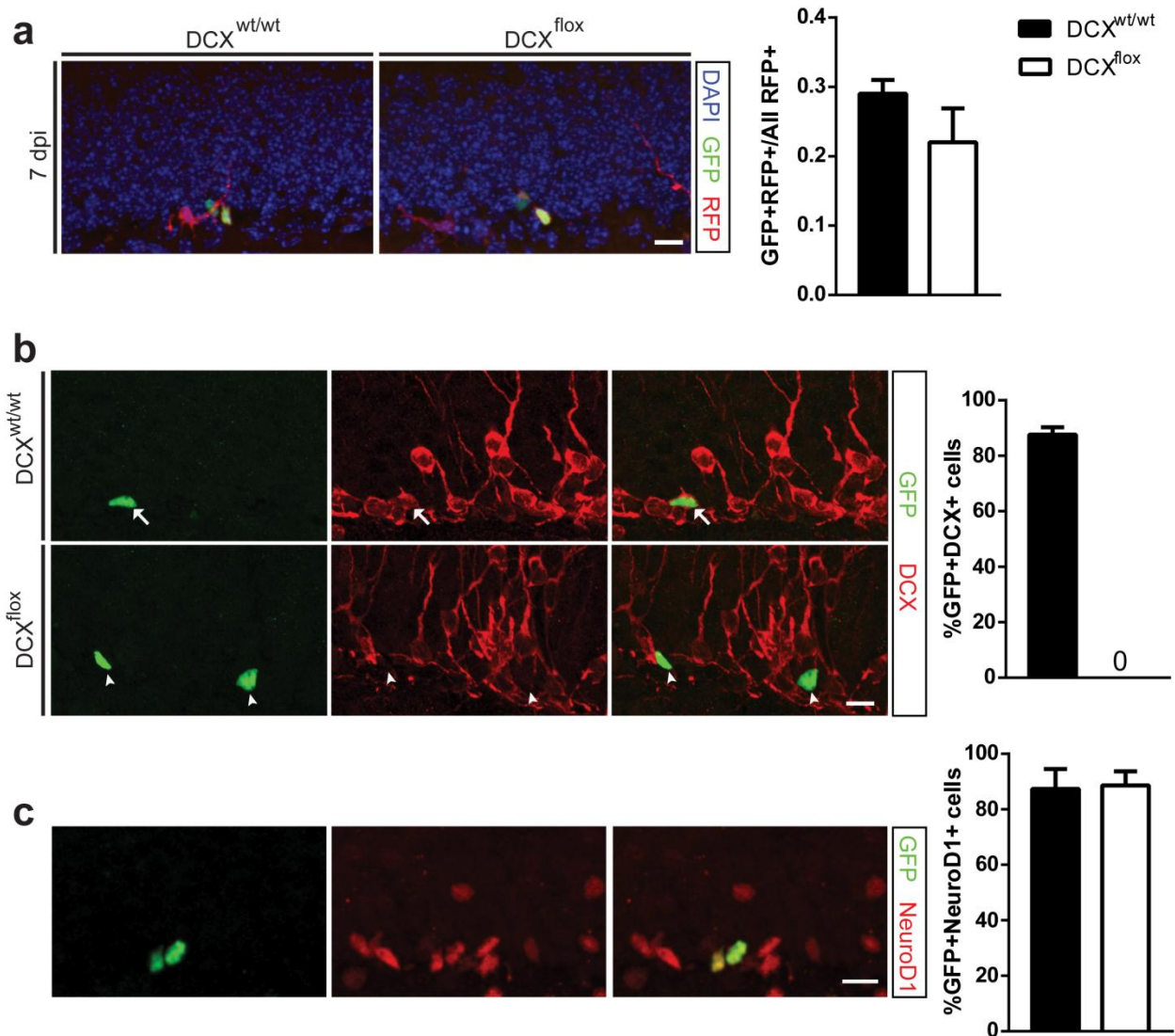


Figure 2. Retroviral knockout of DCX abolishes DCX expression in vivo but does not affect cell survival at 7 dpi. a) Representative image and quantification showing the survival ratio of infected NPCs is the same in WT and floxed DCX (DCX^{flox}) mice at 7 dpi. b) Knockout of DCX was confirmed by no detectable GFP and DCX co-labeled cells in the DCX-null NPCs in DCX^{flox} mice (arrows = co-labeled cells, arrowhead = GFP+ DCX-). c) Proportion of GFP-Cre NPCs expressing the immature marker NeuroD1 is similar between WT and DCX^{flox} mice. N= 3 mice per genotype with the total number of cells analyzed in b) 113 WT cells and 111 KO cells; c) 164 WT cells and 135 KO cells. Scale bars = 10 μ m.

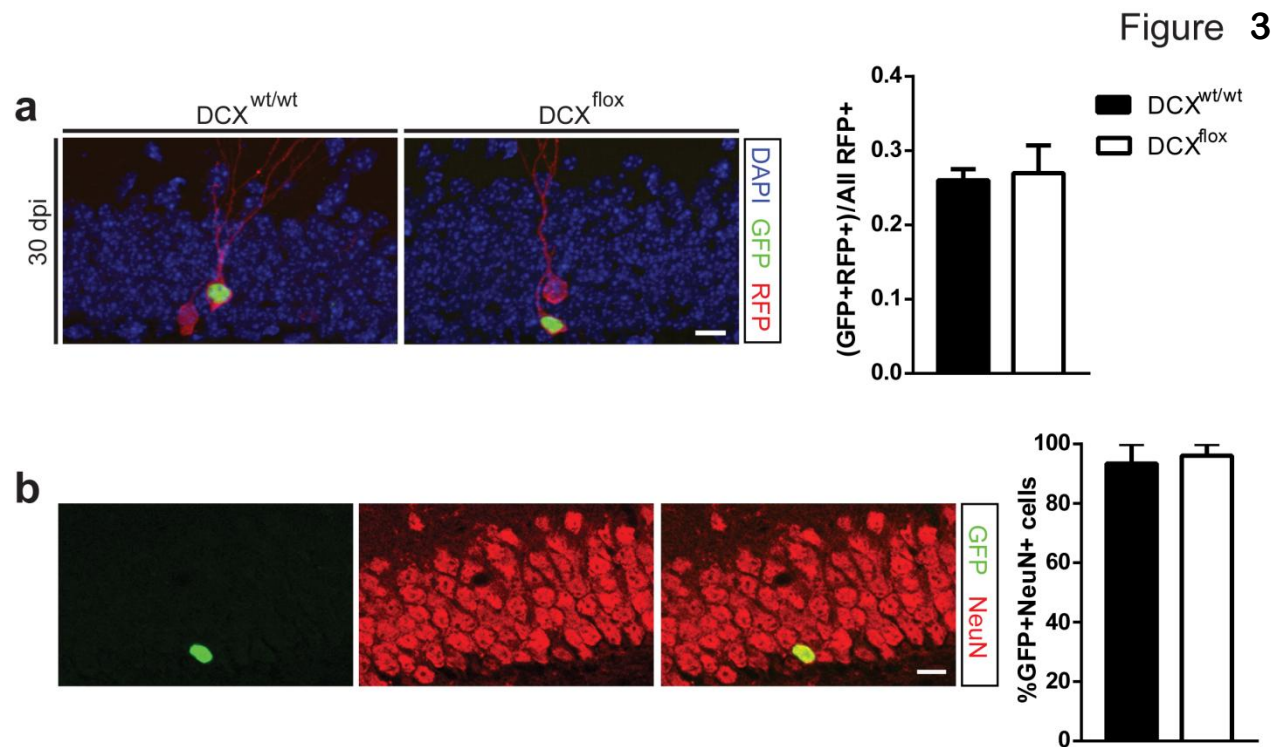


Figure 3. Retroviral knockout of DCX does not affect the survival or fate of adult-generated neurons at 30 dpi. a) Representative image and quantification showing the survival ratio is the same in WT and floxed DCX (DCX^{flox}) mice at 30dpi. b) Neuronal fate was not affected in DCX-null cells, with no difference in the proportion of GFP-labeled cells expressing the mature neuronal marker NeuN. N= 3-5 mice per genotype with the total number of cells analyzed in b = 44 WT cells and 59 KO cells. Scale bars = 10 μm.

By using a retroviral approach to knockout DCX we cannot exclude the possibility that DCX was expressed in the NPCs before the knockout, since retroviruses infect dividing cells, including ones that already express DCX (Ge et al., 2006; Ceizar et al., 2016). This raises the possibility that the lack of phenotype in the DCX-null cells may be in some cases attributed to prior DCX expression. In order to address this possibility, we also created an inducible *GlastCreER^{T2}* x floxed DCX mouse to test if removal of DCX prior to DCX expression in NPCs altered the phenotype of the maturing NPCs. In this inducible mouse, tamoxifen was injected in order to prevent DCX expression from the *Glast*-expressing neural stem cells. At 4 weeks after tamoxifen injection the mice were given a pulse of BrdU to label the NPCs and mice were sacrificed 10 days later, a time point when there is a significant proportion of immature cells that express DCX (Ninkovic et al., 2007). As expected there was a reduction in the total number of DCX cells in the tamoxifen treated *GlastCreER^{T2}* x floxed DCX mice (**Fig 4a**). To quantify the reduction, the proportion of BrdU+ NPCs expressing DCX was examined 10 days after BrdU labeling. As expected, there was a significant reduction of DCX+ cells in the tamoxifen-injected floxed mice (**Fig 4b**). Although less BrdU+ cells expressed DCX, the total number of BrdU+ cells was similar between the vehicle and tamoxifen treated groups (**Fig 4c**). This data therefore supports our combined retroviral data which together suggest that knockout of DCX does not affect the survival of the developing NPCs in the adult hippocampus.

Figure 4

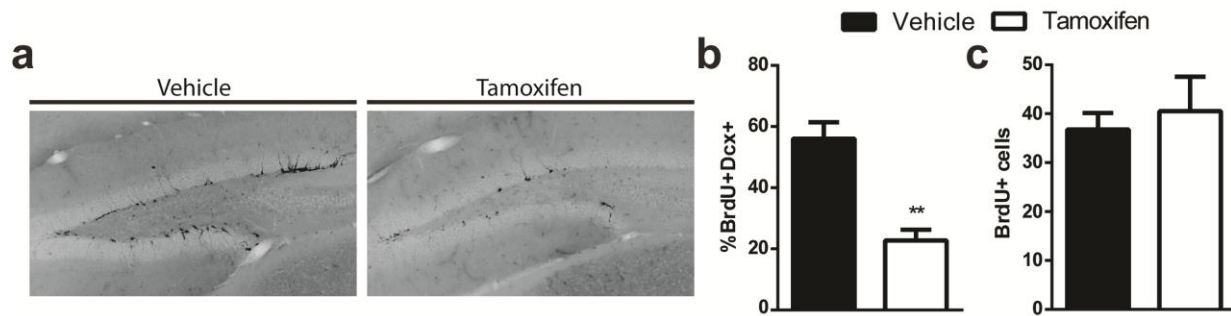


Figure 4. $GlastCreER^{T2}$ x $floxDCX$ inducible knockout of DCX does not impact survival of dividing NPCs. a) Representative image showing less DCX+ cells in the knockout mice four weeks after tamoxifen treatment. b) The proportion of cells expressing both BrdU and DCX is significantly reduced in tamoxifen treated group. c) The overall number of 10-day old BrdU+ cells is similar between the inducible knockout mice and control. N = 4 mice per group. **p<0.005

Discussion

Our findings show that either reducing DCX expression by the retroviral shRNA knockdown approach or knocking out DCX by retroviral delivery of Cre does not alter the survival of adult-generated neurons. We confirm using the $\text{GlastCreER}^{\text{T2}}$ x floxDCX inducible mouse that NPC survival is unaffected even when the knockout is initiated in the GLAST-expressing neural stem cells, which ultimately give rise to the DCX expressing progeny. Additionally we find knocking down or removing DCX does not change the neuronal fate of the maturing NPCs.

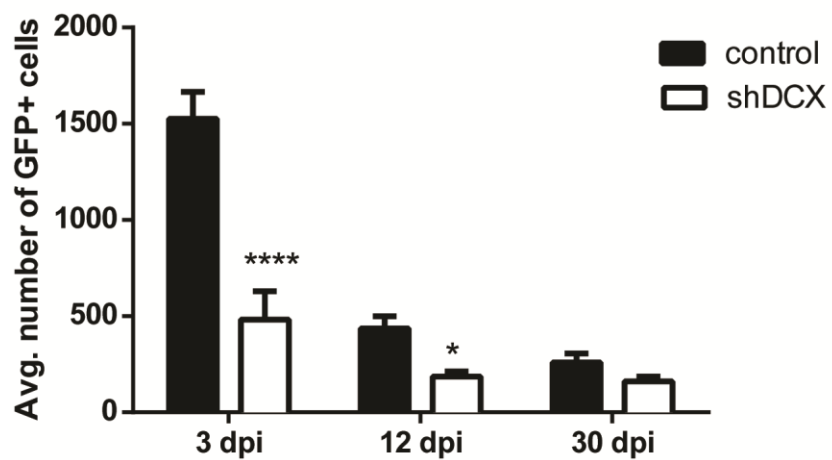
Our converging data that adult-generated NPCs in the hippocampus can develop into neurons in the absence of DCX is in agreement with recent results obtained in a constitutive DCX knockout mouse studied by Germain et al. (2013) and after a knockdown of DCX using micro-RNA in neurogenic cells by Merz and Lie (2013). The DCX knockout mice showed absence of DCX while not altering adult hippocampal neurogenesis. Since the KO of DCX was constitutive, the lack of phenotype could be attributed to compensatory mechanisms, as have been previously identified by differences observed between embryonic and adult-specific knockout models (Urban and Guillemot, 2015). However, Merz and Lie (2013) also identified no significant effects on adult neurogenesis following reducing the levels of DCX specifically in the adult dividing NPCs using a micro-RNA knockdown approach. The lack of deficits following knockdown of DCX however can be confounded by the preservation of some DCX protein expression. Therefore to more extensively examine the function of DCX in the dentate NPCs we used a retroviral and inducible transgenic mouse approach to knockout DCX from the NPCs. In this case, the DCX-null NPCs showed similar survival compared to WT. Thus unlike the regional and age-dependent roles of DCX proposed within the SVZ-RMS-OB, within the neurogenic cells of the dentate DCX does not appear to be required for cell survival.

Both the shDCX-GFP and DCX-null NPCs generated NeuN+ mature granule neurons suggesting that DCX is also not essential for neuronal fate commitment, *per se*, during adult neurogenesis. The lack of requirement for DCX for neuronal differentiation was also supported by Merz and Lie (2013) who found that a reduction in DCX within NPCs did not affect the fate of NPCs in mice that had free access to running wheels. Our work extends this study by showing DCX is dispensable for neuronal fate during basal hippocampal neurogenesis, which is notable given that running may enhance the expression of factors that could render the newly generated neurons resistant to loss of DCX. Furthermore, the role of DCX in modulating differentiation is likely cell-type specific. For example, DCX is a differentiation-based treatment *in vivo* for glioma, where DCX induces terminal differentiation of glioma cells via a DCX/GFAP (glial fibrillary acidic protein) pathway (Santra et al., 2010).

Our results showing no hippocampal neurogenic deficits from both DCX knockdown and knockout approaches in the adult NPCs is also notable given a variety of studies have reported conflicting results between DCX knockdown and knockout approaches. This is especially well documented in studies examining the functional role of DCX in neocortical development, where shRNA-mediated knockdown of DCX results in deficits, whereas germ line DCX knockout models or micro-RNA mediated DCX knockdown show no neocortical deficits (Corbo et al., 2002; Bai et al., 2003; Gotz, 2003; Baek et al., 2014). Recently this conflicting data has been resolved through the discovery that the neocortical deficits associated with the shRNA-mediated knockdown of DCX can be attributed to off-target effects associated with noncoding microRNAs (Baek et al., 2014). In our analysis of adult shRNA knockdown of DCX, the survival of Ctrl-GFP+ and shDCX-GFP+ cells was calculated by normalizing absolute cell numbers to the average number of cells labeled at 3dpi since there were significantly fewer shDCX-GFP+ cells

at 3 dpi compared to Ctrl-GFP+ cells. The reduction in number of shDCX-GFP cells at this early time point we hypothesized to be attributed to differences in viral transduction efficiency, despite using similar amounts and viral titers for infection. It is unlikely that this effect is due to the off-target effects of DCX family shRNA, given that off-target miRNA effects can also occur within the scrambled controls (Baek et al., 2014). Thus this study reveals that shRNA knockdown of DCX and inhibition of DCX expression, does not affect the development of hippocampal NPCs. Our conclusion leaves us with the question as to why adult-generated dentate NPCs would expend energy to transiently express DCX for no critically functional role? Since DCX is considered a structural protein and is also expressed in non-neurogenic cells, it is possible that DCX is not required for cell survival or neuronal maturation, but may have other roles such as in structural plasticity (Bonfanti and Nacher, 2012). Another possibility is that we observed no phenotype following a knockout of DCX due to compensation by other redundant genes. In support of this hypothesis, double knockout mutants for *Dcx* and *Lissencephaly gene 1* show a severe neuronal migration phenotype that is not observed in single *Dcx* knockout mice (Pramparo et al., 2010). Similarly, double knockout for *Dcx* and *Doublecortin-like kinase* show a severe neuronal migration deficit not observed in the single *Dcx* knockout mice (Koizumi et al., 2006a). Additionally, Doublecortin-like, a splice variant of DCLK, is also expressed in the adult dentate where it localizes with DCX in the immature neuronal population and thus is a possible candidate to also compensate for DCX (Saaltink et al., 2012). This hypothesis may suggest rapid changes in expression of these genes even in the presence of a conditional adult-generated knockout. Future studies are required to determine whether within the developing neurons in the adult dentate there is a genetic interaction that may in part compensate for DCX and contribute to the normal development of adult-generated dentate neurons in the absence of DCX *in vivo*.

Supplementary 1



Supplementary 1. Counts of Ctrl-GFP+ and ShDCX-GFP+ cells at 3, 12 and 30 dpi. There were significantly fewer shDCX-GFP+ cells at 3 and 12 dpi compared to Ctrl-GFP+ cells. N = 3-6 mice per group. ****p<0.0001, *p<0.02.

CHAPTER 3:

Knockout of Autophagy-related gene 5 (*Atg5*) delays the maturation and reduces the survival of adult-generated neurons in the hippocampus

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Cell Death & Disease

Contribution of co-authors

Yanwei Xi: Performed half of virus experiments, contributed to data analysis and draft of manuscript.

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**Knockout of Autophagy-related gene 5 (*Atg5*) delays the maturation and reduces
the survival of adult-generated neurons in the hippocampus**

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Abbreviated title: The requirement of Atg5 for adult neurogenesis

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Abstract

Autophagy is an evolutionarily conserved lysosomal degradation pathway that plays important roles in cell maintenance, expansion and differentiation. Removal of genes essential for autophagy from embryonic neural stem and precursor cells reduces the survival and inhibits neuronal differentiation of adult generated neurons. No study has modified autophagy within the adult precursor cells, leaving the cell autonomous role of autophagy in adult neurogenesis unknown. Here we demonstrate that autophagic flux exists in the adult dividing progenitor cells and their progeny in the dentate gyrus. To investigate the role of autophagy in adult hippocampal neurogenesis, we genetically deleted autophagy-related gene 5 (*Atg5*) which reduced autophagic flux and the survival of the progeny of dividing precursor cells. This significant reduction in survival of adult-generated neurons is accompanied by a delay in neuronal maturation, including a transient reduction in spine density in the absence of a change in differentiation. The delay in cell maturation and loss of progeny of the *Atg5*-null cells was not present in mice that lacked the essential pro-apoptotic protein Bax (Bcl-2-associated X *protein*) suggesting that *Atg5* deficient cells die through a Bax-dependent mechanism. Additionally, there was a loss of *Atg5*-null cells following exposure to running, suggesting that *Atg5* is required for running-induced increases in neurogenesis. These findings highlight the cell autonomous requirement of *Atg5* in the survival of adult-generated neurons.

Introduction

In the adult brain, neurogenesis allows for the continuous development of adult-generated neurons in response to physiological and pathological stimuli. The neural progenitor cells (NPCs) within the neurogenic niche of the subventricular zone (SVZ) and subgranular zone (SGZ) give rise to adult-generated neurons within the olfactory bulb and dentate, respectively (Ming and Song, 2011; Aimone et al., 2014; Lepousez et al., 2015). The ability of the NPCs to proliferate, differentiate and integrate into circuitry to modify behavior makes understanding these cells and the factors that regulate these processes critical to develop new therapies. This is especially important for a number of diseases such as neurodegenerative diseases including Parkinson's and Huntington's that are associated with reduced adult neurogenesis, as well as regenerative medicine strategies for recovery after stroke (Bond et al., 2015; Jessberger and Gage, 2015; Zhang and Chopp, 2015).

Two groups have found that *in vivo* macroautophagy, (hereafter referred to as autophagy) can regulate adult neurogenesis by examining the effect of deleting autophagy-related genes (ATGs). Yazdankhah et al. (2014) found Ambra1 and Beclin1 heterozygous embryonic knockout mice have less proliferating NPCs in the SVZ and an associated reduction in neurogenesis in the olfactory bulb. Wang et al. (2013) found conditional removal of *FIP200* (focal adhesion kinase (FAK) family interacting protein of M_r 200K, also known as ULK1, an Atg1 homologue-interacting protein) from embryonic NPCs progressively depletes the number of postnatal NPCs, as well as reduces neurogenesis and increases astrogenesis. In contrast in the embryo, Lv et al. showed that a specific knockdown of the autophagy-related gene 5 (*Atg5*) increases proliferation and inhibits neuronal differentiation of embryonic NPCs during cortical development. These data suggest that embryonic and adult NPCs are altered when autophagy-related genes are

deleted in the embryo. However it remains unknown whether autophagy, independent of effects in the embryo, is directly required for NPCs and their progeny in the adult.

Here we tested the functional role of autophagy specifically in the adult brain by removing the autophagy-related gene 5 (*Atg5*) from dividing NPCs. We found that autophagic flux occurs in adult NPCs and that removal of *Atg5* is associated with a reduction in autophagic flux. Additionally we find that *Atg5*-null cells have a significant reduction in survival, as well as a delay in neuronal maturation. The reduction in neurogenesis occurred in the absence of altering proliferation or cell lineage. Furthermore, inactivation of *Bax* restored neurogenesis in the absence of *Atg5*, implicating *Bax* functions downstream of ATG5 to regulate the survival of adult-generated neurons. Finally we showed *Atg5* dependent signaling is required for running-induced increases in the survival of the adult developing NPC.

Results

Autophagic flux in hippocampal NPCs and their progeny

Autophagy is a highly dynamic process that includes the induction, initiation and elongation of the isolation membrane, forming the autophagosome that is cleared after fusion with the lysosome. The expression of autophagosome marker microtubule-associated protein 1 light chain 3 (LC3) and phosphatidyl-ethanolamine (PE)-modified LC3 (LC3-II) has been shown in the SVZ NPCs and their progeny in the rostral migratory stream and olfactory bulb (Wang et al., 2013; Yazdankhah et al., 2014).

Within the dentate gyrus the cellular distribution of autophagosomes within NPCs and their progeny remain unknown. Thus, in order to test if autophagic activity occurs in the NPCs and their progeny as they mature within the SGZ, we utilized the monomeric mCherry-EGFP-LC3

tagged protein, which has recently been used to measure autophagic flux within the brain in mature neurons (Matus et al., 2014). Based on the sensitivity of the GFP fluorescent signal, but not mCherry fluorescent signal, to the acidic conditions of the lysosome lumen, this approach allows for detection of autophagosomes (GFP and mCherry expressing puncta (GFP+mCherry+)) and autolysosomes (puncta only express mCherry (GFP-mCherry+)).

We created a mCherry-EGFP-LC3 retrovirus to infect and birthmark the dividing NPCs and examined expression of mCherry-EGFP-LC3 protein at 3, 7, 14 and 30 days post infection (dpi). Infected cells were identified by their pronounced expression of cytoplasmic non-nuclear GFP staining and mCherry expressing puncta indicative of autolysosomes and suggestive of rapid autophagic flux (Figure 1a). Autolysosomes were clearly visible in the soma and processes of infected cells. At 3-14 dpi quantification of the number of autolysosomes present in labeled cells revealed no significant difference in the total number of autolysosome per infected cell, or in the distribution of the autolysosomes in either the soma or developing processes (Figure 1b). We further confirmed the infected cells were NPCs and their progeny by identifying that the immature neuronal marker doublecortin (DCX) was expressed in $94\pm 7\%$ and $93\pm 7\%$ of the infected cells at 3 and 7 dpi, respectively (Figure 1d). By 30 dpi there was a significant reduction in number of autolysosomes in the processes, but not within the soma (Figure 1b,c). Together these finding suggests that autophagy is active in the NPC throughout its development and appears more active in the developing process in the cells that are less than 1 month old.

Figure 1

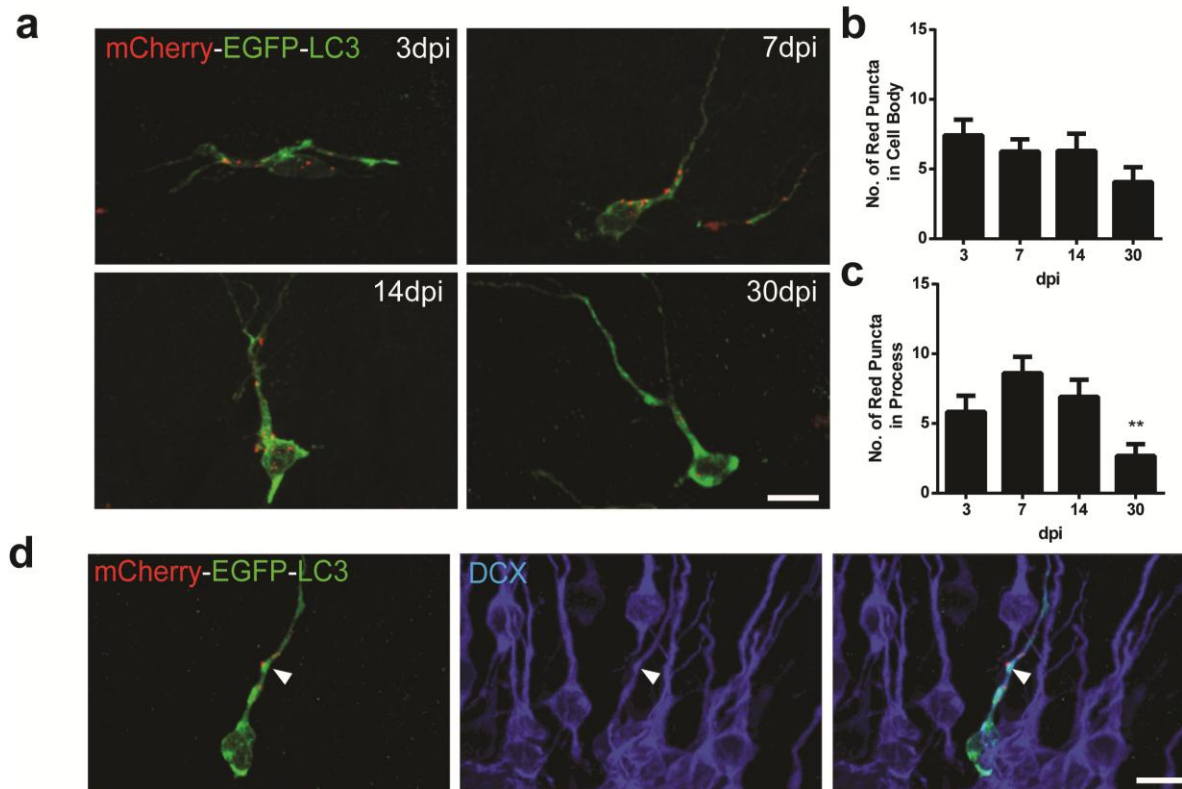


Figure 1 Retroviral labeling with mCherry-EGFP-LC3 shows autophagic activity within NPCs present in the SGZ of the DG. **a)** Representative images of tandem mCherry-EGFP-LC3 retrovirus infected cells showing mCherry⁺ autolysosomes present during the course of NPC maturation at 3, 7 14, and 30 days post injection (dpi). The autolysosomes were quantified within **(b)** the soma; as well as **(c)** the processes extending from the cell body. (n=2-3 animals per group, 7-15 cells/animal, One-way ANOVA, ** p<0.005 compared to 7dpi) **(d)** Representative image at 7 dpi shows DCX⁺ cells expressing autolysosomes (arrowhead pointing to autolysosomes present on the dendrite of DCX expressing neuron). Scale bars = 10 μ m.

Atg5 is required for survival of the progeny of NPCs

To determine if autophagy is essential for the survival of NPCs and their progeny, we used a retrovirus-mediated gene transfer approach to knockout *Atg5* from the dividing NPCs in the adult brain. To determine if loss of *Atg5* from NPCs would affect autophagic flux, the number of autolysosomes were counted in the *Atg5*^{+/+} and *Atg5*^{flox/flox} mice that were co-injected with the GFP-Cre and mCherry-EGFP-LC3 retroviruses. Cells infected with mCherry-EGFP-LC3 alone expressed GFP in the cytoplasm (Figure 2a arrowhead, higher magnification in 2b), whereas cells infected with both viruses expressed GFP throughout the nucleus and cytoplasm because the GFP expression in GFP-Cre infected cells is nuclear due to a nuclear localization signal (Figure 2a arrow, higher magnification in 2c). Quantification of the *Atg5*-null and *Atg5*^{+/+} infected cells revealed significantly less autolysosomes in the *Atg5*-null cells both in the cell body (Figure 2d) and the process (Figure 2e).

In order to fate map and assess the survival of the *Atg5*-null NPCs, a mixture of retroviral GFP-Cre (*CAG-GFP-Cre*) and control RFP (*CAG-RFP*) (Tashiro et al., 2006) was used to infect the dividing NPCs in the SGZ of the *Atg5*^{flox/flox} mice and wild-type (*Atg5*^{+/+}) littermates. This approach was used since it allows for the comparison of the ratio of the *Atg5*-null infected double-labeled GFP-Cre and RFP expressing (GFP+RFP+) cells to all control RFP expressing (RFP+) cells, as similarly published by our group and others (Tashiro et al., 2006; Jessberger et al., 2008; Jagasia et al., 2009; Schnell et al., 2014; Ceizar et al., 2016). As expected in the *Atg5*^{+/+} mice there was no significant difference in survival ratio between 3 and 60 dpi. In contrast, in the *Atg5*^{flox/flox} mice there was a reduction in the survival over time, with post-hoc analysis revealing a significant reduction in survival between 3 and 7 dpi. The significant reduction in survival of *Atg5*-null cells at 3 to 7 dpi was associated with a significant reduction in survival

ratio between the *Atg5*^{flox/flox} mice and the control *Atg5*^{+/+} mice at 30 and 60 dpi (Figure 2 f,g). The reduced survival ratio outcome was also supported by the cell counts for average number of Cre-GFP and RFP infected cells, which revealed a significant reduction in number of Cre-GFP infected cells at 7 dpi in the *Atg5*^{flox/flox} mice compared to the control *Atg5*^{+/+} mice. The reduction in number of *Atg5*-null (GFP+) cells occurred in the absence of any difference in the number of control RFP-infected cells at any time point (Supplemental Figure 1). Thus, overall the results suggest that *Atg5* is required for survival of progeny of the NPCs during the critical window of 3 to 7 days. These results suggest that the significant reduction in survival of *Atg5*-null NPCs is mediated by loss of autophagy.

The progeny of *Atg5*-null NPCs have a delay in neuronal maturation in the absence of a change in fate

To examine if the loss of *Atg5* altered the maturation or fate of the NPCs, we quantified the expression of different cell lineage markers in the *Atg5*-null cells in the *Atg5*^{flox/flox} mice versus the *Atg5*-expressing cells in the control *Atg5*^{+/+} mice. At 3 dpi there was no difference in the proportion of *Atg5*-null and *Atg5*-expressing cells that were dividing, as measured by GFP+ cells expressing the cell cycle marker, Ki67 (Supplemental Figure 2). Examination and quantification of the *Atg5*-null and *Atg5*-expressing cells that expressed the immature neuronal marker DCX varied significantly between 3 and 30 dpi (Figure 3a,b). At 3 dpi there was a similar proportion of *Atg5*-null and *Atg5*-expressing cells that expressed DCX. At 7 dpi there was a significant reduction in the proportion of surviving *Atg5*-null cells that expressed DCX. In contrast at 30 dpi there was a significant increase in the proportion of surviving *Atg5*-null cells that expressed DCX. This finding raised the hypothesis that *Atg5*-null adult-generated cells may have a delay

Figure 2

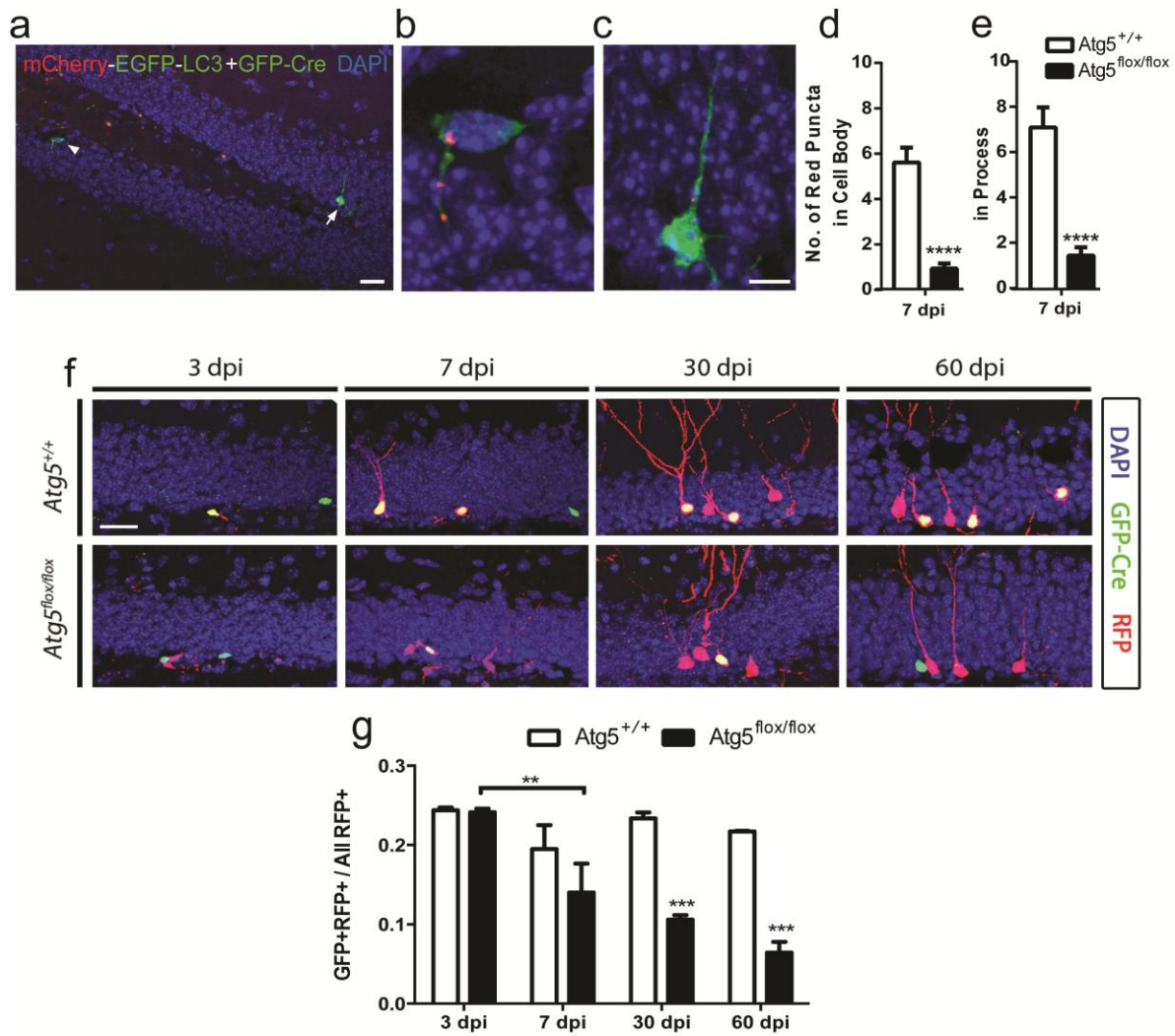


Figure 2 Retrovirus-mediated removal of Atg5 from dividing NPCs reduces the number of autolysosomes and decreases cell survival. (a) Representative image of the dentate gyrus showing two retroviral infected cells. A cell infected with mCherry-EGFP-LC3 virus is identified by cytoplasmic GFP expression, which is also identifiable in (b) at higher magnification. In contrast a cell infected with both mCherry-EGFP-LC3 and GFP-Cre is identified by cytoplasmic EGFP and nuclear GFP expression, respectively, which is also identifiable in (c) at higher magnification. Scale bar = 20um in (a) and 5um in (b,c). The number of autolysosomes in the (d) cell body and (e) processes of Atg5-null cells in Atg5^{flox/flox} mice was significantly reduced compared to Atg5-WT cells in Atg5^{+/+} mice at 7 dpi. (n=3 Atg5^{+/+} mice, n=2 Atg5^{flox/flox} mice; n=7-13 cells/animal; cell t(50)=5.7, p<0.0001; for processes t(50)=5.0, p<0.0001). (f) Representative confocal images of GFP-Cre+ (green), RFP+ (red) and double-labeled (yellow; GFP+RFP+) cells at 3, 7, 30 and 60 dpi of retroviruses (CAG-GFP-Cre and CAG-RFP) into the dentate gyrus of wild-type (WT, Atg5^{+/+}) and floxed Atg5 (Atg5^{flox/flox}) mice. Scale bar= 20um (g) The survival ratio expressed as GFP+RFP+ (yellow) over all RFP+ (red + yellow) cells at 3-60 dpi showing less survival of Atg5-null cells at 30 and 60 dpi. (n=2-5 animals per group, Two-way ANOVA, Bonferroni posthoc).

in their neuronal maturation. In support of this hypothesis there was a significant reduction in the proportion of *Atg5*-null cells at 7 dpi that expressed the mature neuronal marker NeuN (Figure 3c,d). This effect was transient and almost all NPCs expressed NeuN at 30 and 60 dpi. In further support of the *Atg5*-null cells having a transient delay in maturation, the proportion of 30 dpi *Atg5*-null cells that expressed both DCX and NeuN was significantly increased (Figure 3e,f). By combining our analysis of the number of surviving *Atg5*-null and *Atg5*-expressing cells between 3 and 60 dpi (Figure 2) and average percentage that become neurons at 60 dpi (NeuN+) (Figure 3c), we can further estimate that removal of *Atg5* reduced the number of new neurons by approximately two-fold (neuronal survival rate between 3 and 60 days: 53% WT and 27% *Atg5*^{fl/fl}). These results therefore support that *Atg5* has a pro-survival role and is required for the temporal development of the young immature neuron as it develops into an adult generated neuron.

Figure 3

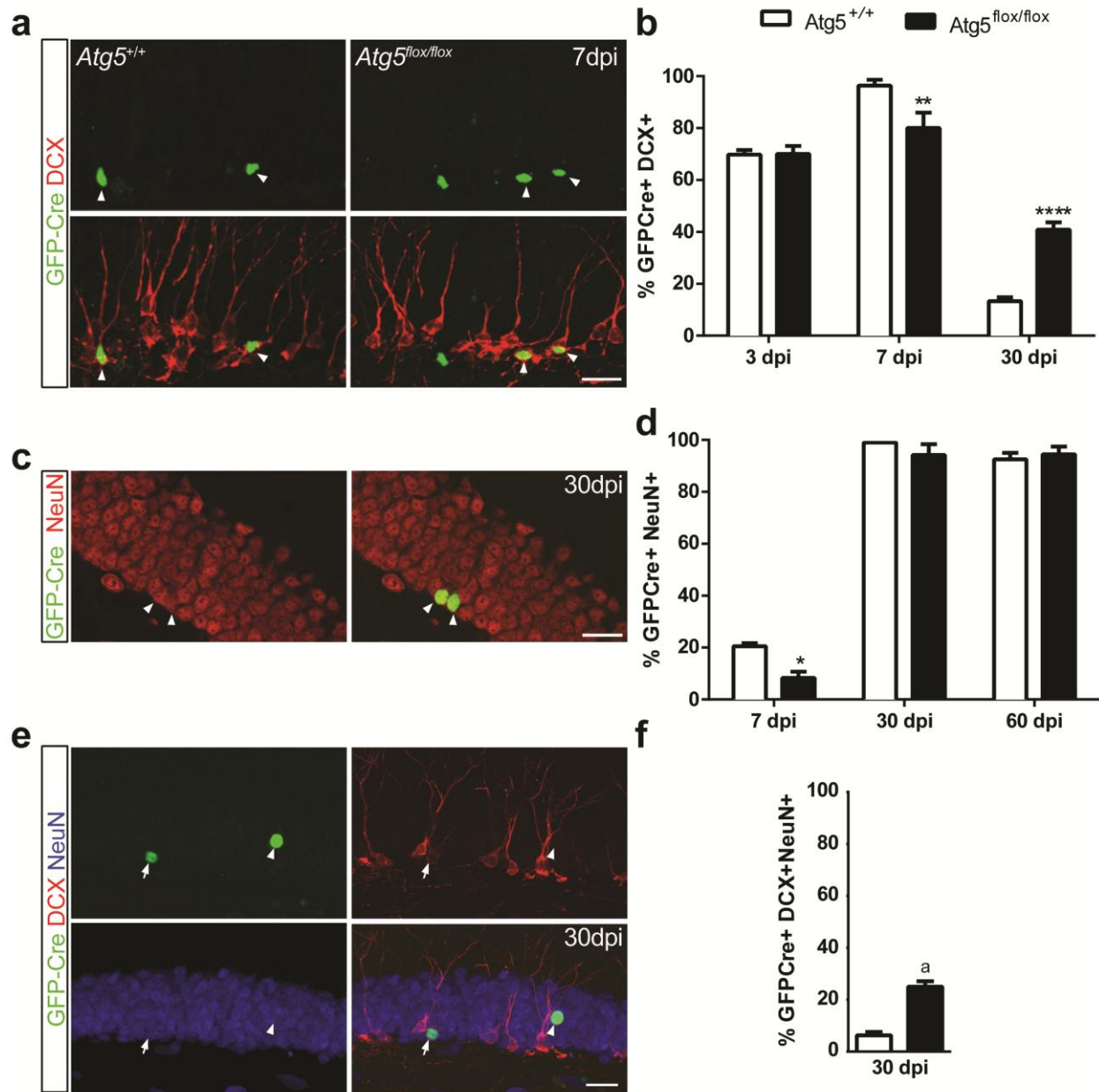


Figure 3 The progeny of the surviving Atg5-null NPCs have a delay in neuronal maturation in the absence of a change in fate. (a) Representative image of GFP-Cre+ cells at 7 dpi colabeled with DCX (arrowheads GFP+DCX+ colabeled cells). (b) Quantification of GFP-Cre+ expressing DCX showing proportionally less Atg5-null cells express DCX at 7 and more at 30 dpi. (n=3-5 animals per group, n=17-23 cells/animal, 2-Way ANOVA, Bonferroni post hoc, ** p<0.01, **** p<0.0001) (c) Representative image showing two GFP-Cre+ cells expressing NeuN at 30 dpi. (d) Quantification of GFP-Cre+ expressing NeuN showing less Atg5-null cells express NeuN+ cells at 7 dpi. (n=3-5 animal per group, n=17-23 cells/animal, 2-Way ANOVA, Bonferroni post hoc, * p<0.05) (e) Representative image of GFP-Cre+ (green) cells expressing DCX (red) and/or NeuN (blue) (Arrowhead = GFP-Cre+NeuN+DCX- and arrow = GFP-Cre+NeuN+DCX+) (f) Quantification showing more Atg5-null cells co-expressed DCX and NeuN at 30 dpi. (n=4 *Atg5^{+/+}*, n=5 *Atg5^{flx/flx}* mice, n=17-23 cells/animal, ^at(7)=7.0, p=0.0002) Scale bars = 20um.

The progeny of Atg5-null NPCs have a transient reduction in spine density

The significant reduction in survival of Atg5-null cells was associated with a delay in neuronal maturation. Given autophagy has been recently shown to regulate embryonic spine pruning (Tang et al., 2014) and our observation of autolysosomes present in the dendrites of adult-generated hippocampal cells (Figure 1), we examined whether removal of *Atg5* altered dendritic outgrowth and spine density in adult generated cells (Figure 4). Sholl analysis and examination of the total dendritic length revealed no differences in the Atg5-null cells compared to Atg5-expressing cells (Figure 4a-c). In contrast there was a significant reduction in spine density in the Atg5-null versus Atg5-expressing cells at 30 dpi (Figure 4d-e). The reduction in spine density was transient and was not significantly different by 60 dpi (Figure 4e). These findings correlate with the delay in neuronal maturation observed by lineage analysis (Figure 3) and further support that *Atg5* is required during the development of the immature neurons.

Figure 4

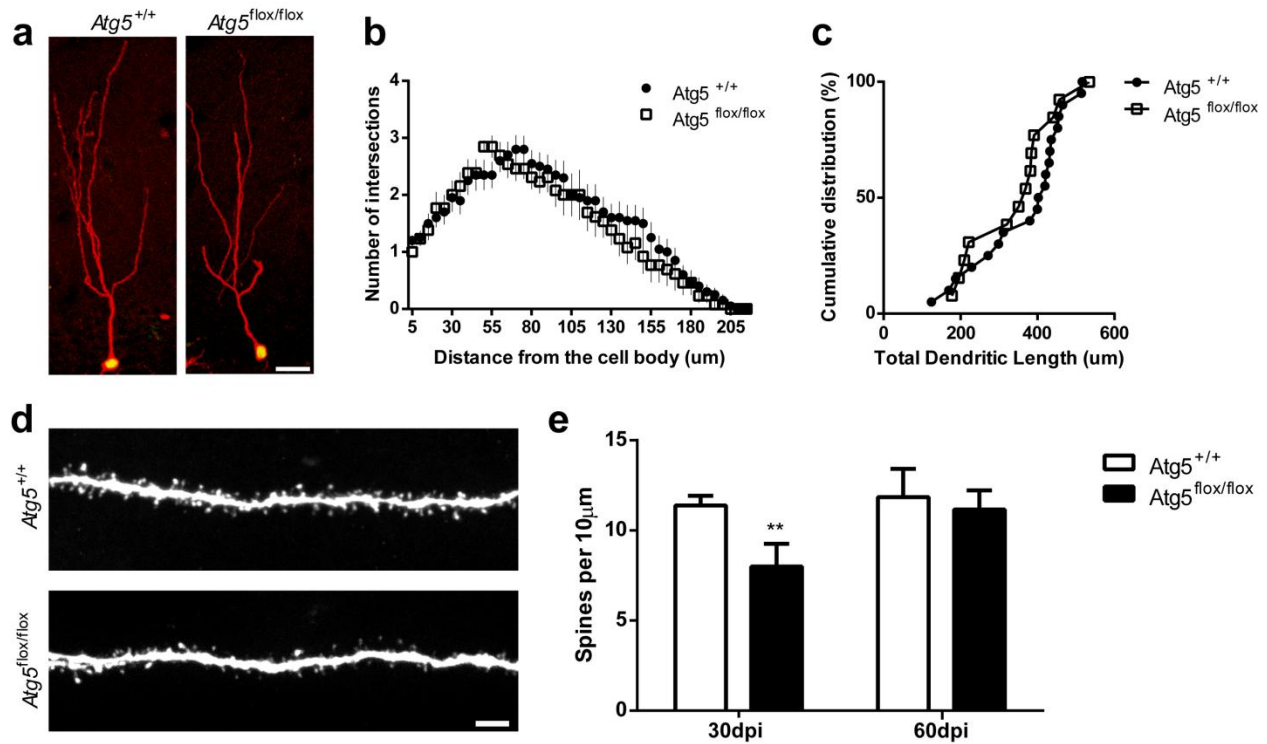


Figure 4 Surviving Atg5-null cells show normal dendritic development but delay in the development of spines. (a) Representative confocal images showing WT and Atg5-null neurons (GFP+RFP+) at 30 dpi. Scale bar = 20 μ m (b) Sholl analysis of dendritic arbors in WT and Atg5-null neurons shows no difference in the complexity of dendrites (n=2 animal per group, 13-20 dendrites/animal). (c) Cumulative frequency distribution of WT and Atg5-null cells shows no difference in total dendritic length (n=2 animal per genotype, 6-13 cells/animal) (d) Representative image of spines along a dendritic segment from WT and Atg5-null neurons at 30 dpi. Scale bar = 2 μ m. (e) Quantification of the number of spines per 10 μ m reveals a reduction in spine density in Atg5-null neurons at 30 dpi. (n=2 $Atg5^{+/+}$, n=4 $Atg5^{flox/flox}$ mice; n=3-7 dendrites/animal) ** p < 0.01

Inactivation of Bax restores *Atg5* induced deficits in neurogenesis

The significant reduction in number of Atg5-null cells lead us to hypothesize that the Atg5-null cells are dying likely by apoptosis. Indeed the number of adult-generated neurons is well known to be only a fraction of the number of dividing NPCs due to apoptotic cell death that occurs during neurogenesis (Dayer et al., 2003; Tashiro et al., 2006; Platel et al., 2010; Sierra et al., 2010). Therefore we tested if blocking apoptosis would prevent the loss of maturing Atg5-null cells. Specifically, we determined if virally infected Atg5-null cells would have enhanced survival in mice that lacked the essential apoptotic protein, *Bax* (Sun et al., 2004). Consistent with a Bax-dependent process mediating apoptosis in adult NPCs in development, we have previously reported that embryonic *Bax* knockout (*Bax*^{-/-}) mice have a significant increase in average number of virally labeled cells compared to WT control mice, and can completely rescue apoptotic Bcl-2-mediated cell death of the maturing NPCs (Ceizar et al., 2016). Therefore we bred embryonic *Bax* knockout (*Bax*^{-/-}) mice with *Atg5*^{flox/flox} mice to create *Atg5*^{+/+}*Bax*^{-/-} and *Atg5*^{flox/flox}*Bax*^{-/-} littermate mice and the labeled cells were examined 30 days after infection with the dual retroviral labeling strategy. In the absence of *Bax* there was no difference in the survival of Atg5-null or Atg5-expressing retroviral-transduced cells (Figure 5a,b). Similarly there was no difference in the percentage of the Atg5-null or Atg5-expressing cells that expressed DCX at 30 dpi (Figure 5c). This is in sharp contrast to the delay in maturation and significant increase in the proportion of surviving Atg5-null cells that expressed DCX at 30 dpi in the presence of *Bax* (Figure 3a). Together these findings support removal of *Bax* can rescue both the loss of the Atg5-null cells and their delay in maturation supporting that Atg5 functions in the maturing NPCs upstream of the *Bax* apoptotic pathway.

Figure 5

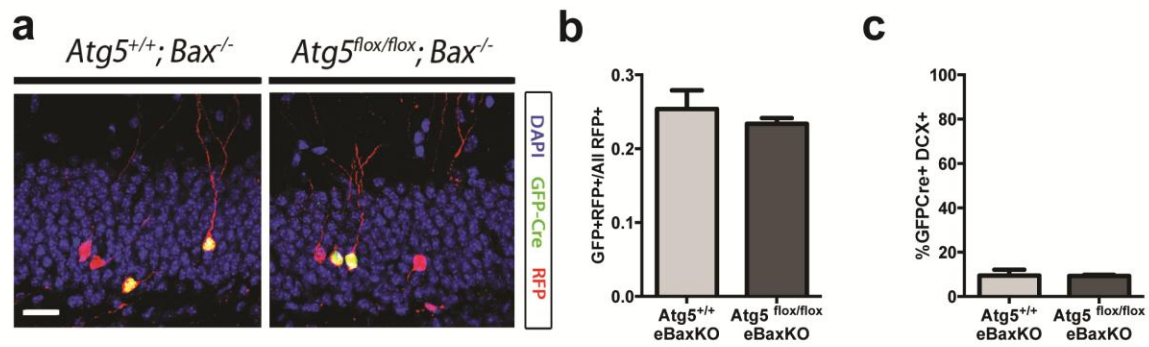


Figure 5 Reduction in survival and maturation of Atg5-null NPCs is Bax-dependent. (a) Representative images and (b) quantification of 30 day old retrovirus infected cells in the *Atg5^{+/+};eBaxKO* and *Atg5^{flox/flox};eBaxKO* mice showing no difference in survival ratios at 30dpi (n=3 animals/group). (c) Quantification of GFP-Cre⁺ cells showing no difference in proportion of cells that express DCX at 30 dpi in the *Atg5^{+/+};eBaxKO* and *Atg5^{flox/flox};eBaxKO* mice (n= 2 animals/group, 20-21 cells/animal). Scale bar = 20μm.

Atg5 is required for exercise-induced increase in survival of NPCs

Running has been shown to increase autophagy within the brain (He et al., 2012) therefore we tested if Atg5 was required for exercise-induced increases in neurogenesis. As expected the *Atg5^{flox/flox}* and the WT (*Atg5^{+/+}*) mice that had free access to a running wheel had an increase in the number of control non-Cre infected (RFP⁺) cells compared to mice that had access to a locked wheel (Figure 6a). Examination of the WT mice revealed that there was significant increase in Cre-infected (GFP⁺) cells in mice given access to the running wheel (Figure 6b). Analysis of the survival ratio further showed in the WT mice a significant increase in survival ratio (Figure 6c). In contrast, the *Atg5^{flox/flox}* mice that were exposed to either a locked or running wheel had a significantly less Atg5-null (GFP⁺) cells compared to WT mice (Figure 6b). Additionally, the *Atg5^{flox/flox}* mice compared to WT mice had a significant reduction in the survival ratio in the locked or free running wheels (Figure 6c). These changes occurred in the context of similar distances ran between the WT and *Atg5^{flox/flox}* mice (WT 210,836 ± 32,164m vs. *Atg5^{fl/fl}* 159,909 ± 27,434m). Therefore, overall these results suggest that running cannot rescue the Atg5-null survival phenotype, with Atg5 being required for running-induced increases in NPCs.

Figure 6

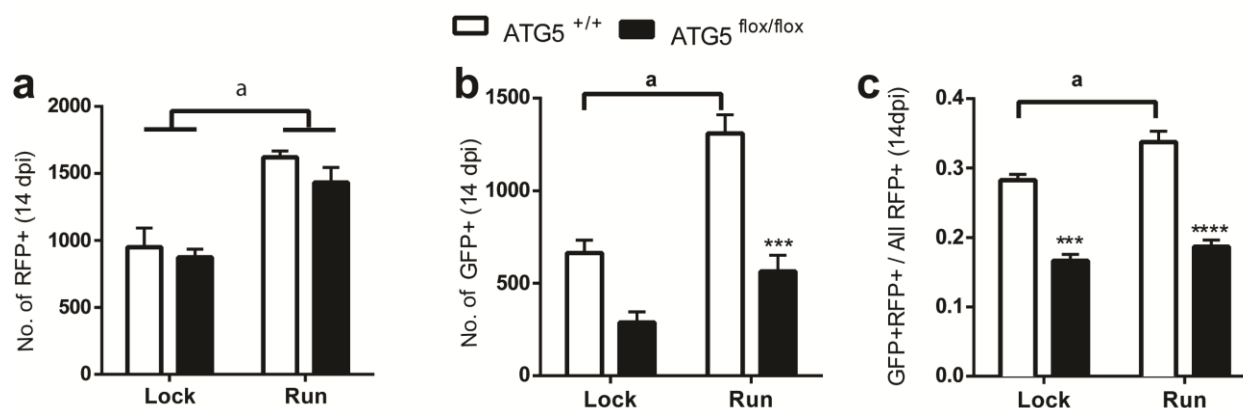


Figure 6 Atg5 is required for Exercise-Induced Increase in Developing NPCs (a) Mice with free access to running wheel compared to a locked wheel showed an increase in the number of non-Cre infected (RFP⁺) cells in both the Atg5^{flox/flox} and WT mice. (b) Number of GFP-Cre⁺ cells were increased in the Atg5^{+/+} run vs. Atg5^{+/+} lock mice and comparable in Atg5^{flox/flox} run vs. Atg5^{flox/flox} lock. (c) The survival ratio is higher in the Atg5^{+/+} run vs. Atg5^{+/+} lock mice and comparable in Atg5^{flox/flox} run vs. Atg5^{flox/flox} lock. (For a-c, n=4 Atg5^{+/+} lock, n=3 Atg5^{flox/flox} lock, n=4 Atg5^{+/+} run, n=6 Atg5^{flox/flox} run), Bonferroni post hoc, ***p<0.0002, ****p<0.0001 a denotes significant difference when compared to lock group of same genotype.

Discussion

Here we demonstrate that autophagy is active in the progeny of the NPCs and that *Atg5* is required for survival of the progeny of the adult NPCs. Autophagic flux is present in the progeny of the hippocampal NPCs, with autolysosomes being detected within the soma and dendrites. Functionally, retroviral infection of Cre in *Atg5^{flox/flox}* mice reduces autolysosomes in *Atg5*-null cells and is associated with a significant loss of the survival of the progeny of the NPCs during the first 7 days of their maturation. The reduction in survival is accompanied by a delay in the maturation of the adult-generated neuron including a transient reduction in spine density in the absence of altering proliferation or neuronal fate. *In vivo* complementation experiments with *Bax* knockout mice indicate that the reduction in *Atg5*-null immature neurons occurs upstream of a *Bax*-mediated death. Moreover, running, which is a known inducer of autophagy, was insufficient to reverse the significant survival phenotype of the *Atg5*-null NPCs.

These findings suggest that *Atg5* is one of the gatekeepers that controls which NPCs in the adult brain survive during their development into new functional neurons.

These findings add to the recent work of others that have examined autophagy and adult neurogenesis *in vivo* (Wang et al., 2013; Lv et al., 2014; Yazdankhah et al., 2014) and show for the first time that autophagy has a pro-survival role during the development of adult-generated hippocampal neurons. Our conclusions further provide evidence that 3-7 day old cells undergo a critical period for survival that requires *Atg5*. We also show the pro-survival effect of *Atg5* in the NPCs can occur independent of effects in the embryonic or early postnatal brains, independent of altering cell proliferation, as well as independent of effects in the adult stem cell population since this retrovirus targets dividing NPCs. To delineate whether *Atg5* is required in

the stem cells future studies will require an inducible system to target the slowly dividing stem cells.

Removal of *Atg5* in the NPCs also resulted in a significant delay in their maturation. The timing of the delay in maturation was observed between 1 week and 1 month post viral infection. During this time it is well known that there is a requirement of neuronal activity to promote cell survival through activity-dependent mechanisms, including the NPCs having a lower threshold for long-term potentiation (LTP) (Schmidt-Hieber et al., 2004; Ge et al., 2007; Lin et al., 2010; Lepousez et al., 2015). Therefore the delay in maturation may contribute to the reduction in the survival of *Atg5*-null cells. This is an exciting possibility since a variety of studies have identified that neuronal activity can regulate autophagy through a variety of mechanisms, such as activity-dependent retrograde transport of autophagosomes and presynaptic AMPA receptor degradation (Katsumata et al., 2010; Shehata et al., 2012; Shehata and Inokuchi, 2014; Wang et al., 2015). Together these findings lead to the hypothesis that autophagy and neuronal activity may co-regulate the survival of the maturing NPCs.

The reduction in survival and delay in maturation of *Atg5*-null cells occurs independent of affecting the differentiation of the dividing NPCs into mature dentate neurons. Almost all of the *Atg5*-null NPCs that survive develop into NeuN expressing neurons within two months. This is surprising since *in vivo* studies examining autophagy and neurogenesis support that at least, Ambra1, Beclin1 and FIP200, are required for neuronal differentiation in the progeny of adult NPCs (Wang et al., 2013; Yazdankhah et al., 2014). There are many possible explanations that could account for this difference. One hypothesis is that the retrovirus transduced dividing NPCs could be past the stage of neuronal lineage commitment. This explanation however seems unlikely since a decrease in neuronal lineage in retroviral transduced NPCs was recently

published (Mu et al., 2012). A more likely hypothesis may be that the requirement of autophagy for differentiation may be different within the embryo versus adult NPCs and their progeny. This could arise from intrinsic differences in the embryonic or adult cell populations and in compensation that occurs in the embryonic versus adult neurogenic niches. This is supported by the variability in phenotypic outcomes reported in the *Atg5* ablation *in vivo* mouse models. Conditional removal of *Atg5* at E10.5 within the nestin-expressing stem and NPCs induced neurodegeneration and intracellular protein accumulation, in the absence of gross differences in differentiation (Hara et al., 2006). More recently, inducible knockdown of *Atg5*, or overexpression of *Atg5* in the cortical NPCs at E13 suggested *Atg5* was essential to promote neuronal lineage commitment (Lv et al., 2014). Using the same methodology at E16 this group also recently reported that *Atg5* promotes astrocyte differentiation within the cortex (Wang et al., 2014). In contrast, we find removal of *Atg5* does not alter the fate of the dividing NPC progeny.

Our study also demonstrates that removal of BAX can rescue the loss of the *Atg5*-null cells supporting that *Atg5* functions upstream of the BAX apoptotic pathway in the immature neurons. Despite autophagy and apoptosis being distinct cellular processes, the protein networks that control their regulation and execution are highly interconnected (Rubinstein and Kimchi, 2012). For example, *Atg5* can induce apoptosis through cleavage of the non-conjugated form of *Atg5* by calpains, which then directly interacts with the Bcl-2 family member, B-cell lymphoma-extra large (Bcl-xL) (Yousefi et al., 2006). Although the both *Atg5* and truncated *Atg5* do not directly bind BAX, it remains unknown if the truncated *Atg5* can promote apoptosis through activation of Bax-Bak-like molecules by the inactivation of Bcl-xL. Since truncated *Atg5* promotes apoptosis and we have found deletion of *Atg5* promotes the death of the NPC progeny it is unlikely that truncated *Atg5* is involved in the death of the *Atg5*-null cells. This is also

supported by our recent finding that calpain-1 and calpain-2, the proteases that cleaves Atg5, have no deficits in adult neurogenesis (Amini et al., 2013).

Together these data suggest Atg5 may be working through the canonical autophagy pathway to regulate the survival of the developing NPCs. If this is true then inducing autophagy should be able to rescue the survival phenotype. This could happen through an Atg5-dependent conventional or Atg5-independent pathway (Nishida et al., 2009). We show that running requires Atg5 in the NPCs to increase the survival of NPCs and thus cannot rescue the Atg5-null survival phenotype. Further studies will be required to elucidate in more detail the role of autophagy in exercise-induced neurogenesis in the brain. For example, this study examines the effect of voluntary running raising the questions about whether our findings will generalize to all forms of exercise? Additionally to directly test the hypothesis that the Atg5-null survival deficits that occur in naïve and running mice are Atg5 dependent, an inducible NPCs mouse model is required to elucidate whether a gain-in-function experiment can rescue the survival deficits of the Atg5-null cells.

In conclusion, our findings support a cell-autonomous requirement for Atg5 in adult immature neurons. This finding provides new information about the regulation of survival of the NPC progeny, which is important for designing therapeutic strategies aimed at promoting endogenous repair and improving the success of neural transplants. Our findings also have additional implications due to exponential growth of the development of therapeutics that inhibit and induce autophagy as an intervention for various types of human disorders (Kroemer, 2015). For example, the autophagic inhibitors that are being tested as cancer therapeutics may have unexpected deleterious side effects of reducing neurogenesis. However, on the converse the

autophagic inducers that are being designed for neurodegenerative diseases could exert additional beneficial effects through promotion of adult neurogenesis.

Materials and Methods

Animals. The C57BL/6 mice were purchased from Charles River (male, 7-12 weeks). The mouse lines that were genotyped according to previously published protocols included the floxed *Atg5* mice from RIKEN (Hara et al., 2006) and *Bax* knockout mice from Jackson Laboratories (Knudson et al., 1995). A range of 2-9 mice was included in each experimental group. Animal procedures were performed within the guidelines of the Canadian Council on Animal Care and were approved by the University of Ottawa Animal Care Committee.

Mice were housed in a 12-hour light/dark cycle with free access to food and water. For exercise experiment (Figure 6), mice were singly housed with free access to a low profile wireless running wheel or a locked wheel (Med Associates) for 1 week prior to, and 2 weeks post retroviral infection.

Retrovirus and stereotaxic injection. The retroviral vectors *CAG-GFP-Cre* and *CAG-RFP* and the corresponding packing and envelope constructs were generously provided by Dr. Fred Gage (The Salk Institute). The retroviruses were generated as previously described (Tashiro et al., 2006). The titers of *GFP-Cre* and *RFP* viruses were determined by live titrating using 293T cells and ranged between 2 and 4 X 10⁸ infectious units (IU) per ml. The *GFP-Cre* and *RFP* virus was injected in a 1:1 ratio (volume 1.5ul), except for mice used for phenotyping, which were injected with only *GFP-Cre* (volume 1ul). The mCherry-EGFP-LC3B plasmid (Addgene #22418) was used to produce retroviruses as described above and 1.5 ul was injected into the dentate. In order to target the SGZ, all retroviruses were bilaterally injected into the

dentate gyrus (-1.7mm anterior/posterior, ± 1.2 mediolateral, -2.4mm dorsoventral from Bregma) of mice (7-9 weeks old) during stereotaxic surgery. mCherry-EGFP-LC3B retrovirus was also co-injected with the GFP-Cre retrovirus in both Atg5^{+/+} and Atg5^{flox/flox} mice to determine effect of Atg5KO on autophagic puncta. Infected cells were distinguished based on either nuclear GFP expression (GFP-Cre infected), cytoplasmic GFP expression (mCherry-EGFP-LC3B infected) or both nuclear and cytoplasmic GFP (GFP-Cre & mCherry-EGFP-LC3B dual infected) cells.

Tissue processing and immunohistochemistry. Mice were anesthetized and transcardially perfused with cold phosphate buffered saline (PBS, pH7.4) followed by 4% paraformaldehyde in PBS. Brains were removed and post fixed for 1 hour in 4% paraformaldehyde and then transferred into 30% sucrose with 0.1% sodium azide in PBS. Brains were sectioned coronally into 30 μ m slices on a freezing microtome and stored in PBS with 0.1% sodium azide. Histology was performed using free-floating methodology with sections being washed in PBS and incubated in 0.1% Tween-20 and 0.1% Triton X-100 in PBS with corresponding primary antibodies at 4°C overnight. The following primary antibodies were used: chicken anti-GFP (GFP-1020, Aves, 1:5000), rabbit anti-dsRed (632496, Clontech, 1:5000), rabbit anti-Ki67 (275R-14, Cell Marque, 1:100), goat anti-DCX (sc-8066, Santa Cruz, 1:500), and mouse anti-NeuN (MAB377, Millipore, 1:500). On day 2, sections were washed in PBS, incubated with corresponding Cy2, Cy3 and Cy5-conjugated IgG antibodies (Jackson ImmunoResearch, 1:500) for 1 hour at room temperature, stained using 4',6-diamidino-2-phenylindole (DAPI, 10236276001, Roche, 1:10000, 3min), and washed in PBS.

Cells in the SGZ were counted using an Olympus BX51 fluorescent microscope using unbiased histological approaches in every 9th serial section throughout the dentate, as previously described (Lagace et al., 2007). The quantification of co-labeled cells was performed using a

Zeiss LSM510-META confocal microscope, as previously described (Lagace et al., 2007). For mCherry-EGFP-LC3 puncta counts, the images were acquired at the confocal microscope using 63X objective and all puncta were counted irrespective of their size within the infected cells. For spine analysis, dual-labeled cells were imaged at 63x (oil immersion) with a Quorum Spinning-disk confocal microscope at emission wavelengths of 406, 490, and 561. MetaMorph automation and image acquisition software (Molecular Devices) was used to create a high resolution three dimensional representation of spines throughout the visible dendritic arbor using 0.5 μm Z-plane optical sectioning in combination with a tile-scan module. Images were subsequently stitched and flattened in MetaMorph and exported to NeuronStudio (CNIC, Ichan School of Medicine at Mount Sinai) to measure neurite length. Spines were manually quantified from a single neurite that spanned the hippocampal molecular layer (top of the granule cell layer to the hippocampal fissure) per cell in Fiji image processing software (ImageJ). Spine density (spines / 10 μm) was calculated as the quotient of the number of spines over neurite length multiplied by 10. Although not specifically quantified, spine density was observed to be similar for any individual cell throughout the molecular layer.

Statistics. All data are reported as mean $\pm$ s.e.m. and statistical analysis performed using GraphPad Prism (v6.0) software. Experiments with two groups were analyzed by the two-tailed *student's t-test*. Analyses of three or more groups were performed using an ANOVA test followed by a Bonferroni *post hoc*. Statistical significance was defined as $P < 0.05$.

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Conflict of Interest

The authors declare no competing financial interests.

List of abbreviations

Autophagy-related gene 5 (*Atg5*)

Bcl-2–associated X *protein* (*Bax*)

Neural progenitor cells (NPCs)

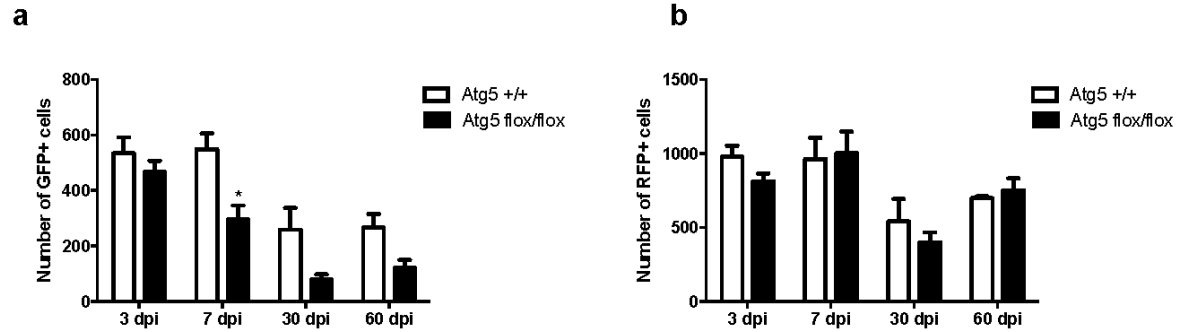
Subventricular zone (SVZ)

Subgranular zone (SGZ)

Microtubule-associated protein 1 light chain 3 (LC3)

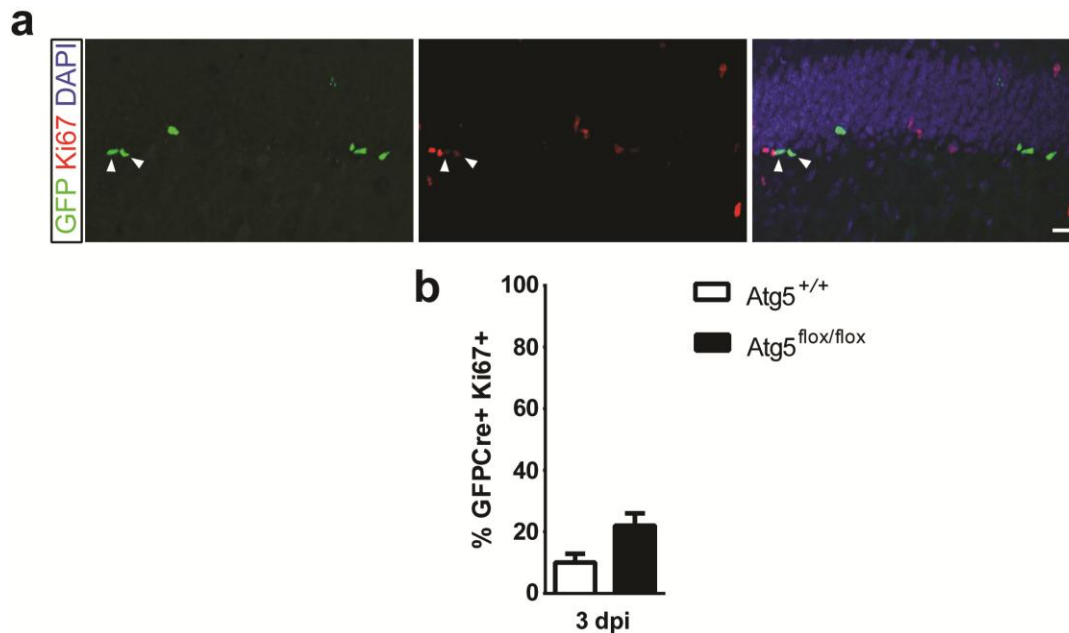
Doublecortin (DCX)

Supplementary Material



Supplemental Figure 1 Retrovirus-mediated removal of *Atg5* from dividing NPCs.

(a) Average number of GFP-Cre+ (green) and (b) control RFP+ (red) cells at 3, 7, 30 and 60 dpi of retroviruses (*CAG-GFP-Cre* and *CAG-RFP*) into the dentate gyrus of wild-type (WT, *Atg5*^{+/+}) and floxed *Atg5* (*Atg5*^{flox/flox}) mice. (n=2-5 animal per group, Two-way ANOVA, Bonferroni posthoc * $p < 0.05$ *Atg5*^{flox/flox} versus *Atg5*^{+/+} at 7 dpi).



Supplemental Figure 2 Retrovirus-mediated removal of *Atg5* from dividing NPCs does not alter proportion of NPCs in cell division.

(a) Representative confocal images of GFP-Cre+ (green), Ki67+ (red) and double-labeled (arrowheads yellow; GFP+Ki67+) cells in the dentate gyrus of a floxed *Atg5* (*Atg5*^{flox/flox}) mice (blue=DAPI nuclear counterstain) scale bar=20um (b) Percentage of GFP-Cre+ cells that are labeled with Ki67 shows no difference in percentage of dividing cells between wild-type (WT, *Atg5*^{+/+}) and floxed *Atg5* (*Atg5*^{flox/flox}) mice. t-test, $t(6)=2.1$, $p > 0.05$

CHAPTER 4:

Adult hippocampal neurogenesis occurs in the absence of *Presenilin 1* and *Presenilin 2*

In submission

Contribution of co-authors

Jagroop Dhaliwal: Performed all virus and inducible experiments, performed data analysis and generated draft of the manuscript.

Timal Kannangara: Performed electrophysiology experiments and data analysis.

Michael Vaculik: Contributed to inducible experiments and analysis.

Keren L. Kumar: Contributed to generation and maintenance of mouse breeding.

Amanda Maione: Contributed to inducible experiments.

Jie Shen: Collaborator. Provided the floxed PS1 and germline PS2 KO mice.

Diane C. Lagace: Contributed to the design, analysis, interpretation of results and writing the manuscript

Adult hippocampal neurogenesis occurs in the absence of *Presenilin 1* and *Presenilin 2*

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The authors declare no competing financial interests.

Abstract

Presenilins (*PS1* and *PS2*) are essential components of the gamma-secretase complex and presenilin mutations are the major cause of familial-Alzheimer's disease (FAD). Forebrain-specific presenilin knockout mice have revealed the presenilins to be essential for neuronal survival and learning & memory in the adult brain. These findings prompted us to investigate the role of presenilins in the context of adult hippocampal neurogenesis. To specifically target proliferating neural progenitor cells (NPCs) in the subgranular zone (SGZ) of the adult dentate gyrus, we used a retroviral approach to express GFPCre fusion protein to remove *PS1* in floxed *PS1* mice on a *PS2* KO background and follow the fate of the labeled NPCs. Surprisingly, we report no defects in NPC survival or differentiation post ablation. The infected NPCs also developed into mature neurons in the dentate without any apparent defects in morphology. Finally, we tested, using NestinCreER^{T2} inducible mice, the requirement of *PS1* in neural stem cells (NSCs). Surprisingly, ablation of *PS1* from type1 radial glia- like NSCs and their progeny showed no change in NSC proportion or on neurogenesis. These results suggest that the NSCs and NPCs can develop in the absence of presenilins and thus the presenilins are not required for cell autonomous regulation of adult hippocampal neurogenesis.

Introduction

Neurogenesis occurs throughout the lifespan in the adult mammalian brain and has important implications for cognitive functions. Within the subgranular zone (SGZ) of the hippocampus, there are neural stem cells (NSCs) that give rise to intermediate neural progenitor cells (NPCs) which differentiate to form immature and subsequently mature granule neurons that can integrate into the preexisting neuronal network (Ming and Song, 2011). In the human it is estimated within the hippocampus there is a production of 700 new neurons per day that can replace ~35% of the total hippocampal population (Spalding et al., 2013). This large capacity for generation of new neurons in the adult human brain continues to fuel hope for the development of novel therapeutic strategies to delay the onset of cognitive deficits associated with neurodegenerative disorders by enhancing neurogenesis prior to the onset of progressive neuronal loss. This has been particularly explored for Alzheimer's disease (AD) since AD is associated with compromised neurogenesis, which is associated with cognitive deficits, such as difficulty learning new information and memory loss (Mu and Gage, 2011). Additionally, increasing evidence suggests that molecular players in AD, such as the presenilins, Notch 1 and amyloid precursor protein (APP) regulate the development of new hippocampal granule neurons (Ables et al., 2010; Mu and Gage, 2011; van Tijn et al., 2011).

Mutations in the *Presenilin* (*PS1* & *PS2*) genes are the major cause of early onset familial AD (FAD) through a loss-of-function mechanism (Shen and Kelleher, 2007; Xia et al., 2015). The presenilins are essential components of the multiprotein γ -secretase complex, responsible for the proteolytic cleavage of the amyloid precursor protein (APP). Loss of *presenilin 1* (*PS1*) in mice causes perinatal lethality (Shen et al., 1997) that is accompanied by neurogenesis defects including a reduced neural progenitor population caused by premature cell differentiation and

reduced Notch signaling (Handler et al., 2000; Yang et al., 2000; Kim and Shen, 2008). Loss of *presenilin 2* (*PS2*) in mice causes little detectable phenotypes (Steiner et al., 1999), yet the dual loss of *PS1* and *PS2* causes embryonic lethality and has a more severe phenotype than loss of *PS1* alone (Donoviel et al., 1999).

There have been conflicting findings on whether presenilins alter adult neurogenesis as tested by conditional knockout or knockdown models, as well as studies overexpressing the FAD-associated mutants in the adult brain. One debated issue is whether *PS1* plays a role within the adult NSCs and NPCs. For example, lentiviral knockdown of *PS1* in NPCs enhances differentiation (Gadadhar et al., 2011) yet expressing FAD-linked *PS1* mutant exclusively in adult NPCs does not alter neurogenesis (Veeraraghavalu and Sisodia, 2013). Second, there are discrepancies between studies surrounding whether *PS1* plays a role in naïve versus enrichment-induced neurogenesis. For instance, adult conditional forebrain *PS1* KO mice have no deficits in neurogenesis under naïve condition, however enrichment-induced neurogenesis is suppressed (Feng et al., 2001). Similarly, *PS1* FAD mutant mice have no phenotype when housed in standard condition, yet have impaired NPC proliferation when exposed to an enriched environment (Choi et al., 2008). These findings are at odds with other studies that have reported *PS1* FAD mutants have impairments in proliferation and survival of NPCs independent of enrichment induced neurogenesis (Wang et al., 2004; Wen et al., 2004; Chevallier et al., 2005; Zhang et al., 2007). Thus, it remains unclear whether *PS1* has a cell-autonomous effect during adult neurogenesis since no study has directly examined the effect of ablating *PS1* in the NPCs.

To determine the cell intrinsic requirement of *PS1* during adult neurogenesis, we ablated *PS1* from dividing NPCs using a retrovirus and from NSCs and their progeny using an inducible mouse model. Both approaches used mice that were on a *PS2* KO background. Our data support

that *PS1* & *PS2*-null NSCs and NPCs can proliferate and differentiate into functional, mature granule neurons in the dentate gyrus. Collectively, our data thus provides strong evidence that *PS1* and *PS2* are not essential for cell autonomous regulation of adult hippocampal neurogenesis.

Methods

Animals and Tamoxifen administration

The generation and characterization of Nestin-CreER^{T2} mice has been previously described (Lagace et al., 2007). Floxed *PS1* and germline knockout *PS2* mice (*PS2*KO) were obtained from Dr. Jie Shen (Wines-Samuelson et al., 2010) and were maintained on a mixed (C57BL/6 and 129/SvEv) genetic background. Inducible NestinCreER^{T2} R26R-YFP (*PS1*^{nKO}*PS2*^{KO}) and (*PS1*^{WT}*PS2*^{KO}) mice were used for experiments. Animals were group-housed (2-5 per cage) and were maintained on a 12h light-dark cycle with free access to food and water. To induce CreER^{T2} mediated recombination, 5 week old male & female mice were administered tamoxifen (160 mg/Kg, dissolved in 10%EtOH/90% sunflower oil) i.p., daily for 5 days. All experiments were approved by the University of Ottawa's animal care, in accordance with the Guidelines of the Canadian Council on Animal Care.

Genetic labeling & manipulation with retroviruses

Retroviral expression plasmids used to express GFPCre and/or RFP in proliferating cells were provided by Dr. Fred Gage and have been previously described (Tashiro et al., 2006). High titers of retroviruses (4×10^8 units/ml) were produced by co-transfection of the GFPCre or RFP expression plasmids, VSVG and the packaging plasmid into HEK293T cells followed by ultracentrifugation of the viral supernatant.

Adult (6-9 weeks old) *PS1*^{fl/fl}*PS2*^{KO} & *PS1*^{WT}*PS2*^{KO} mice were anaesthetized (2% Isoflurane) and a 1:1 mixture of CAG-GFPCre & CAG-RFP retroviruses were bilaterally

injected into the dentate gyrus (1.5ul per injection at 0.2ul/min) at the following coordinates: anteroposterior -1.7mm from bregma, lateral +1.2/-1.2mm, ventral -2.4mm. Mice were sacrificed at 12 and 30 days post-infection (dpi) for cell counts, phenotyping and dendritic analysis.

For running experiment (Figure 5), mice were singly housed with free access to a low profile wireless running wheel or a locked wheel (Med Associates) for 1 week prior to, and 2 weeks post retroviral infection.

Immunostaining, histology and Confocal analysis

Coronal brain sections (30um thick) were stained free floating. Sections were first washed in 1XPBS followed by block (3% Normal Donkey Serum, 0.1% Tween-20, 0.1% Triton X-100) for 45 mins. The following primary antibodies were used: chicken anti-GFP (AVES, 1:5000), rabbit anti-DsRed (Clontech, 1:5000), mouse anti-NeuN (Millipore, 1:500), goat anti-DCX (Santa Cruz, 1:500), goat anti-Sox2 (Santa Cruz, 1:500), goat anti-Nestin (R&D, 1:500), mouse anti-GFAP (Millipore 1:500), rabbit anti-Ki67 (cell marque, 1:250). Sections were incubated with DAPI (Roche, 1:10000) or DRAQ5 (Cell Signaling, 1:10000) nuclear dyes for 10 mins followed by washing in 1XPBS and mounting.

All brains were cut on a freezing microtome and 30um thick tissue was collected in 1 in 9 series. Every 9th 30um coronal sections throughout the SGZ were counted by an observer blind to the experimental groups at 400X magnification on the Olympus BX51 fluorescent microscope. For quantification of total YFP+ counts, total Ki67+ and DCX+ counts, 1 out of 9 well with collected brain tissue was used. The number counts obtained were multiplied by 9 (i.e.1 in 9 series) and then by 2 (since one brain hemisphere was counted) to obtain total count estimate for entire brain. For counts of retrovirus labeled GFP+RFP+ and RFP+ cells, the final

counts were only multiplied by 9 and were not doubled since both brain hemispheres were counted for all retrovirus labeled cells.

For phenotyping analysis, all images were acquired on the Zeiss LSM 510 META confocal microscope using multi-track, sequential scanning configuration. Z-series stacks of confocal images were analyzed and rendered in three-dimensions (3D) using the ZEN 2009 software. For analysis of dendritic structure of GFP+RFP+ double positive neurons 3D projection images were semi-automatically traced with ImageJ software using the NeuronJ plugin. A minimum of 20 cells from each genotype were traced. Sholl analysis was performed using the Sholl analysis ImageJ plugin from Ghosh lab (<http://labs.biology.ucsd.edu/ghosh/software/>). Briefly, the analysis was performed by counting the number of times a series of concentric circles (at 5um intervals) centered at the cell soma crossed the dendrites of individual cells. A minimum of 5 individual double transduced (GFP+RFP+, yellow) cells each, from 4 animals of each genotype were analyzed.

Electrophysiology

Adult mice were deeply anesthetized with isoflurane (Baxter Corporation), and transcardially perfused with ice-cold, oxygenated choline-based artificial cerebrospinal fluid (choline-aCSF), containing the following: 119 Choline-Cl, 2.5 KCl, 4.3 MgSO₄, 1.0 NaH₂PO₄, 1.0 CaCl₂, 11 glucose, and 26.2 NaHCO₃ (pH 7.2-4). Mice were then decapitated and the brain was quickly removed. Coronal slices (300 µm) containing the full extent of the dentate gyrus were generated using a vibratome (Leica VT1000S). Brain sections were then transferred to an incubation chamber filled with oxygenated artificial cerebrospinal fluid (aCSF), containing the following: 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 1.0 NaH₂PO₄, 2.5 CaCl₂, 11 glucose, and 26.2

NaHCO₃ (pH = 7.2-4). Slices were initially maintained at 30°C, then allowed to recover at room temperature for at least 1 hour.

Whole-cell recordings

Slices were transferred to a recoding chamber and perfused with oxygenated aCSF (2 mL/minute) at room temperature. A Zeiss Axio Examiner Z1 microscope was used to visually target GFP-positive cells. Borosilicate recording pipettes (4-8 MΩ, World Precision Instruments) were backfilled with either a cesium- or potassium-based intracellular solutions for voltage- and current-clamp experiments, respectively. The cesium internal solution contained the following (in mM): 115 Cs-methanesulfonate, 0.4 EGTA, 5 tetraethylammonium-Cl, 2.8 NaCl, 20 HEPES, 3 Mg-ATP, 0.5 Tris-GTP sodium salt hydrate, 10 Na-phosphocreatine, and 5 QX-314 (Abcam), (pH 7.2–7.3, 280–290 mOsm/L). The potassium internal solution contained the following (in mM): 115 K-gluconate, 20 KCl, 10 HEPES, 4 Mg-ATP, 0.5 Tris-GTP sodium salt hydrate and 10 Na-phosphocreatine (pH 7.2–7.3, 280–290 mOsm/L). Voltages were uncompensated and liquid junction potentials were left uncorrected. All whole-cell recordings were acquired at 2 kHz (sampled at 10 kHz) using an Axon Multiclamp 700B amplifier and Axon Digidata 1440A digitizer (Molecular Devices). R_m was obtained directly from Clampex 10.2 software, in response at 5 mV step. V_{Rest} was obtained with the Axon Multiclamp 700B set to zero-current mode ($I=0$). Synaptic currents were acquired using the cesium-based internal solution, and elicited by positioning a borosilicate stimulating pipette (3-5 MΩ, World Precision Instruments) into the middle third of the dentate gyrus molecular layer and producing currents from an Iso-Flex stimulus isolator controlled by a Master-8 pulse generator (both products from A.M.P.I). These experiments were conducted in the presence of bicuculline methiodide (20 μM; Tocris Bioscience).

For a subset of cells, two-photon imaging was performed to visualize cell morphology.. Imaging was conducted using a Ti:Sapphire pulsed laser tuned to 850 nm (MaiTai-DeepSee, Spectra Physics) coupled to a Zeiss LSM710 multiphoton microscope with a 20x (1.0 NA) objective.

All electrophysiological recordings were analyzed using Clampfit (Molecular Devices) and OriginPro 8.5 (OriginLab). Results were processed for statistical analysis using Excel (Microsoft), and OriginPro 8.5 statistical software. AMPA:NMDA ratio was calculated at +40 mV as previously described (Beique et al., 2006). Briefly, the AMPA current value was obtained at +40 mV, during the peak current time of the AMPA response at -70 mV, whereas the NMDA current value was obtained at +40 mV, at 3 x decay time constant of the AMPA current at -70 mV.

Statistics

All data are reported as mean $\pm$ S.E.M. and statistical analysis performed using GraphPad Prism (v6.0) software. Experiments with two groups were analyzed by the two-tailed *student's t-test*. Analyses of three or more groups were performed using an ANOVA test followed by a Tukey's *post hoc*. Statistical significance was defined as $P < 0.05$.

Results

Presenilin-2 (PS2) embryonic knockout mice exhibit no changes in adult hippocampal neurogenesis

PS1 and *PS2* have overlapping functions in the developing and in the adult brains (Wines-Samuelson and Shen, 2005). *PS2* KO mice, unlike *PS1* KO mice, survive to adulthood and allow for the assessment of adult hippocampal neurogenesis. To assess the proliferating cells

and newborn immature neurons in the dentate gyrus, brain sections from adult germline *PS2* KO mice were stained for Ki67 or doublecortin (DCX), respectively. Cell counts revealed the *PS2*^{KO} and *PS2*^{WT} mice had similar numbers of Ki67+ and DCX+ cells in the SGZ (**Fig 1a, b**). These results suggest *PS2* is not essential for adult hippocampal neurogenesis. Thus, for all subsequent experiments that required inducible ablation of *PS1*, the multi-transgenic mice were created by breeding with the embryonic *PS2* KO strain.

Figure 1.

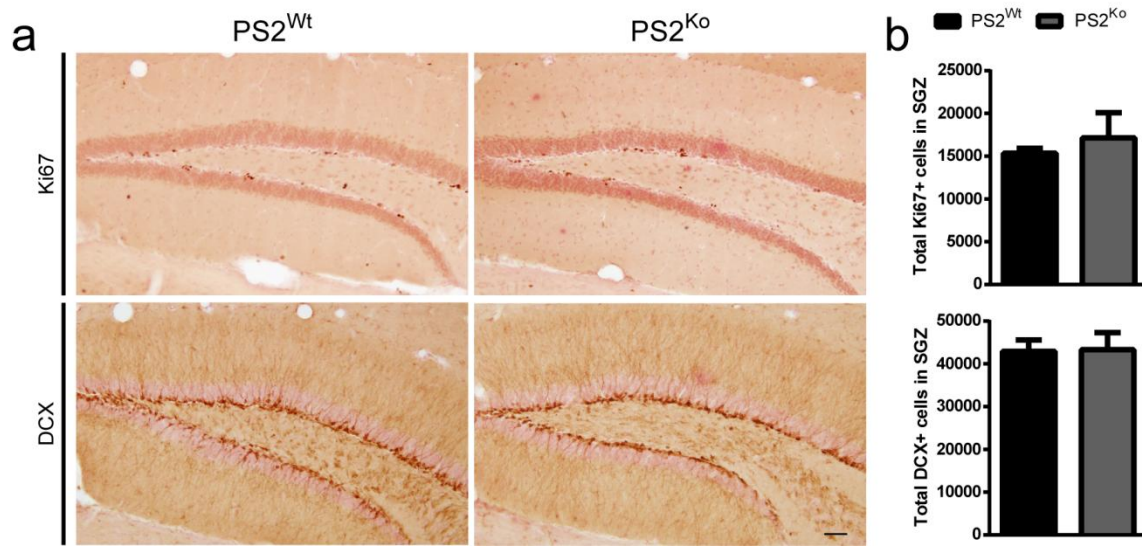


Figure 1. *PS2* knockout does not affect adult neurogenesis. a) Immunohistochemical staining of adult brain tissue from *PS2*^{Wt} and germline *PS2*^{Ko} mice showing dividing Ki67+ NPCs (top panel) and DCX+ neuroblasts & immature neurons (bottom panel). b) Total counts of Ki67+ and DCX+ cells in the SGZ of the dentate gyrus show no difference between the two groups (n= 8 mice).

***PS1* & *PS2* null NPCs differentiate into functional, mature granule neurons**

To track and fate map *PS1* & *PS2*-null NPCs, we conditionally ablated *PS1* from dividing NPCs using a retroviral approach in the dentate gyrus of *PS1^{fl/fl}* and *PS1^{WT}* mice that were kept on a *PS2* KO background (*PS1^{fl/fl}PS2^{KO}* & *PS1^{WT}PS2^{KO}*). Two retroviruses consisting of 1) nuclear GFPCre and 2) control RFP reporter were mixed 1:1 and injected in the dentate gyrus. This controlled for any variations in the site of injection and virus titer, as we and others have previously described (Tashiro et al., 2006; Jagasia et al., 2009; Xi et al., 2016). Survival was expressed as the fraction of double transduced (GFPCre+RFP+, yellow) cells over all RFP+ cells at both 12 days and 30 days post infection (dpi). In agreement with others, at 12 day and 30 days, virally infected cells are expected to display morphological characteristics of immature and mature neurons, respectively (Zhao et al., 2006). We observed the expected time-dependent decrease in the number of virally labeled cells in both genotypes (**supplementary 1**), however there was no change in cell survival between *PS1^{WT}PS2^{KO}* vs. *PS1^{fl/fl}PS2^{KO}* mice at both time points examined (**Fig 2a,b**).

Next, we asked if NPC- specific ablation of *PS1* would affect neuronal differentiation in the dentate. Surprisingly, phenotyping of the GFPCre+ cells for DCX and NeuN at 12 and 30 dpi, respectively, showed the majority of *PS1*& *PS2*-null cells becoming immature and mature neurons at levels comparable to the *PS1*WT cells (**Fig 3a-d**). In addition, morphological assessment of *PS1* & *PS2*-null cells showed similar level of dendritic complexity as *PS1*WT cells at 30dpi (**Fig 3 e,f**).

Figure 2.

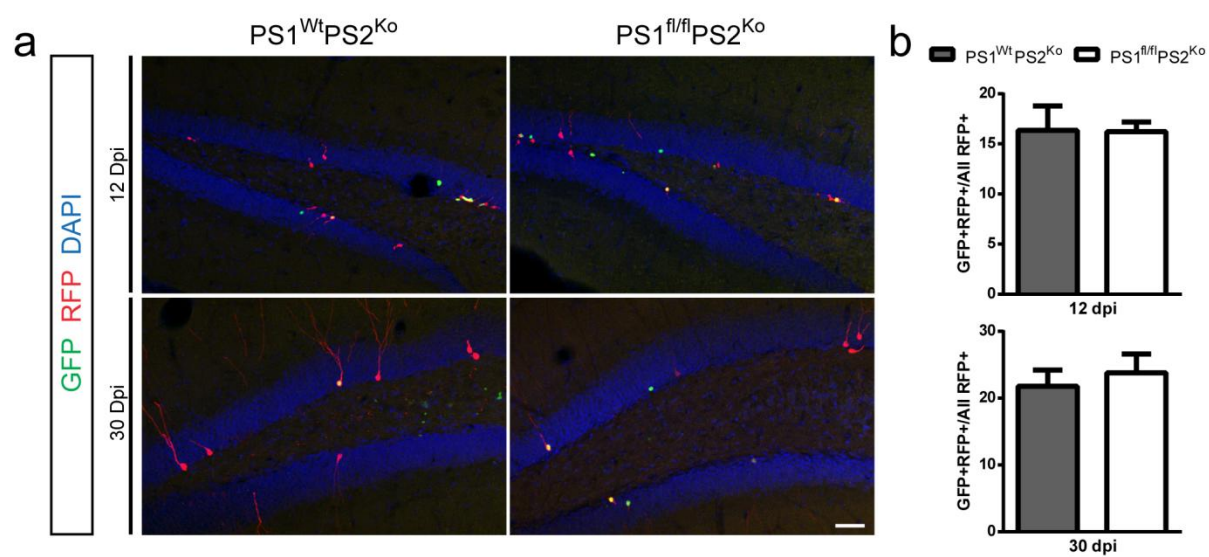


Figure 2. Retroviral ablation of *PS1* does not affect survival at 12 & 30dpi. a) Representative image of virus labeled cells showing GFPCre only (Green), RFP only (red) and GFPCre+RFP+ (yellow) double labeled NPCs at 12 and 30dpi. B) Quantification of survival shows no change in survival ratio (GFP+RFP+)/Total RFP+ at either 12 or 30DPI (n= 9 at 12dpi, n= 15 at 30dpi).

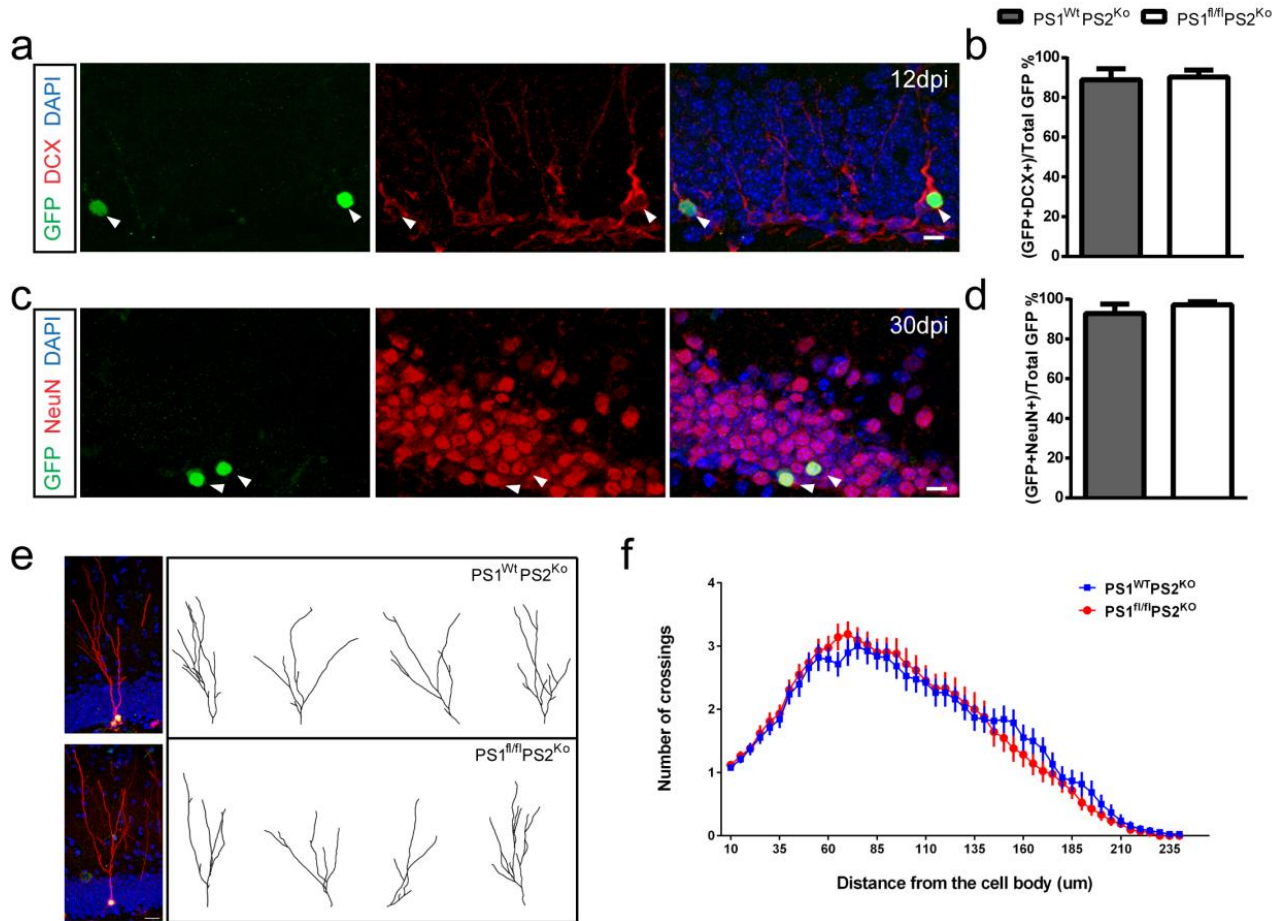


Figure 3. Retrovirally labeled NPCs can differentiate into mature granule neurons in the absence of *PS1* and *PS2*. a) Representative image of GFPCre+ cells also expressing DCX (arrowheads) at 12dpi. b) Quantification of the proportion of GFPCre+DCX+ NPCs among all GFPCre+ cells shows no difference at 12 DPI. c), Representative image of GFPCre+ cells that are also NeuN+ at 30DPI d)Quantification of the proportion of GFPCre+NeuN+ NPCs among all GFPCre+ cells shows nearly all GFPCre+ cells generated mature granule neurons. e) Sample projections of Z-series stack shown on left with dendritic traces on the right to illustrate the dendritic complexity of GFPCre+RFP+ (yellow) neurons at 30 DPI. f) Sholl analysis of dendritic complexity shows no difference between *PS1*wt and *PS1* & *PS2*-null cells.

Previous reports examining the CA3-CA1 regions of forebrain-specific *PS1* and *PS2* double knockout mice showed that presenilins modulate the function of NMDA receptors (Zhang et al., 2010). To investigate this function and to confirm if the virally labeled *PS1* & *PS2-null* cells were functionally integrating into the hippocampal circuit, we examined if there were cell autonomous alterations of NMDA receptors by performing whole-cell electrophysiology on *PS1* & *PS2-null* newborn neurons at 6-8 weeks post retroviral labeling in the dentate gyrus. AMPA:NMDA ratio revealed no difference between *PS1*-WT and *PS1* & *PS2-null* cells (**Fig 4c,d**). Additionally, *PS1* & *PS2-null* cells also demonstrated intact passive and active properties (**Fig 4b,e,f**). Together these results show NPCs can form functional neurons in the dentate gyrus in the absence of the presenilins.

Running induced neurogenesis occurs in *PS1* & *PS2* null NPCs

Exposure to enriched environment has previously shown to unmask the role of *PS1* in adult hippocampal neurogenesis in both *PS1*KO and mutant models (Feng et al., 2001; Choi et al., 2008). Thus, to determine if *PS1* served a cell-intrinsic function in enrichment induced neurogenesis, we allowed mice free access to either a running or a locked wheel in their homecage for 3 weeks and retrovirally ablated *PS1* at the end of week 1. Both *PS1^{fl/fl}PS2^{KO}* & *PS1^{WT}PS2^{KO}* ran similar distances (105201m±31875m vs. 93594m±16017m). Runners showed a significant increase in the number of virally labeled GFPCre+RFP+ cells, regardless of whether the cells were WT or KO for *PS1* ($p < 0.05$; **Fig 5a,b**). Furthermore, there was no change in the survival ratio between the two groups (**Fig 5c**). Together, these results show *PS1* is not an essential cell-intrinsic regulator of enrichment induced neurogenesis.

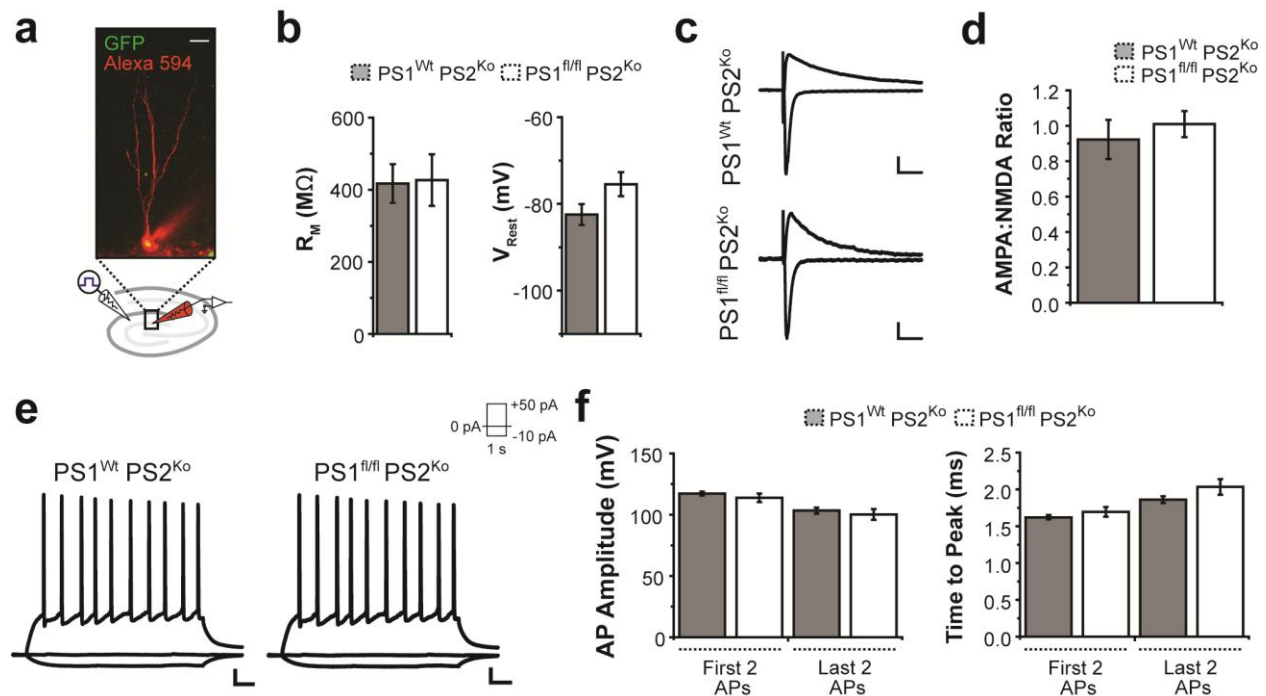


Figure 4 Electrophysiological properties of *PS1*-null cells. a) Representative two-photon image of a GFPCre+ cell filled with Alexa 594 dye. b) Membrane Resistance (R_M) and resting membrane potential (V_{Rest}) were similar between the two genotypes. c) Current traces of glutamate receptor mediated currents held at -70 and +40 mV. d) AMPAR:NMDAR ratios were similar between $PS1^{WT}PS2^{KO}$ and $PS1^{fl/fl}PS2^{KO}$ in seven-eight week old virus labeled cells. e) Voltage traces of action potentials in response to direct current injection. f) Action potential amplitude and the time to peak were comparable between the two genotypes.

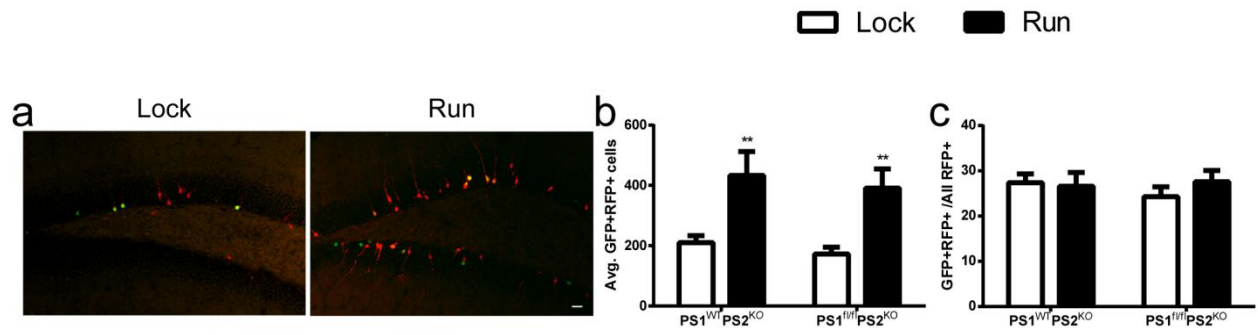


Figure 5. Running induced neurogenesis occurs in the absence of *PS1* and *PS2*. a) Representative image comparing the number of retrovirally labeled cells between locked wheel and running wheel conditions, b) Runners showed a significant increase in the number of virus infected RFP+ and GFP+ cells compared to non-runners (locked wheels) at 14 DPI for both groups. c) There was no significant change in the survival ratio. N= 18 mice lock wheels & 14 mice running wheel. Error bars represent $\pm$ SEM.

***PS1* is not essential for the development of Type-1 radial glia-like NSCs and their progeny**

In the developing brain presenilins are essential for stem cell maintenance as their knockout leads to the depletion of progenitor cell pool and premature neuronal differentiation (Kim and Shen, 2008). We questioned if ablation of *PS1* from type-1 radial glia-like NSCs and their progeny in the adult hippocampus could lead to a cell autonomous depletion of stem cells and reduced neurogenesis over time. To address this, we generated the nestinCreER^{T2}x RosaYFP-inducible *PS1* knockout mice on a germline *PS2* null background (*PS1nKO*) using a published nestinCreER^{T2} line (Lagace et al., 2007). This line was used since it shows higher expression specificity compared to some of the other NestinCreER^{T2} lines and unlike the retroviral ablation of *PS1* from dividing NPCs, this mouse allows for specific removal of *PS1* from all nestin-expressing stem cells and their progeny within the neurogenic niche (Lagace et al., 2007). (Sun et al., 2014).

Post-tamoxifen, both *PS1*WT and *PS1* & *PS2*-null YFP+ cells showed an accumulation over time, however there was no difference between the two experimental groups at either timepoint examined (**Fig 6a, b**). To determine the effect of *PS1* KO on the stem population, YFP+ cells were phenotyped for GFAP+ and Nestin+ expression that is normally found in the radial processes of NSCs (**Fig 7a arrowhead & orthogonal view in 7b**). Both *PS1*WT and *PS1nKO* mice showed a similar proportion of radial NSCs at 12 days post-tamoxifen (**Fig 7c**). There was no change in the proportion of Ki67+ proliferating (**Fig 7d, e**) and the proportion of DCX+ immature neurons at 12 days (**Fig 7f, g**) post-tam. Finally, *PS1* & *PS2*-null cells generated DCX+NeuN+ neurons at 30 days post-tam at similar proportion as *PS1*WT cells (**Fig 7h, i**). Together, these results show that, unlike in the developing brain, presenilins do not regulate neural stem cells and neurogenesis in the adult.

Figure 6

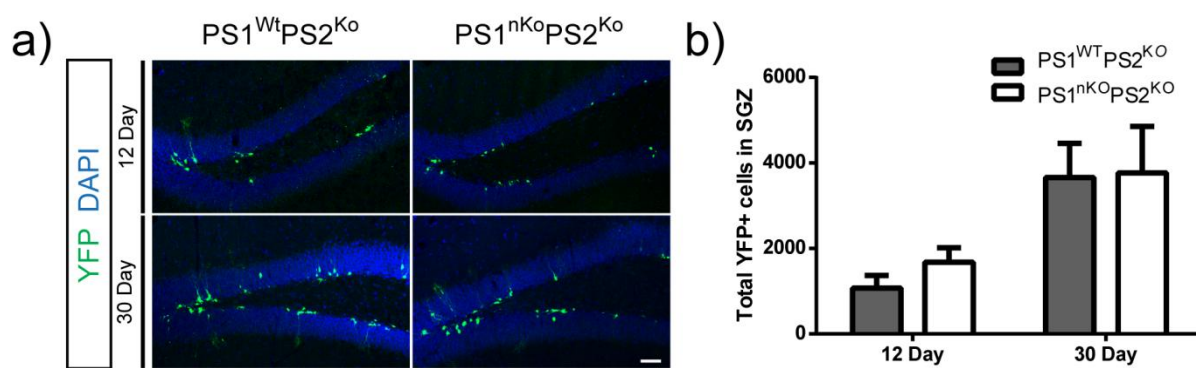


Figure 6. Ablating *PS1* from type 1 radial stem cells and progeny does not affect the total number of YFP+ progeny. a) Representative images comparing the number of YFP+ cells between the two genotypes at 12 and 30 days post-tamoxifen. b) Quantification of the total number of YFP+ cells shows an accumulation of recombined cells at 30 days post-tam, however there is no difference between the genotypes. N= 22 mice (12 day), 21 mice (30 day).

Figure 7

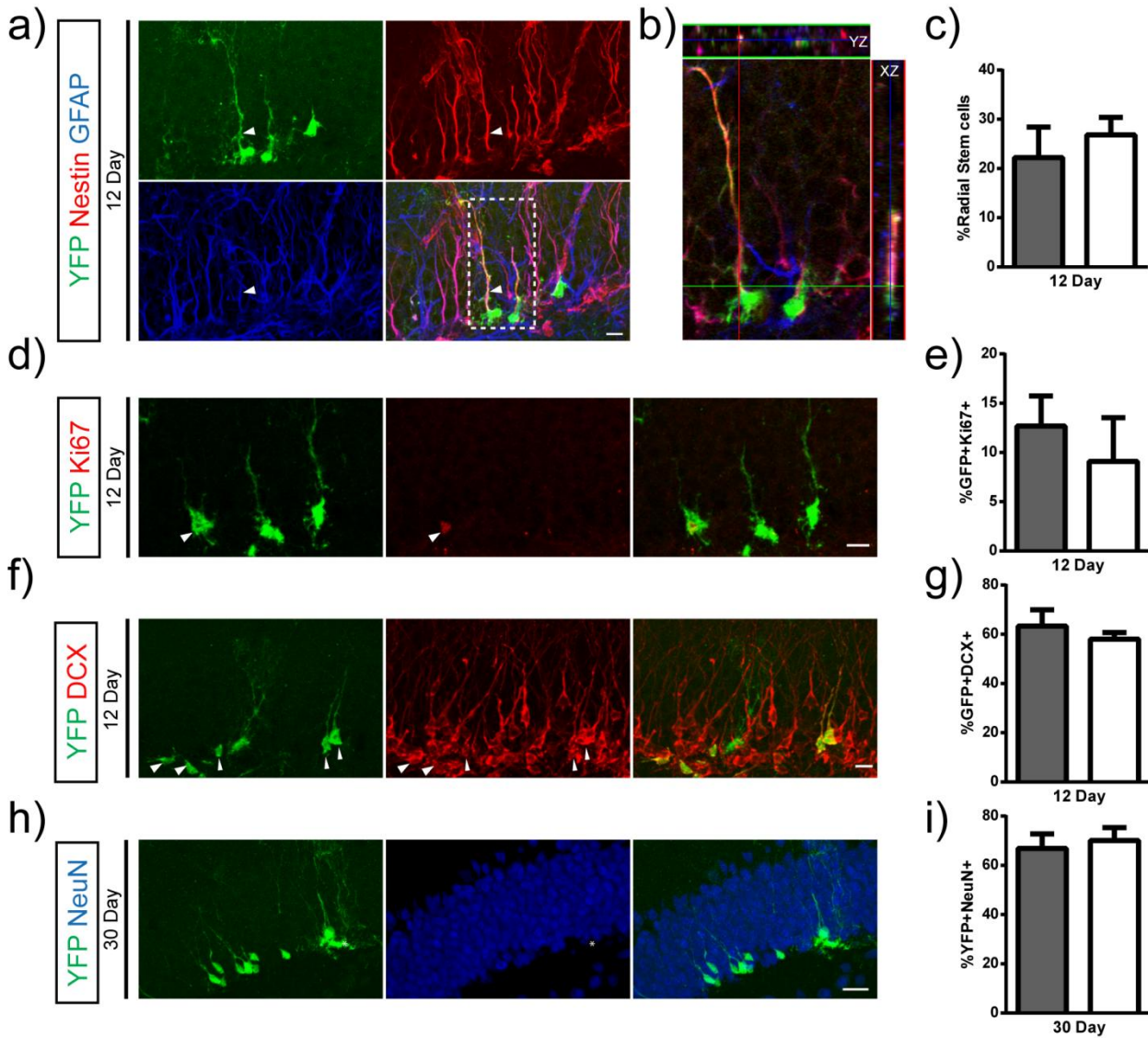


Figure 7. Ablating *PS1* from type 1 radial stem cells and progeny does not affect neurogenesis. a) Representative image of YFP+ cells expressing NSC markers Nestin and GFAP (arrowhead). A higher magnification, orthogonal view of the cells (dashed box) is shown in b). c) Quantification of proportion of YFP+Nestin+GFAP+ cells among all YFP+ population shows no change between genotypes. d) Representative image of YFP+ cells expressing cell division marker Ki67 (arrowhead). e) Quantification of proportion of YFP+Ki67+ cells among all YFP+ population shows no change between genotypes. f) Representative image of YFP+ cells expressing neuroblast and immature neuron marker DCX (arrowheads). g) Quantification of proportion of YFP+DCX+ cells among all YFP+ population shows no change between genotypes. h) Representative image of YFP+ cells expressing post-mitotic neuronal marker NeuN at 30 days post-tamoxifen. i) Quantification of proportion of YFP+NeuN+ cells among all YFP+ population shows no change between genotypes.

Discussion

In this study we ablated *PS1* exclusively in NSCs and in NPCs to investigate whether presenilins are critical for adult neurogenesis within the dentate gyrus. Unexpectedly, we did not detect any alteration in neurogenesis in any of our presenilin knockout models. This includes no changes in stem cell maintenance, proliferation and differentiation following inducible ablation of *PS1* from the nestin-expressing NSCs and their progeny. There was also no change in the survival, differentiation, and integration of adult-generated granule neurons after retroviral mediated removal of *PS1* from dividing NPCs. Additionally, removal of *PS1* did not affect running induced enhancement of neurogenesis. The failure to identify a phenotype in our *PS1* knockout models is not due to compensation by *PS2* since all of our experiments were completed on a *PS2*-null background using the germline *PS2* KO mice. Based on these findings, we conclude that the presenilins are not key mediators of adult hippocampal neurogenesis which argues against the hypothesis that compromised neurogenesis underlies the cognitive decline seen in FAD models associated with loss of presenilins.

Our findings also show the electrophysiological properties of *PS1* & *PS2*-null newborn, adult-generated neurons remain unmodified. Newborn NPCs mature and primarily receive excitatory input from the entorhinal cortex at 60 days post viral labeling (Vivar and van Praag, 2013). By stimulating the afferent connections coming from the entorhinal cortex and recording from *PS1* & *PS2*-null cells, we observed no change in the AMPA:NMDA ratio compared to *PS1*Wt cells, suggesting that post-synaptic ablation of *PS1* does not alter glutamatergic synaptic function in newborn cells. This is comparable to the role of presenilins in the CA3-CA1 network of the hippocampus where both cell-autonomous and trans-synaptic mechanisms regulate presenilin- dependent NMDAR functions (Zhang et al., 2010). Specifically, ablation of

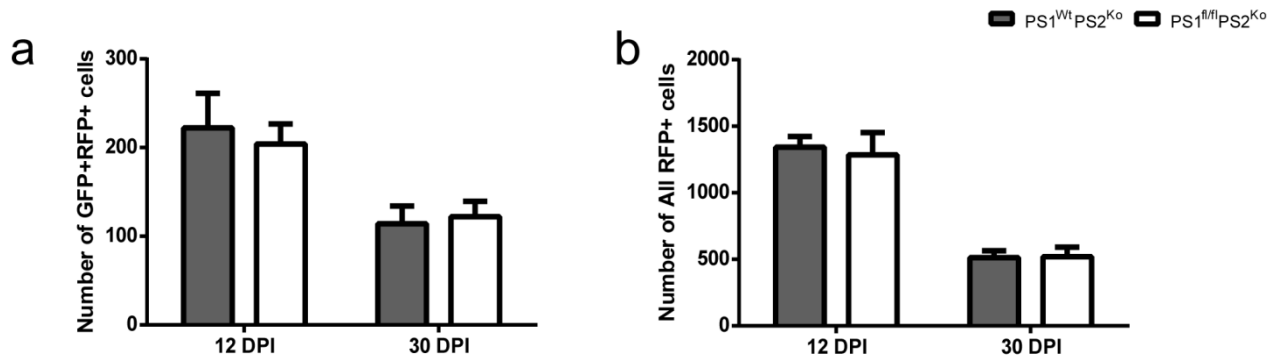
presenilins in either pre-synaptic CA3 or post-synaptic CA1 neurons alone is insufficient to produce NMDAR deficits, whereas both pre-and post-synaptic presenilin ablation leads to defects in NMDAR function. Thus, analogous mechanisms regulating presenilin-dependent NMDAR functions might be hypothesized in the entorhinal cortex- dentate gyrus network. In order to test this hypothesis future work would be required in which the effect of presynaptic ablation of *PS1* and *PS2* in the entorhinal cortex together with post-synaptic ablation in the newborn NPCs is assessed.

Our results strongly support that *PS1* and *PS2* are not cell intrinsic mediators of adult hippocampal neurogenesis. It is not surprising that *PS2* has no effect on neurogenesis alone, given that *PS2* does not have a significant role in performing presenilin functions. This is supported by evidence showing, for example, that lack of *PS2* does not alter processing of APP (Herreman et al., 1999) or germline *PS2* KO mice have no gross abnormalities in the developing brain (Donoviel et al., 1999) . Unlike *PS2*, the experiments showing no cell-intrinsic role of *PS1* are very surprising, given that *PS1* regulates Notch1 activity during early development and the Notch signaling pathway is critical for the maintenance of adult hippocampal NSCs (Ables et al., 2010). Our results argue against presenilins modulating Notch *in vivo* in the adult, thus our study adds presenilins to the growing list of regulators that have differential roles during embryonic versus adult neurogenesis (Urban and Guillemot, 2015; Gotz et al., 2016).

Our results provide evidence against the idea that loss of *PS1* contributes to dysfunction of adult neurogenesis which, in turn, impairs cognitive function in AD (Hollands et al., 2016). We show this by dissociating cell-intrinsic modulation of adult neurogenesis and the presenilins. Still, from this study, we cannot preclude the possibility that presenilins have a non-cell autonomous role in regulating neurogenesis. In support of this, *PS1* and *PS2* double knockout

mice (*Psen* cDKO) with presenilins inactivated specifically from postmitotic neurons, show increased cell density in the dentate gyrus that is likely associated with enhanced neurogenesis (Wines-Samuelson et al., 2010). Also, the neurogenesis deficits seen during enrichment in mice overexpressing *PS1* mutation in neuronal and non-neuronal cells, can be rescued if the mutant transgene is ablated from forebrain neurons, supporting a non-cell autonomous mechanism (Veeraraghavalu and Sisodia, 2013). Thus it maybe that *PS1* expression in cells surrounding the NSCs and NPCs in the niche could regulate neurogenesis. Therefore, it remains for future experiments to help identify the cell populations and mechanism responsible for a potential non cell autonomous role of *PS1* in adult neurogenesis by separately targeting either mature granule neurons or glial cells in the niche for *PS1* ablation.

Supplementary Material



Supplementary Figure 1. Total counts of retroviral labeled cells at 12 and 30dpi for both genotypes.

(a) Average number of GFP-Cre+RFP+ (yellow) and **(b)** all RFP+ (red+yellow) cells at 12 and 30 dpi of retroviruses (*CAG-GFP-Cre* and *CAG-RFP*) into the dentate gyrus of $PS1^{WT}PS2^{KO}$ & $PS1^{fl/fl}PS2^{KO}$ mice shows expected decrease at 30dpi.

CHAPTER 5: DISCUSSION

Summary of findings

The dynamic process of adult neurogenesis is orchestrated by a vast number of intrinsic and extrinsic regulators that function at different times during the lifespan of the NSCs that develop into adult-generated granule neurons in the hippocampus. This thesis examined three different potential regulators (*Dcx*, *Atg5*, *PS1* & *PS2*) at specific times during the process of adult neurogenesis. The results suggest that *Dcx* and *PS1* & *PS2* are not required for the development of adult generated hippocampal neurons, which is in contrast with their role during embryonic development. We also discovered that *Atg5* is a regulator for survival of adult generated cells, independent of controlling proliferation and differentiation of NPCs. In the following discussion I have individually summarized and further developed a common theme that can be extracted from these independent findings highlighting for example, the differential requirement of these genes in the embryonic versus the adult brain.

The lack of requirement for *Dcx* in adult hippocampal neurogenesis

Dcx has been considered a gold-standard marker for immature neurons in the field of adult neurogenesis, yet the intrinsic function of *Dcx* in the adult hippocampal NPCs and immature neurons has been elusive. Our work using three different genetic manipulation approaches produced the common finding that *Dcx* had no effect in modifying cell survival or differentiation for newborn granule neurons (Dhaliwal et al., 2016). Knockdown or complete ablation of *Dcx* using a retroviral strategy to alter NPCs did not change survival or affect differentiation. Additionally inducible ablation of *Dcx* in GLAST-expressing NSCs did not alter proliferation or survival of *Dcx*-null cells. Our results support the findings of others (Merz and Lie, 2013) and highlight the functional differences in region-specific requirements of DCX in the brain.

In the developing brain, *Dcx* is primarily associated with neuronal migration (Gleeson et al., 1999; Corbo et al., 2002). Similarly, in the adult forebrain, *Dcx* supports the migration of newborn NPCs generated in the SVZ that migrate a considerable distance along the RMS to reach the OB (Koizumi et al., 2006b; Belvindrah et al., 2011). One unique feature of neurogenesis in the DG is limited migration. NPCs migrate tangentially in direct contact with blood vessels and upon maturation extend radial dendritic processes and exhibit limited radial migration in the DG (Sun et al., 2015). Unlike in the adult forebrain, altered migration pattern of newborn cells in the DG does not seem to critically impact their function in the naïve brain. This, for example, has been shown following disruption of cyclin dependent kinase 5 (*Cdk5*) which induced abnormal migration in the absence of any changes in synaptic integration of newborn cells in the DG (Jessberger et al., 2008). Although we did not quantify the position of *Dcx*-null cells within the DG, subtle differences in spatial organization of *Dcx*-null cells in naïve condition are unlikely to have significant impact on overall functional integration.

Dcx was found not to be required for adult neurogenesis under naïve condition, but this does not exclude the possibility that *Dcx* could have a more significant role in neurological conditions associated with aberrant migration and integration of NPCs within the hippocampus. Mesial temporal lobe epilepsy (mTLE) is an intractable form of epilepsy where seizure activity arises from the hippocampus and other mesial temporal lobe structures (Liu et al., 2007). Notably, animal models of mTLE show a net increase in neurogenesis however, seizure induced newborn neurons ectopically migrate into the hilus and show aberrant integration into the dentate network (Jessberger and Parent, 2015). As a consequence of this ectopic migration and integration, mTLE is suggested to progress to the clinical syndrome of epilepsy (Jessberger and Parent, 2015). In fact, as few as 9% of aberrantly connected newborn granule neurons are

sufficient to induce spontaneous seizures and reducing these aberrant, seizure-generated neurons can reduce the frequency of spontaneous recurrent seizures (Jung et al., 2004; Pun et al., 2012; Jessberger and Parent, 2015). Given that *Dcx* can modify neuronal migration in the embryo and adult forebrain, a reasonable hypothesis would be that *Dcx* may cell-intrinsically regulate ectopic neuronal migration during mTLE in the adult DG. In this context, future experiments may consider reducing or ablating *Dcx* within seizure-generated NPCs in order to inhibit ectopic migration of these NPCs into the hilus. Reducing aberrant migration might also prevent aberrant integration thus, providing a potential therapeutic target of treating mTLE.

Different functions of presenilins in adult vs. embryonic neurogenesis

The absence of a neurogenic phenotype following cell intrinsic ablation of *PS1* & *PS2* was perhaps one of the more surprising results in this thesis. Under naïve condition, retroviral ablation of *PS1* did not affect NPC survival, differentiation or maturation in to granule neurons. *PS1* was also not essential for running induced enhancement of neurogenesis. Ablation of *PS1* from NSCs and their progeny using the NestinCreER^{T2} mice showed *PS1* was dispensable for NSC maintenance, proliferation and differentiation. Potential compensation by *PS2* was eliminated as a reason for these findings since knockout of *PS2* itself did not alter neurogenesis. Together these findings suggest adult hippocampal neurogenesis can occur in the absence of *PS1* & *PS2*. These findings thus adds to the growing body of literature suggesting profound differences in the mechanism of action of key proteins shared in neurogenesis at the embryonic and adult stages (Gotz et al., 2016).

The lack of the phenotype after ablation of *PS1* & *PS2* was surprising due to their striking role in the developing brain where loss of *PS1* leads to a significant depletion of the NSC/NPC

pool due to early exit from cell cycle and premature differentiation into neurons (Kim and Shen, 2008). The primary culprit behind this phenotype is described to be a blockade of Notch signaling (Handler et al., 2000). Notch is one of the substrates of gamma-secretase cleavage of which PS1 is an active component, and is required for maintenance of embryonic neural stem cells (Hitoshi et al., 2002). Functional analysis of Notch1 and the Notch-pathway genes in adult NSCs have revealed ablation of Notch1 or its downstream transcriptional effectors such as RBPJ κ deplete the NSC pool and suppress hippocampal neurogenesis, similar to its effects in the embryo (Ables et al., 2010; Ehm et al., 2010; Lugert et al., 2010). We found that the adult NSC population after ablation of *PS1* & *PS2*, was not modified, which given the known effects of Notch signaling in adult neurogenesis, suggests that within the adult NSCs there is a presenilin-independent notch activation pathway (Berechid et al., 2002). An alternative explanation to why ablation of *PS1* & *PS2* had no impact on adult NSCs could be due to the combination of presenilin interactions with Notch and the Wnt/ β -catenin pathway. It is known that *PS1* possesses gamma-secretase-independent activities which includes *PS1* being a negative regulator of the Wnt/ β -catenin pathway (Soriano et al., 2001). Canonical Wnt signaling is known to stimulate NSC proliferation and self-renewal and regulate hippocampal neurogenesis (Lie et al., 2005; Qu et al., 2010). Thus, a combination of reduced Notch signaling and active Wnt/ β -catenin could counteract each other producing no net change in overall adult neurogenesis with *PS1* & *PS2* ablation. This hypothesis could be dissected in the future through analysis of the NSC to determine the relative contribution of gamma-secretase-dependent and independent functions of the presenilins. Lastly, different requirement of *PS1* & *PS2* in embryonic versus adult may also be attributed to the inherent differences between embryonic and adult NSCs. One of the differences lies in the rate of proliferation. For example, it can be argued that embryonic

NSCs are more similar to adult NPCs rather than adult NSCs, since both embryonic NSCs and adult NPCs have a fairly similar, short cell cycle ranging between 10-18 hours and 22-27 hours, respectively indicating rapid cell divisions (Lange and Calegari, 2010; Brandt et al., 2012).

In contrast, the majority of adult NSCs in the hippocampus are largely quiescent and contribute to long-term neurogenesis by self-renewing and transitioning between quiescent and activated states (Bonaguidi et al., 2011). Thus, cell-intrinsic regulation by *PS1* & *PS2* might be critical during early developmental period of corticogenesis when cells constituting the different cortical layers need to be generated, whereas in the adult NSCs *PS1* & *PS2* might be dispensable for the generation of NPCs.

Our results showing no change in adult neurogenesis when presenilins are removed, combined with the known loss of *PS1* function in FAD-linked *PS1* mutations, argue against the hypothesis that *PS1*-mediated decline in adult hippocampal neurogenesis is an underlying cause of cognitive decline for FAD. Indeed many of the changes in adult neurogenesis reported in different *PS1*-mutant models are correlative in nature (van Tijn et al., 2011). More recent evidence suggests *PS1* mutations affect neurogenesis through cell non-autonomous mechanisms (Veeraraghavalu and Sisodia, 2013). Since our study only examined cell-intrinsic regulation, it remains to be determined if removal of presenilins from mature neurons or glial cells can induce a decline in neurogenesis.

The rationale for studying presenilins and adult neurogenesis is based on the hope to develop new treatments to reduce cognitive decline associated with AD, most notably for patients that have FAD-*PS1* mutations. The question is whether this approach is going to be successful. It is well known that FAD-linked *PS1* mutations cause complete loss of *PS1* function and gamma-secretase activity *in vivo* and affect two parallel pathways (Xia et al., 2015). On one

end, FAD-*PS1* mutations cause synaptic dysfunction, memory impairment and age-dependent neurodegeneration and in a parallel pathway, loss of *PS1* function promotes amyloid pathology by inhibiting gamma-secretase activity (Xia et al., 2015). Thus, both of these pathways converge leading to the development of FAD. Thus single target interventions aimed at, for example, delaying cognitive decline by enhancing neurogenesis may not be effective. Future approaches of drug development to treat FAD may benefit more from restoring *PS1* function and gamma-secretase activity.

Autophagy: An essential regulator for survival

Perhaps the most exciting findings in this thesis is the *in vivo* discovery of autophagy being a regulator of adult NPC survival (Chapter 3). This study used retroviruses to target and genetically manipulate autophagy in the dividing NPCs, however the question remains as to what function might autophagy serve in the adult NSCs? Clues on the role of autophagy and how autophagy related genes can modulate adult neurogenesis have recently started to emerge. For instance, different groups have generated conditional knockout or mutant mice targeting specific genes in the autophagy pathway to examine impact on neurogenesis (Wang et al., 2013; Wang et al., 2016; Wu et al., 2016). In addition, genetic and pharmacological manipulations *in vitro* using adult NSCs isolated from the dentate gyrus have also shed light on mechanisms regulating NSCs, autophagy and neurogenesis (Yu et al., 2008; Chung et al., 2015; Ha et al., 2015) . Here I discuss some of these recent findings by comparing the effect of embryonic versus adult specific deletion of autophagy genes on adult neurogenesis.

Effects of embryonic disruption of autophagy on adult neurogenesis

Wang et al. (2013) was the first to examine the effects of autophagy inhibition on adult neurogenesis using their *FIP200^{fl/fl};GFAP-Cre* (designated *FIP200^{GFAP}* cKO) mouse. In this mouse, the human GFAP promoter drives the expression of Cre recombinase to knockout the autophagy gene *FIP200* in radial glia cells beginning around embryonic day 10.5 (Zhu et al., 2005). The focal adhesion kinase family interacting protein of 200kD (*FIP200*), functions as a component of the multiprotein complex ULK1/Atg13/FIP200/Atg101 required for induction of autophagy in mammalian cells (Hara et al., 2008). The *FIP200^{GFAP}* cKO had a dramatic phenotype including a robust reduction in self-renewing NSCs, NPCs, neuroblasts and adult generated neurons in the olfactory bulb and the dentate gyrus, accompanied by increased apoptosis and astrogenesis.

Ablation of *FIP200* not only inhibited autophagy but also resulted in an accumulation of mitochondria and an increase in levels of reactive oxygen species (ROS) in NSCs. This work was followed by the discovery that inhibiting the elongation phase of the autophagy pathway by ablating *Atg5* or *Atg16L1* using the same GFAP-Cre conditional mice which resulted in very interesting differences on adult neurogenesis (Wang et al., 2016). Similar to the removal of *FIP200*, loss of *Atg5* and *Atg16L1* induced an increase in mitochondrial mass and ROS yet had no effect on adult NSC maintenance, proliferation and differentiation. This difference could be attributed to the significantly higher accumulation of the adaptor protein p62 in *FIP200*-null versus *Atg5* or *Atg16L1*- null cells, that ultimately resulted in superoxide $O_2^{\bullet-}$ accumulation and neurogenesis inhibition. These results support the idea that autophagy helps mitigate cellular stress by means of recycling damaged organelles and proteins as seen in other cell types and during tumorigenesis (Liu et al., 2010; Altman et al., 2011).

In addition to regulating ROS in NSCs, there is also evidence that autophagy genes might directly control pathways involved in adult NSC maintenance, proliferation and differentiation. In support of this, transgenic mice with a hypomorphic mutation in *Atg16L1* show autophagy impairment along with deficits in Notch degradation (Wu et al., 2015). The consequence of hyperactive Notch signaling in these mice is a significant, yet reversible, reduction in proliferation in the adult SVZ. Although it is not known what cells in the SVZ were affected by *Atg16L1* hypomorphic mutation, it is likely active Notch signaling led to repression of proneural gene expression and maintenance of NSCs, as previously described for Notch signaling (Imayoshi et al., 2010). Thus, overall, these few transgenic models suggest that the outcome of modifying autophagy within the embryo on adult neurogenesis is dependent upon the autophagy gene in question, as well as the type of manipulation (conditional knockout versus germline mutation) being made.

Requirement of autophagy in adultborn NSCs

To date, our published work is the only study that has specifically manipulated autophagy *in vivo* in the adult NPCs, independent of embryonic development, and demonstrated the critical role of *Atg5* on adult neurogenesis (Xi et al., 2016). The challenging task of investigating autophagic flux *in vivo* in adult NSCs, how it changes at the NSC-NPC transition and the overall requirement of autophagy in adult-born NSCs remain to be determined. Some hints to address these questions may be derived from few studies that have utilized the *in vitro* adult rat hippocampal neural stem cells (HCN) and established the function of autophagy in context of cell death in this *in vitro* model (Yu et al., 2008; Chung et al., 2015; Ha et al., 2015). In particular, when the cells were cultured under conditions of insulin withdrawal, HCNs underwent

autophagic cell death (ACD) instead of apoptosis (Yu et al., 2008). Importantly, knockdown of *Atg7* suppressed ACD under these conditions. This work was followed by three separate studies using similar growth conditions that showed indirect modulation of autophagy could affect cell death in HCNs (Chung et al., 2015; Ha et al., 2015; Chung et al., 2016). One study identified glycogen synthase kinase 3 β (GSK-3 β) as a positive regulator of autophagic flux and ACD, with knockdown of *Atg7* reversing GSK-3 β induced ACD (Ha et al., 2015). The second study identified calpains as a potential switch between ACD and apoptosis in HCNs, with ACD being enhanced upon inhibiting Calpain 2 and reduced following ectopically expressing Calpain1 (Chung et al., 2015). Finally, Chung et al.,(2016) described the Type 3 Ryanodine Receptor (RyR3) as a mediator of ACD in HCNs with RyR agonist treatment enhancing ACD, whereas this effect could be blocked by *Atg7* knockdown. Together these *in vitro* studies suggest autophagy can be described as a quality control mechanism in the adult HCNs with ACD being a means of eliminating dysfunctional HCNs, for example when insulin signaling is impaired (Hong et al., 2016).

As with other *in vitro* model systems such as neurosphere cultures, one concern in using HCNs is whether they truly model *in vivo* adult NSCs. This is a limitation to the use of the HCN model since these cells in culture can generate all three lineages including oligodendrocytes (Palmer et al., 1997) whereas hippocampal NSCs *in vivo* have a latent tri-lineage potential and do not generate oligodendrocytes under naïve condition unless certain lineage barriers are removed (Bonaguidi et al., 2011; Sun et al., 2015). Thus, there is a need for targeting adult NSCs *in vivo* to determine the requirement for autophagy and autophagy associated genes. As described earlier, adult hippocampal NSCs are heterogeneous ranging from mostly quiescent but also dividing populations. This heterogeneity might reflect distinct populations or different states

of the same hippocampal NSC population (Bond et al., 2015). Thus, future experiments would need to address if and how might autophagy maintain or promote transition between quiescent and activated NSCs *in vivo*. This challenging task could benefit from the use of *in vivo* clonal labeling of adult hippocampal NSCs in order to determine the impact of ablating genes linked with different stages of the autophagy pathway.

Concluding Remarks

Several insights are provided by the studies presented in this thesis. First, genes common to both neurogenic regions in the adult brain can show regional differences in their function, as demonstrated by the progression of hippocampal neurogenesis in the absence of *Dcx* (Dhaliwal et al., 2016) . Second, the role of disease associated genes expressed during embryonic and adult neurogenesis can drastically differ between the two developmental stages, as seen by the lack of cell intrinsic requirement of the Alzheimer's associated *PS1* in adult NSCs and NPCs (Chapter 4). Lastly, autophagy can be an essential regulator of survival during adult neurogenesis, as demonstrated by the requirement of *Atg5* in the development of adult NPCs (Xi et al., 2016). The latter findings have additional implications due to the number of autophagy-enhancing compounds that are being tested for their efficacy for the treatment of neurodegenerative diseases (Nah et al., 2015). These therapeutic manipulations for autophagy induction are backed by pre-clinical studies showing for example, enhancing autophagy can increase the lifespan of the organism (Pyo et al., 2013) as well as provide neuroprotection from α -synuclein toxicity (Decressac et al., 2013). Thus, autophagy inducers designed to treat neurodegenerative diseases may provide the additional benefit of enhancing adult neurogenesis and provide improved cognitive processing that could provide an alternate or additional means for endogenous repair.

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