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CHARECTERISATION OF NEWT NEURAL STEM CELLS DURING DEVELOPMENT AND REGENERATION

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Characterisation of newt neural stem cells during development and regeneration

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ABSTRACT

The adult newt brain has a unique potential to regenerate neurons after injury. Ependymoglial cells that line the ventricular system of the newt brain are critical for neuronal regeneration, since they reenter the cell cycle upon injury and differentiate into neurons. Ependymoglial cells share key features with radial glial cells in mammals. In contrast to mammals, where the majority of radial glial cells disappear during development, the adult newts retain ependymoglial cells. This thesis aimed to characterise ependymoglial cells under homeostatic conditions and after injury, both during development as well as in the adult animal.

In Paper I we characterised ependymoglial cells during development in two newt species, *Notophthalmus viridescens* and *Pleurodeles waltl*. Here we describe the ependymoglia maturation as regards to their proliferation pattern and gene expression profile. Moreover, we correlate the cell cycle length, and exit from the proliferative state to brain maturation and to the acquisition of complex behaviours. The findings also suggest that early cell cycle exit is essential for the persistent presence of ependymoglial cells in adulthood.

In Paper II we evaluated adult newt ependymoglial cells in normal homeostasis and during regeneration following ablation of cholinergic neurons in the forebrain. We find that ependymoglial cells are not a homogenous cell population. Despite their morphological homogeneity, gene expression profile identifies subpopulations among ependymoglial cells. The majority of ependymoglial cells are fast-dividing cells in homeostatically proliferating hotspots, whereas, proliferating ependymoglia in quiescent areas are slowly cycling cells with stem cell features. Neuronal ablation altered the fate of ependymoglial cells, and neurogenic niches with neuroblasts in normally non-germinal regions were created. This study identifies processes of both homeostatic as well as injury-induced neurogenesis in the adult newt brain.

In Paper III we assessed how the production of reactive oxygen species impacts the brain during normal and regenerative neurogenesis. By manipulating environmental oxygen availability, we find that newts could cope with hypoxia and subsequent re-oxygenation. The shifts in environmental oxygen concentration causes initial neuronal loss and subsequent increase in neurogenesis, which is dependent on the production of reactive oxygen species. Also, we find that neuronal regeneration in the homeostatically quiescent midbrain is dependent on the production of reactive oxygen species during constant normoxia. Altogether the data assign a key role to reactive oxygen species in adult neurogenesis in newts and suggests that naturally occurring environmental changes in oxygen concentration might be an evolutionary driving force to replace lost neurons in newts.

LIST OF SCIENTIFIC PAPERS

I. Alberto Joven, Heng Wang, Tiago Pinheiro, **L Shahul Hameed**, Laure Belnoue, and Andras Simon

Cellular basis of brain maturation and the acquisition of complex behaviors in salamanders
Development, 2018, 145: dev160051. doi: 10.1242/dev.160051

II. Matthew Kirkham, **L Shahul Hameed**, Daniel A. Berg, Heng Wang, and Andras Simon

Progenitor Cell Dynamics in the Newt Telencephalon during Homeostasis and Neuronal Regeneration
Stem Cell Reports, 2014, Vol.2, 1-13.
http://dx.doi.org/10.1016/j.stemcr.2014.01.018

III. **L Shahul Hameed**, Daniel A.Berg, Laure Belnoue, Lasse D Jensen, Yihai Cao, and Andras Simon

Environmental changes in oxygen tension reveal ROS-dependent neurogenesis and regeneration in the adult newt brain
eLife 2015;4; e08422. doi: 10.7554/eLife.08422

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LIST OF ABBREVIATIONS

3-AP	3-acetyl pyridine
6-OHDA	6-hydroxydopamine
AF64A	Ethylcholine mustard aziridinium ion
BrdU	Bromo-deoxyuridine
BMP	Bone morphogenetic protein
CNS	Central nervous system
EdU	5-ethynyl-2'-deoxyuridine
FGF	Fibroblast growth factor
GS	Glutamine synthetase
GFAP	Glial fibrillary acidic protein
H_20_2	Hydrogen peroxide
HEt	Hydroethidine
HSCs	Hematopoietic stem cells
HVC	High vocal centre
MSCs	Mesenchymal stem cells
NADPH	Nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidase
NSCs	Neural stem cells
RGCs	Radial glial cells
ROS	Reactive oxygen species
SGZ	Subgranular zone
Shh	Sonic hedgehog
SVZ	Subventricular zone

1 REGENERATION AND NEWTS

1.1 INTRODUCTION

The long-term aspiration of regenerative medicine is to stimulate mechanisms in humans that could lead to the functional repair and/or replacement of lost or damaged tissues and organs. This aspiration also includes the treatment of a number of age-related neurodegenerative disorders which affect our normal daily life such as Parkinson's and Alzheimer's disease. Current therapies available for neurodegenerative disorders may improve the quality of life but do not cure the disease. There is an increase in the number of cases of neurodegenerative disorders, especially in developed countries, which creates both social and economic burden and highlights the need for novel therapies. Interestingly, within the vertebrates, certain taxa, especially urodele amphibians (salamanders), which includes newts shows wide-spread regenerative capabilities (Goss, 1969; Tanaka, 2016; Tsonis et al., 2004). Newts have been extensively studied for their regenerative potential, including their central nervous system (Minelli et al., 1987; Okamoto et al., 2007).

Previously, studies from our laboratory showed that in contrast to mammals, newts are able to regenerate dopamine neurons in the adult midbrain (Berg et al., 2010). Midbrain dopamine neurons are particularly interesting because their degeneration is the major hallmark of Parkinson's disease (Barzilai and Melamed, 2003; Surmeier et al., 2010). Dopamine regeneration in newts proceeds by quiescent ependymoglial cells reentering the cell-cycle as a response to neuronal ablation (Berg et al., 2010).

Notably, after completion of the regenerative events, these ependymoglial cells return to quiescence (Berg et al., 2011). Interestingly, under certain disease conditions mammalian neural stem cells (NSCs) also respond to injury by activation but this process does not lead to the production of a significant number and functional integration of new neurons (Dibajnia and Morshead, 2013). It is important however to point out that evolutionary similarities between newt ependymoglial cells and mammalian NSCs do exist. For example, based on findings in newts, which showed that proliferation of midbrain ependymoglial cells was under the control of dopamine signalling, our lab was able to increase dopaminergic neurogenesis in mice (Hedlund et al., 2016). These data support the view that studies on newts could provide clues on how to manipulate the mammalian brain to improve recovery in neurodegenerative disorders.

Since newt ependymoglial cells play a central role in neuronal regeneration, it is important to gain a mechanistic understanding of their unique regenerative potential. Therefore, this thesis focuses on the detailed characterisation of ependymoglial cells in newts both in the adult brain as well as during their maturation in a developmental context. Understanding the developmental origin of ependymoglial cells, how they mature, acquire quiescence and comparing them to their mammalian counterparts might reveal critical interspecies differences.

In this thesis, efforts were made for a detailed characterisation of ependymoglial cells from their developmental origin to adult stage to understand their unique nature. In the introductory part of this thesis, I give a general overview of the field of regeneration biology with emphasis on regenerative ability in salamanders. The maturation of neural progenitors from embryonic to adulthood and adult NSCs potential to respond to injury are discussed in the following section. Third, I discuss the role of reactive oxygen species in neurogenesis, including evolutionary considerations. In the last part, I summarise the findings of the papers included in this thesis.

1.2 HISTORICAL OVERVIEW OF REGENERATION

The concept of regeneration has fascinated scientists for centuries. One of the earliest dated accounts on regeneration originates from Greek mythology, where Prometheus - the half god half man- was punished by Zeus for disobeying God's order and giving fire to humanity, an act of disobedience. Prometheus was chained to a rock and an eagle pecked his liver every day. The lost part of his liver grew back every night. Myths are bit exaggerated, and in reality, it is not possible to regenerate the liver overnight. However, scientific evidence has proven that human liver can partially regenerate (Chen et al., 1991). Another example from mythology comes from the tale of three hags in the legend of Mercury, where the concept of eye regeneration was coined. Hags had only one eye among them and, if another hag wanted to see, the eyeball had to be passed to the other hags orbit. The story is indeed mythical, but experimental manipulation showed newts have remarkable ability to regenerate the lens repeatedly without any sign of age-related decline (Eguchi et al., 2011; Tsonis et al., 2004). Apart from the Greek mythological beliefs, first scientific discoveries of regeneration were documented by Aristotle (384-322BC), in his book "The history of Animals", he mentioned about the tail regeneration of lizards, however, until the 18th century, there was no scientific report about the regeneration abilities in animals.

The first report on regeneration based on experimental evidence dates back to 1712, when the French scientist Rene-Antoine Ferchault de Reaumur (1683-1757), published a paper about the regeneration of the legs of freshwater crayfish (Dinsmore, 1991). Later that century, another breakthrough occurred in the field of regenerative biology. Abraham Trembley (1710-1784), a Swiss naturalist, discovered regeneration in the polyp, hydra. When he first looked at the polyp, he was curious whether it was an animal or a plant. When he noted that it has step-by-step movement, he predicted it to be an animal. His curiosity for regeneration emerged when he noticed that not all polyps have a similar number of arms. Trembley coined the term hydra after cutting the polyps repeatedly and observed seven-headed polyps, which looked like a monster, the Hydra in from Greek mythology (Dinsmore, 1991).

The earliest studies on regeneration of vertebrates were performed by the Italian scientist Lazzaro Spallanzani (1729-1799). In 1768, he studied pre-metamorphic frogs and toads and demonstrated that they could regenerate the tail. Spallanzani was also the first to describe regeneration of limbs in salamanders after amputation (Tsonis and Fox, 2009). He documented the appearance of a small round stump at the injury site, a structure that subsequently was denoted the blastema and shown to be critical for limb regeneration (Stocum, 1968). Spallanzani had also recorded tail regeneration in the newts.

The discovery that certain adult vertebrates can regenerate large body parts laid a foundation of several studies on the regenerative abilities in adult vertebrate species, and also led to speculations on why only certain species have regenerative potential. Thomas Hunt Morgan (1866-1945), a renowned geneticist and August Weismann (1834-1914) known for his famous 'germ plasm theory', had different views on animal regeneration. Weissmann believed that regeneration is adapted to species, and organs which are prone to injury have evolved a regenerative potential independently (Esposito, 2013). However, Morgan was against this theory; he argued that if regeneration occurs in species that are prone to injury, then how about species/organs which are not prone to injury? Morgan tried to explain this theory by amputating salamander and crab legs, where no natural injury occurred and proved they do regenerate (Morgan, 1901). He considered that regeneration is innate during evolution, which has been lost in most species. Even today, there is an on-going debate about whether the regenerative ability is inherited or adapted. I will discuss this view in detail towards the concluding chapter of the thesis.

1.3 TYPES OF REGENERATION

In his book *Regeneration* in 1901, T.H Morgan divided regeneration processes into two categories: Morphallaxis and Epimorphosis based on whether regeneration required proliferating cells or not (Sunderland, et al., 2009).

In morphallaxis, where regeneration does not require proliferating cells, animals can regenerate by remodelling of existing tissues. From his studies on planarian regeneration Morgan concluded that planarian regeneration occurs by tissue remodelling (Morgan, 1898). He came to this conclusion after monitoring how planarians regenerated from $1/279^{th}$ of tissue. However, recent evidence indicates that planarian regeneration occurs by stem cells called neoblasts, which are the only proliferating cells in planarians (Reddien and Alvarado, 2004; Wagner et al., 2011). Apart from planarian, hydra regeneration was also thought to be mediated by tissue re-organisation. Inhibiting DNA synthesis via hydroxyurea showed that regeneration was independent of mitosis (Cummings and Bode, 1984). However, recent evidence shows that dividing cells, in a structure, which looks like a proliferating blastema is important for hydra regeneration (Chera et al., 2009). Therefore, species thought to regenerate by morphallaxis still require proliferating cells for their regeneration. Morphallaxis is an old term, and currently no known species regenerate without the requirement of proliferating cells.

The requirement of proliferating cells has been lately demonstrated in hydra and planarians as well as among different vertebrates including newts. Among the animals examined so far for their regenerative mechanisms, regeneration occurs through dedifferentiation and transdifferentiation of matured somatic cells as well as by activation of resident stem cells in the adult tissues.

Transdifferentiation is the process where a matured cell type is converted to another cell type without any intermediate stage. In the context of regeneration, transdifferentiation has been conclusively demonstrated during lens regeneration in newts. In newts, only the dorsal iris retains regenerative potential, and upon lens removal, pigment epithelial cells change morphology, proliferate and transdifferentiate into lentoid bodies to form the new lens (Okada, 1991). However, transdifferentiation is not a predominant source of regeneration in other tissues.

During dedifferentiation, the terminally differentiated cells lose their characteristics and acquire an intermediate stage before their redifferentiation. Newt limb regeneration is a typical example of dedifferentiation. Upon injury, the multinucleate muscle fibres, fragment to produce mononucleate cells, which in turn proliferate and contribute to blastema

formation, which leads to regeneration of the limb (Echeverri et al., 2001; Lo et al., 1993; Wang and Simon, 2016). Apart from newts, zebrafish regenerate their heart upon injury, and recent experiments demonstrate that this event is also mediated by dedifferentiation of cardiomyocytes. Lineage tracing of differentiated cardiomyocytes indicates that after injury cardiomyocytes dedifferentiate and reenter the cell cycle to contribute to regeneration (Jopling et al., 2010). The regenerative potential exists in the neonatal mouse heart, and lineage tracing studies indicate that cardiac myocytes contribute to regeneration by dedifferentiation (Porrello et al., 2011). Adult mice, on the other hand, cannot regenerate heart tissue.

Proliferating adult stem cells in organisms also contribute to regeneration. Neoblasts in adult planarians can generate all major cell types needed for regeneration upon injury. If neoblasts are depleted by irradiation, planaria will eventually die, but transplantation of single neoblasts to an irradiated host is sufficient for their regeneration (Wagner et al., 2011). Newt limb regeneration also involves stem cells exemplified by activation of skeletal muscle satellite cells, which reenter the cell cycle and contribute to functional regeneration after injury (Morrison et al., 2006). Zebrafish and newt brain regeneration is also mediated by activation of stem cells present in the brain (Berg et al., 2011; Kizil et al., 2012).

From our current understanding of all organisms with regenerative potential, it appears that there is a pre-requirement of proliferating cells to regenerate and replace damaged tissues. Interestingly, there are species that retain proliferating adult stem cells with a restricted regeneration ability (Alunni and Bally-Cuif, 2016), indicating additional regulatory processes that either promote regeneration in regeneration-competent species or counteract it in regeneration-incompetent species.

1.4 LIFE HISTORY OF NEWTS

Newts belong to the urodele amphibians, also called salamanders. These amphibians retain their tail after metamorphosis. Among the newt species, Japanese fire-bellied newts (*Cynops pyrrhogaster)*, red-spotted newts (*Notophthalmus viridescens*) and Iberian ribbed newts *(Pleurodeles waltl*) are the most commonly used newts in regeneration research (Berg et al., 2011; Hayashi et al., 2013; Ueda et al., 2005; Zaky et al., 2015). Red-spotted newts and Iberian ribbed newts are used in the current study hence I discuss their life cycle in detail.

Newts have a complex life cycle, which is categorised to embryonic, larval, juvenile and adults (Figure 1). Each developmental period has been subdivided into stages based on a set of external characters (Joven et al., 2015; Simon and Odelberg, 2015). Newt larvae mainly use gills and skin for their oxygen uptake and do not have lungs. However, during metamorphosis, newts lose their gills and develop lungs, which are essential during postmetamorphic terrestrial life (Shi De-Li and Boucaut, 1995). Additionally, several external changes occur in newts during metamorphosis, such as skin adaptations to the terrestrial environment.

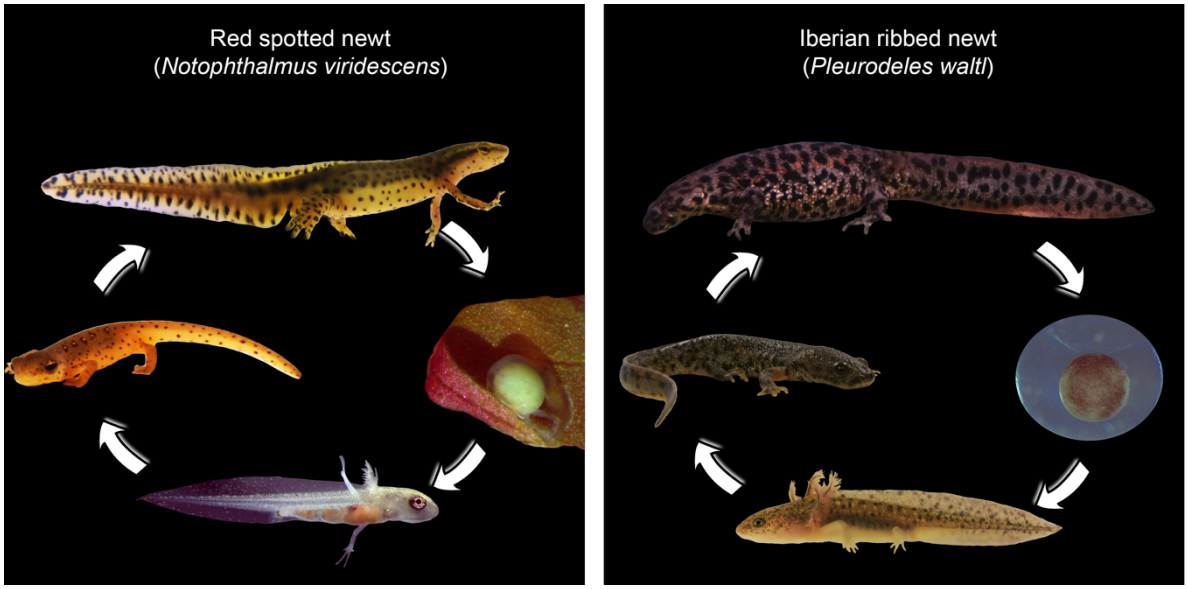

Figure1: Life cycle of red-spotted newt and Iberian ribbed newt
For each panel, an adult is shown on top, egg on the right, aquatic larva on the bottom and post-metamorphic juvenile on the left. In addition to the different external morphology, red-spotted newts are much smaller than Iberian ribbed newts. Photo credit: Alberto Joven.

The red-spotted newts are widely distributed in North-Eastern America. In red-spotted newts, the stages have been described separately for the embryonic and larval periods (Khan and Liversage, 1995). Their postmetamorphic juvenile phase is called eft. During the eft phase, the skin adopts a reddish tone and they live in a terrestrial habitat for about one to three years. After this terrestrial stage, newts return to the water, and the skin colour changes to greenish-grey (Brockes and Kumar, 2005). Adult red-spotted newts breed in water, even during winter under an ice-covered pond (Berner and Puckett, 2010). In the wild, the lifespan of red-spotted newt is up to 15 years (Hillman, 2009).

The distribution of Iberian ribbed newts is from the Iberian Peninsula to Morocco (Joven A et al 2015). Their developmental stages are well-described based on external morphology (Gallien and Durocher, 1957). Iberian newts are larger than the red-spotted newts, and unlike the latter, juveniles can be found in water (Joven et al., 2015). The advantages of the Iberian ribbed newts as a laboratory animal model is their ease of breeding in captivity, availability of large numbers of eggs where a single female can lay from 300 to more than 1000 eggs at a time (Salvador, 2015), and the possibility of genetic manipulation, including transgenesis and gene editing (Elewa et al., 2017; Hayashi et al., 2013; Joven et al., 2018).

Irrespective of their variation in distribution, habitat use and breeding, both red-spotted newts and Iberian ribbed newts retain widespread regenerative capacity. However, variations on regeneration processes between the species do exist (unpublished observations, Simon lab) and comparing inter-species regenerative ability will help us understand regeneration in an evolutionary perspective.

1.5 REGENERATION IN NEWTS

As discussed earlier, the first report on the regenerative ability in newts dates back to Lazzaro Spallanzani during the early eighteenth century. Spallanzani described regeneration of limbs and tail in newts. Experiments spanning the 20th century have shown that newts regenerate almost all body parts and they are called the champions of regeneration. Newts can regenerate lens, jaws, heart, tail, and limb (Brockes, 1997; Ghosh et al., 1994; Iten and Bryant, 1976; Tsonis et al., 2004; Witman et al., 2011).

Apart from the broad regenerative spectrum, newts show no sign of age-related decline in their regenerative ability. The repeated removal of the lens in the same newt for 18 times led to the correct replacement of lens each time. This study demonstrated that repeated injury does not alter the efficiency of regeneration spanning 16 years. Remarkably, the animals aged to 30 years by the end of the experiments did not show any age-related decline in their regenerative abilities (Eguchi et al., 2011).

Apart from the appendages, newts also possess the ability to regenerate injured spinal cord and brain. Spinal cord regeneration has been extensively studied in newts mainly by two injury models: spinal transection and tail amputation. After transection of the spinal cord, newts are able to regenerate and recover hindlimb movement by four weeks (Davis et al., 1990). The paedomorphic salamander, the axolotl, has been extensively studied for spinal cord regeneration after tail amputation. Studies on axolotl, indicate that neural stem cell-mediated proliferation contributes to spinal cord regeneration (Albors et al., 2015; Mchedlishvili et al., 2007).

Adult newts are able to regenerate parts of the brain after mechanical lesioning. In classical experiments in amphibians, the approach was to remove the optic tectum and study the functional outcome. In newts, removal of optic tectum and assessment of the brain till 90 days indicates that they can regenerate the optic tectum (Minelli et al., 1987). In another study, the retinotectal projection pathway was analysed after partial optic tectum removal. This study revealed that newts regenerate the optic tectum and recover most of the retinotectal projections by eight months (Okamoto et al., 2007). Recently, a number of studies on brain regeneration have been performed on newts, and this will be discussed later in Chapter 3 (Section 3.5.4).

2 EMBRYONIC NEURAL STEM CELLS

The adult brain retains the neurogenic potential through adult neural stem cells (NSCs). To understand the origin of adult NSCs and to evaluate their neuronal regeneration potential, it is necessary to study NSCs during development. Mammals have developed more complex brain compared to non-mammalian vertebrates. Specifically with regard to the development of pallium, which give rise to the neocortex in mammals (Butler et al., 2011). Mammalian brain development and the NSCs within have been extensively studied. In this chapter, I give a brief overview of vertebrate brain development and NSCs maturation, with emphasis on mammalian models.

2.1 EARLY CNS SPECIFICATION

During gastrulation, the vertebrate egg separates into three germinal layers, mesoderm, ectoderm and endoderm. Subsequently, the ectoderm is subdivided into the epidermal and neural ectoderm, a process called neural induction (Muñoz-Sanjuán and Brivanlou, 2002). The repression of bone morphogenetic protein (BMP) together with the expression of fibroblast growth factor (FGF) are the two-intracellular signalling pathways important for establishing the neural ectoderm, whereas BMP and Wnt signalling promote the epidermal ectoderm (Stern and Stern, 2005; Wilson et al., 2001). Neurulation is the process by which the neural plate forms and folds into the neural tube due to the action of signalling molecules from the primitive node and notochord, which are two important embryonic signalling centres (Schoenwolf and Smith, 2000). BMP and sonic hedgehog (Shh) signalling play a major role in neural tube patterning, which gives rise to dorso-ventral and anterior-posterior domains (Levine and Brivanlou, 2007). These patterning steps lead to the anterior part of the neural tube developing into the brain and posterior part developing into the spinal cord.

2.2 CNS DEVELOPMENT

During mammalian CNS development, neuroepithelial cells lining the ventricular zone of the neural tube gives rise to neurons and radial glial cells (RGCs). RGCs further contribute to the formation of neurons and glial cells in the CNS (Delaunay et al., 2008).

Neuroepithelial cells at the ventricular zone divide symmetrically to expand their population and they also divide asymmetrically to produce one neuron and one neuroepithelial cell (Haubensak et al., 2004). Neuroepithelial cells maintain apical-basal contact and express cell surface marker prominin-1 on the apical surface (Weigmann et al., 1997). During the onset of neurogenesis, pseudo-stratified neuroepithelial cells transform

into RGCs. This transition starts around the embryonic stage E9-E10 in mice (Götz and Huttner, 2005). There are several molecular changes leading to the transformation of neuroepithelial cells to RGCs. The transformed RGCs readily start expressing the glial markers such as glutamate aspartate transporter (GLAST), brain lipid binding protein (BLBP), glutamine synthetase (GS), vimentin and radial glial cell marker-2 (RC2) (Akimoto et al., 1993; Anthony et al., 2004; Feng et al., 1994; Shibata et al., 1997). Moreover, RGCs maintain only adherent junctions and lack tight junctions (Kriegstein and Alvarez-Buylla, 2009). Generally, the RGCs divide symmetrically or asymmetrically. The result of symmetrical division leads to expansion of RGCs pool and the asymmetric division gives rise to one-RGCs and neurons or intermediate progenitor cells (Noctor et al., 2004). The asymmetric division of RGCs occurs at the ventricular zone, and intermediate progenitor cells present in the embryonic subventricular zone (SVZ) divide symmetrically to produce either two neurons or two intermediate progenitor cells (Miyata, 2004; Noctor et al., 2004).

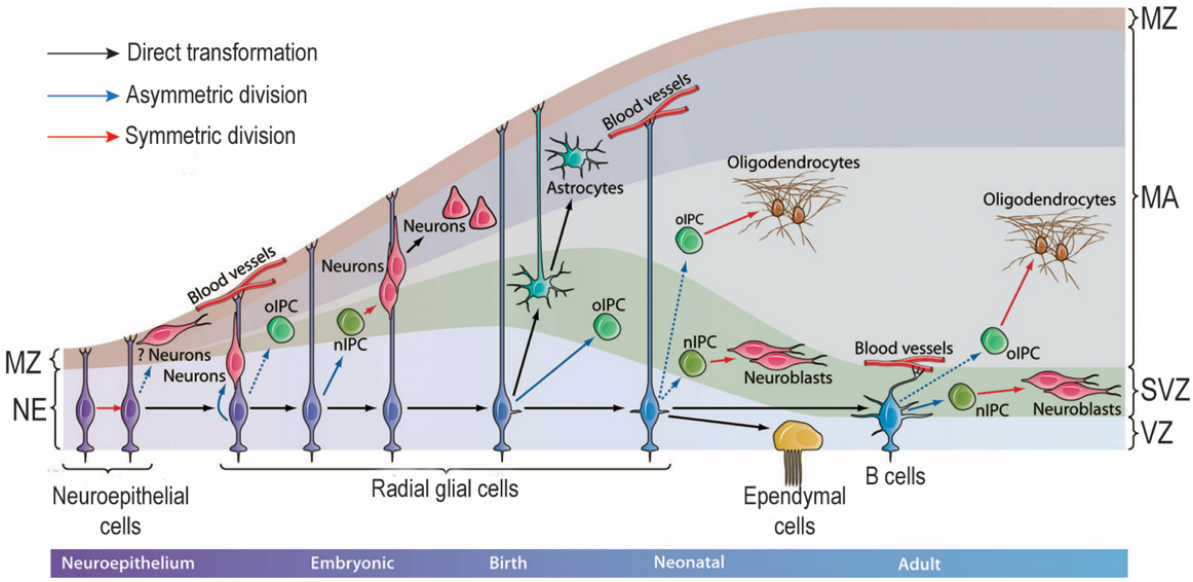

Figure 2: Maturation of embryonic neural stem cells

In early development, neuroepithelial cells give rise to neurons and radial glial cells (RGCs). As development proceeds, RGCs self-renew and also produce intermediate progenitor cells (IPCs). The IPCs, self-renew or differentiate into either neurons or oligodendrocytes. Soon after birth, RGCs regress their processes and the majority differentiate to astrocytes. Some of the RGCs are retained as ependymal cells and neural stem cells in the adult brain. MA-mantle; MZ-mandel zone; NE-neuroepithelium; VZ-ventricular zone, SVZ-subventricular zone. Reprinted with permission from the publisher, Elsevier. (Kriegstein and Alvarez-Buylla, 2009).

In the late stage of brain development, RGCs switch from neurogenesis to gliogenesis, which denotes the production of oligodendrocytes and astrocytes. In mice, oligodendrocytes appear around E16 in the cortex. $NG2^+$ oligodendrocyte progenitor cells arise from Nkx-2.1^+ RGCs, differentiate to produce oligodendrocytes in the CNS (Goldman and Kuypers, 2015; Kriegstein and Alvarez-Buylla, 2009). Oligodendrocyte progenitor cells express markers such A2B5, NG2, PDGFαR and mature oligodendrocyte expresses myelin basic protein (Miron et al., 2011) In the cortex, the presence of astrocytes has been recorded around E17. Astrogenesis is a complex process where numerous factors induce their specification, these include cardiotrophin-1, leukemia inhibitory factor and ciliary neurotrophic factor (Bonni et al., 2007; Koblar et al., 1998; Miller and Gauthier, 2007). Astrocytes express a wide variety of markers, which include GS, BLBP, and GFAP (glial fibrillary acidic protein). Recently, live *in vivo* imaging of transgenic mice carrying green fluorescent protein (GFP) under the control of a human promotor GFAP (*hGFAP: GFP)* revealed that a majority of astrocytes in the cortex are generated by the proliferation of local astrocytes (Ge et al., 2012). Moreover, most of the RGCs in postnatal brain disappear along with the emergence of astrocytes (Figure 2) (Noctor et al., 2004).

In non-mammalian vertebrates, similar to mammals, neuroepithelial cells and RGCs are NSCs in early development. Interestingly, in *Xenopus* two waves of neurogenesis occurs during development (Thuret et al., 2015). The first neurogenic wave occurs around neurulation and afterwards neurogenesis gradually declines till the premetamorphic stage. Subsequently, the second wave of neurogenesis starts before metamorphosis (Raucci et al., 2006; Thuret et al., 2015). An intriguing difference in zebrafish is that, when compared to other vertebrates, the zebrafish telencephalon folds outwards (eversion) and NSCs lie in the outer layer (Folgueira et al., 2012). Importantly, most of the non-mammalian vertebrates retain radial glial-like cells in their adulthood (Becker and Becker, 2015). How the RGCs persist into adulthood in non-mammalian vertebrates is not clear, and this particular aspect merits further investigation.

2.3 MATURATION OF EMBRYONIC NEURAL STEM CELLS

In mammals, maturation of NSCs is characterised by the conversion of neuroepithelial cells to RGCs and disappearance of RGCs in postnatal stage (Figure 2). A number of distinctive changes occur during the maturation process and these changes are necessary for the progression of development.

During development, early neuroepithelial cells have the potential to produce all types of neurons and glial cells. However, as development proceeds, neuroepithelial cells transform

to RGCs and RGCs fate choice becomes restricted. For example, transplantation of mid-hindbrain cells from E10.5 to E13.5 mice ventral telencephalon demonstrated that cells from E10.5 could differentiate and integrate. However, E13.5 mid-hindbrain cells do not integrate into ventral telencephalon at stage E10.5 indicating that they are more restricted in lineage by E13.5 (Olsson et al., 1997). In ferrets, cortical layer VI and IV neurons are formed at stage E30 and stage E36, respectively. Transplantation of NSCs from stage E36 to E30 reveals that NSCs from E36 do not migrate and integrate into layer VI, indicating a lineage restriction of NSCs fate (Desai and McConnell, 2000).

Besides lineage restriction of NSCs, an increase in cell cycle length also has been noticed during embryonic development. In neuroepithelial cells at embryonic stage E11, the cell cycle length is around 8 hours. In contrast, at E16 the cell cycle lengthens up to 18.4 hours in the corresponding RGCs. The gradual lengthening of the cell cycle length is mainly due to an increased length of G1 phase (Takahashi et al., 1995). Interestingly the increase in cell cycle length correlates with neurogenesis. Lineage tracing studies with the Tis-21 promoter, a pan-neurogenic progenitor marker, showed that NSCs which self-renew had shorter cell cycle length, whereas, longer cell cycle length correlates with neurogenesis (Calegari, 2005). Irrespective of increase in cell cycle length associated with neurogenesis, it has been noted that there is an increase in NSCs quiescence during development (Faiz et al., 2005). Quiescence of NSCs seems to be important for maintaining NSCs in adulthood. Recent lineage tracing analysis of embryonic NSCs showed that fast cycling cells exhaust during development and cells which acquire quiescence were retained in adult stem cell niche. A large number of quiescent cells has been noted between E13.5-E15.5, and it is likely that cells which acquire quiescence before E17.5 are retained in the adult stem cell niche (Fuentealba et al., 2015). Stem cell exhaustion has been noted in aging disorders and also studies have shown that hyperactivation of adult NSCs can lead to its depletion in adult (Sierra et al., 2015). Thus, maintaining quiescent state during ontogeny could be a way of keeping the NSCs in the adulthood.

Detailed information about the maturation of NSCs and their lineage restriction in non-mammalian vertebrates is lacking. Nevertheless, both the increase in cell cycle length and occurrence of quiescence has been noted in amphibians (Thuret et al., 2015). Notably, the majority of adult progenitors retained in non-mammalian vertebrates resemble RGCs. Therefore, it is intriguing to understand the mechanistic basis for the maturation of RGCs and their retention throughout the adulthood among regenerative vertebrates.

2.4 NEURONAL SUBTYPE SPECIFICATION

In the developing brain, different classes of neurons are produced in a unique temporal pattern from common or lineage-restricted progenitors (Bartolini et al., 2013; Lledo et al., 2008). The mammalian cortex is highly complex and organised in layers. Neurons are added in an inside to outside manner in mammals; the deep layer neurons VI-V are added initially, followed by the superficial layer neurons IV-II (Parnavelas, 2000; Rakic, 1974). Interestingly, in birds, reptiles, and amphibians the pallial neurons appear to be produced in an outside to inside manner (Moreno and González, 2017; Suzuki and Hirata, 2014). Nevertheless, a number of factors are known to regulate the cell sub-type specification during cortical neurogenesis in mammals, which include Pax6, Cux2, Svet1, Fezf2, Otx-1, and Ctip2 (Kohwi and Doe, 2013).

Given the nature of investigations in the papers of this thesis, here I will focus mainly on dopaminergic and cholinergic specification. These neuronal subtypes have been implicated in a number of behavioral processes and are also known to be affected in certain neurodegenerative disorders (see below). The origin of these cells and what kind of intrinsic factors regulate their development could be beneficial to gain insight into the regeneration capacities of these neuronal populations.

2.4.1 Dopaminergic neuronal specification

Dopamine is a neurotransmitter implicated in decision making, fear processing and in the reward system (Martinez et al., 2008). Dopaminergic neurons are present throughout the brain as a group of nuclei called A1-A16. The nuclei A8-A10 are located at the ventral mesencephalon, including the substantial nigra (A9) and the ventral tegmental area (A10) (Bonilla et al., 2008; Vogt Weisenhorn et al., 2016). Striatal dopaminergic projections from the ventral midbrain are important for decision-making and the nucleus accumbens is involved in fear conditioning (Levita et al., 2002). Degeneration of dopaminergic neurons and a behavioural deficit has been noted in Parkinson's disease. Especially dopaminergic neurons in the substantial nigra and in the ventral tegmental area are affected in Parkinson's (Sulzer and Surmeier, 2013). Therefore, in this section, I focus on ventral midbrain dopaminergic neuron specification.

In mouse, ventricular zone RGCs from floor plate induces dopaminergic neuronal production around E9.5 (Bonilla et al., 2008). Both Shh and FGF8 are crucial for early dopaminergic neuronal specification (Ye et al., 1998). Shh-induced Lmx1a expression in ventricular NSCs is critical for inducing dopaminergic neurogenesis in the midbrain (Andersson et al., 2006). Moreover, TGF-β and Wnt-1 also appear to be necessary for midbrain dopaminergic neurogenesis (Farkas et al., 2003; Prakash, 2006). Other factors

such as Otx2 also play a crucial role in progenitor proliferation and differentiation (Vernay, 2005). Dopaminergic precursor cells gradually become postmitotic and exit the cell cycle around E10.5 to E13.5. During this period, maturation and differentiation of dopaminergic neurons occur. A number of factors, Nurr1, Pitx-3, Lmx1b, and Engrailed1/2, have been shown to be involved in differentiation and survival of dopaminergic neurons (Bissonette and Roesch, 2016; Hegarty et al., 2013; Smidt et al., 2000). Tyrosine Hydroxylase (TH), which catalyses the rate-limiting step in dopamine biosynthesis, is one of the markers for identifying mature dopaminergic neuronal population and it is expressed around E14.5 (Specht et al., 1981).

2.4.2 Cholinergic neuronal specification

Acetylcholine is a neurotransmitter produced by cholinergic neurons, and the loss of these neurons in basal forebrain has been reported in many forms of neurodegenerative disorders, including Alzheimer's disease (Nyakas et al., 2011; Schliebs and Arendt, 2011). The cholinergic system is also implicated in the regulation of behaviour. Acetylcholine release in the ventral tegmental area is thought to regulate reward and addiction behaviour. Furthermore, hypothalamic regulation of acetylcholine has been shown to control food intake and endocrine functions (Picciotto et al., 2012).

Cholinergic neurons are grouped in nuclei named Ch1-Ch8 present throughout the brain. The forebrain contains the nuclei Ch1-Ch4 which send projections to the hippocampus, olfactory bulb and cortex (Allaway and Machold, 2017). Forebrain cholinergic neurons arise from Nkx2.1 expressing NSCs from the ventral telencephalon. Nkx2.1 start appearing around E10.5 in the ventral telencephalon (Butt et al., 2008). Intrinsic determinants, such as transcription factors Lhx7/L3, Gbx-1, and Gbx-2 are important for the cholinergic neuronal specification in the forebrain (Allaway and Machold, 2017; Chen et al., 2010; Fragkouli et al., 2005). In addition, extrinsic factors BMP, NGF, and BDNF have been implicated in cholinergic differentiation and neuronal survival (Allaway and Machold, 2017; Higgins et al., 1989; Lopez-Coviella et al., 2005). The majority of the basal striatal cholinergic neurons are formed between E12-E17 in rats (Phelps et al., 1989).

3 ADULT NEURAL STEM CELLS

3.1 HISTORICAL PERSPECTIVE OF ADULT NEUROGENESIS

A view that no new neurons are added into the postembryonic mammalian brain prevailed in the field of neuroscience for decades. This view also implied that cellular plasticity is restricted in the adult vertebrate brain. The usage of ^{3}H-thymidine added new dimensions to the field of experimental biology. In 1950's methods were developed to incorporate ^{3}H-thymidine into the DNA of proliferating cells and the resultant radiation from a cell was detectable using autoradiography. This technological advancement paved the way to the identification of proliferating cells in the mouse spleen and gastrointestinal tract (Hughes et al., 1958) as well as in the rat liver (Grisham, 1962). The first report on neurogenesis in the brain using autoradiographic method was reported by Altman and Das (Altman and Das, 1965). In a series of studies conducted in rat brain, they concluded that the postnatal brain has the potential for cellular proliferation. Chasing of ^{3}H-thymidine-incorporating cells indicated a possibility of neurogenesis in the dentate gyrus of the hippocampus and in the olfactory bulb (Altman, 1969, Altman and Das, 1966, 1965). Despite these seminal findings, their reports did not get much attention until 1980's.

During early 1980's Goldman and Nottebohm used a similar autoradiography method to study neurogenesis in the canaries. They showed the presence of newly formed neurons in the high vocal centre (HVC) and that the HVC is critical for song learning and production (Goldman and Nottebohm, 1983). In contrast, claims on mammals lacking adult neurogenesis using ^{3}H-thymidine came from studies on monkeys (Rakic, 1985). However, further studies have been performed in reptiles and in birds using ^{3}H-thymidine, and reported on-going neurogenesis occurring in these species (Alvarez-Buylla and Nottebohm, 1988; Garcia-Verdugo et al., 1989). Furthermore, studies in 1990's concluded that there is ongoing neurogenesis in the mammalian brain, including humans (Cameron et al., 1993; Eriksson et al., 1998; Lois and Alvarez-Buylla, 1994).

The introduction of Bromo-deoxyuridine (BrdU), an analogue of thymidine, which stably incorporates into the DNA during replication, and could be detected by colorimetric and fluorescence microscopy has advanced the field of cell cycle analysis (Gratzner, 1982; Gratzner et al., 1975). The BrdU labelling methodology also enables the identification of the progeny of proliferating cells by double immunostaining. However, BrdU at high concentrations can be toxic and some reports claim that it can also label cells that undergo apoptosis or DNA repair (Cooper-Kuhn and Georg Kuhn, 2002; Kuan, 2004; Sekerková et al., 2004).

In the early 1990's primary cell culture systems were developed from adult mouse brain. Isolated cells from the striatum has shown self-renewing potential and differentiated into neurons and astrocytes in *in vitro* (Reynolds and Weiss, 1992). The availability of cell culture systems added new dimensions and further advancement in the area of neurogenesis to evaluate neural stem cells (NSCs). With the recent development of genetic manipulation technologies, it has become possible to understand the potential and function of NSCs even at the single cell level (Bonaguidi et al., 2011; Dulken et al., 2017; Renault et al., 2009).

Adult neurogenesis has been identified in a number of vertebrates including non-mammalian species. In mammals, only two regions display extensive neurogenesis in adults (see further below). Although mammals retain neurogenic potential in these regions, this in itself provides no evidence that the mammalian brain could regenerate the lost neurons. Activation of endogenous NSCs appears to be necessary for regeneration to proceed in fish and salamanders. Hence, gaining insights into species-specific features of adult NSCs and understanding how they respond to injury might give us indications about the regenerative potential of these species, and which attributes of NSCs could be manipulated to promote regeneration in mammals.

3.2 MAMMALIAN NEURAL STEM CELLS

In the adult mammalian brain two distinct niches, subventricular zone (SVZ) and subgranular zone (SGZ) retain the ability of homeostatic neurogenesis (Alvarez-Buylla and Lim, 2004; Ma et al., 2005). The cells located in these unique niches are multipotent and responsive to mitotic stimuli. Like embryonic NSCs, they self-renew and generate different types of brain cells under certain stimuli. In the following sections, I discuss the characteristics of these cells located in these niches, and the developmental origins of NSCs.

3.2.1 Origin of adult neural stem cells

As discussed earlier (Chapter 2), RGCs form around E10.5 and produce neurons and astrocytes in the mammalian embryonic brain. During the early postnatal stage, most of the RGCs have vanished and a small population of radial glial-like cells is retained in the hippocampus (Kriegstein and Alvarez-buylla, 2009). During the early postnatal stage, the bipolar RGCs that contact the pial-ventricular surface show regression of the radial processes and become unipolar cells (Figure 2). Regression of the radial process is correlated with the occurrence of increased astrogenesis. Tracing of RGCs in ferret

postnatal brain with DiI revealed that RGCs retain DiI initially but later it was predominately present in astrocytes (Voigt, 1989). The loss of RGCs in rodent brain is also linked with the appearance of more astrocytes. Recent retroviral labelling of RGCs and tracing indicates that majority of RGCs become astrocytes in the postnatal brain (Noctor et al., 2008).

Early lineage analyses showed that RGCs in embryonic stage gives rise to adult NSCs in the mammalian brain. Labelling of RGCs at P0 with eGFP (enhanced green fluorescent protein) and analysing them at different time point indicates that NSCs located in the SVZ niche originated from RGCs (Merkle et al., 2004.). In a recent study, *hGFAP:GFP* labelled RGCs where traced from the embryonic stage until adulthood revealed that NSCs located at the SVZ originate from embryonic RGCs. Cells which are predominantly quiescent during embryonic stage E13.5-E15.5 give rise to adult NSCs in rodents (Fuentealba et al., 2015). The origin of adult NSCs in the hippocampus is still unclear. During embryonic development, the dentate neuroepithelium generates both granule neurons and adult hippocampal NSCs (Gonçalves et al., 2016; Li and Pleasure, 2005). However, a recent study proposed that adult NSCs in the hippocampus originate from sonic hedgehog (Shh)-responding RGCs from the ventral hippocampus. Using Gli-1-CreERT2 mice, tamoxifen-mediated induction and analysis from E15.5 until adulthood identified Shh-responding RGCs at E17.5 in ventral hippocampus that give rise to adult hippocampal NSCs (Li et al., 2013)

Interestingly, embryonic RGCs are retained in adulthood in the CNS of many vertebrates and serve as major source of stem cells. Fish and amphibians also retain radial glial-like cells even in the adult stage, and this aspect is discussed in detail later in this chapter.

3.2.2 Subventricular zone

The SVZ is located along the lateral walls of the lateral ventricles (Alvarez-Buylla and García-Verdugo, 2002; Doetsch and Alvarez-Buylla, 1996) and contains four major cell types, which were defined using lineage tracing in mice (Figure 3a). The cells directly lining the ventricle are called ependymal cells (type-E cells). These cell types are in a quiescent state during normal homeostasis. Type-E cells express markers CD-24 and S100b (Carlén et al., 2009), and possess long motile cilia, which contribute to cerebrospinal fluid movement (Sawamoto et al., 2006). Another cell type, the type-B cells are considered as the NSCs of the SVZ. Type-B cells are slowly proliferating and express markers such as GFAP, GLAST, and BLBP (Alvarez-Buylla and García-Verdugo, 2002; Doetsch et al., 1999; Garcia et al., 2004; Lee et al., 2012). Based on their location and ventricular contact,

two populations of type-B cells exist; type-B1 and type-B2 cells. Type-B1 cells have apical processes that contact the ventricle and basal process in touch with blood vessels and have a single non-motile primary cilium. Type-B2 cells do not have ventricular contact and are located deeper in the tissue than type-B1 cells (Gonzalez-Perez, 2012). Recent studies have found that type-B cells are either in a quiescent or active state (Codega et al., 2014). The active form of type-B cells expresses another intermediate filament protein Nestin (Codega et al., 2014; Lim and Alvarez-Buylla, 2014). Active type-B cells also produce transient amplifying cells, type-C cells (Doetsch et al., 1999). Type-C cells express EGFR and Ascl1 as markers (Ihrie and Álvarez-Buylla, 2011). The type-C cells divide approximately three times and give rise to type-A cells which, in rodents, migrates through the rostral migratory stream (RMS) to the olfactory bulb via a tube of astrocytes. Before final terminal differentiation into neurons, type-A cells usually divide one or two times. The neuroblasts that originate from SVZ migrate tangentially up to a distance of 5 mm in rodents (Lim and Alvarez-Buylla, 2014; Zhao et al., 2008), and these cells become GABAergic granule neurons and dopaminergic periglomerular neurons in the olfactory bulb (Ming and Song, 2011). Interestingly, neurogenesis in the olfactory bulb in human is very limited compared to rodents (Bergmann et al., 2012).

3.2.3 Subgranular zone

The SGZ is located between the granule cell layer and the hilus in the dentate gyrus of the hippocampus (Palmer et al., 2000). The SGZ retains two major cell types in adult, type-I cells that have long radial processes and retains markers of RGCs such as GFAP, SOX2, BLBP, Vimentin, and Nestin (Fukuda et al., 2003; Nicola et al., 2015; Seri et al., 2004). Like type-B cells in SVZ, type-I cells are slow dividing and multipotent stem cells and about 1-2% of these cells were found to incorporate BrdU during a ten-hour pulse labelling (Encinas et al., 2011). Type-II cells have short processes and they differ from type-1 by lacking expression of GFAP. Type-II cells exist in two forms; type-IIa which express Nestin but are negative for Dcx (doublecortin), and type-IIb cells, which express Dcx and lack expression of both GFAP and Nestin (Steiner et al., 2006). However, type-IIa cells express Sox-2 (Suh et al., 2007). About 60% of type-II cells are transient amplifying progenitors, proliferate actively and they go through approximately 2.5 division to give rise to type-III cells (Figure 3b) (Encinas et al., 2011). Type-III cells (neuroblasts) express markers of PSA-NCAM and Dcx. Expression of certain markers by type-III cells overlaps and it is difficult to get a clear picture of which markers are exclusively specific to each subtype. It has been shown that PSA-NCAM is expressed by type-II cells in certain conditions. During conversion of type-II cells to neuroblasts, the majority of the newborn cells die through apoptosis (Sierra et al., 2010). It takes approximately four to seven weeks

for the neuroblasts to differentiate into glutamatergic granule cells in the granule cell layer (Kempermann et al., 2004; Sun et al., 2015). In bats, the only flying mammal, proliferation analysis by PCNA expression indicated lack of proliferating cells in the dentate gyrus (Amrein et al., 2007).

Although at a population level these NSCs from SVZ and SGZ appear homogenous they oscillate between quiescence and activation. Also, characterisation of these cells with functional analysis indicates that they are a heterogeneous population. This aspect will be discussed later in this chapter.

3.3 NEURAL STEM CELLS IN NON-MAMMALIAN VERTEBRATES

The extent of adult neurogenesis differs among vertebrates, especially in non-mammalian species. Among the non-mammalian vertebrates, the teleost fish show widespread neurogenic regions, whereas, reptile and birds display more restricted seasonal neurogenesis (Chapouton et al., 2007; Grandel and Brand, 2013; Kaslin et al., 2008).

3.3.1 Birds and Reptiles

In birds, proliferation zones are mostly restricted to the ventricular zone of the telencephalon. In songbirds, ^{3}H-thymidine-mediated autoradiography studies have identified that radial glial cells that line the ventricular zone are able to proliferate and produce newborn neurons which migrate and integrate predominately into the HVC (Alvarez-Buylla and Kirn, 1997; Goldman and Nottebohm, 1983). The ultrastructural analysis further identified three major cell types, type-E cells, type-B cells, and type-A cells, located in the ventricular zone (Figure 3c). The type-B cells retain radial glial-like cell morphology and act as stem cells and, type-E cells are identified as ependymal cells, while type-A cells are immature neuroblasts (García-Verdugo et al., 2002). Other than songbirds, the adult ring dove displays widespread neurogenesis in the telencephalon. Interestingly, in this species, an age-related decline in neurogenesis has been noticed. In comparison to three-month-old, eight-year-old birds showed a significant reduction in neurogenesis (Ling et al., 1997).

In reptiles, neurogenesis occurs in all major subdivisions of the adult telencephalon and occurs to a lesser extent in the cerebellum (Font et al., 2001; Kaslin et al., 2008). Most cells in the ventricular zone are radial glial-like cells. Similar to birds, three major cell types have been identified in the reptilian ventricular zone: migrating (type-A) cells, radial glial

(type-B) cells, and ependymal (type-E) cells (Figure 3d) (García-Verdugo et al., 2002). Type-B and type-E cells virtually share all the requirements for radial glial cells except that type-B cells have single cilium while type-E cells have 15 to 20 cilia (García-Verdugo et al., 2002; Pérez-Cañellas and García-Verdugo, 1996). In comparison to mammalian brain, no evidence of neuronal death has been reported during neurogenesis in the reptile (Font et al., 2001).

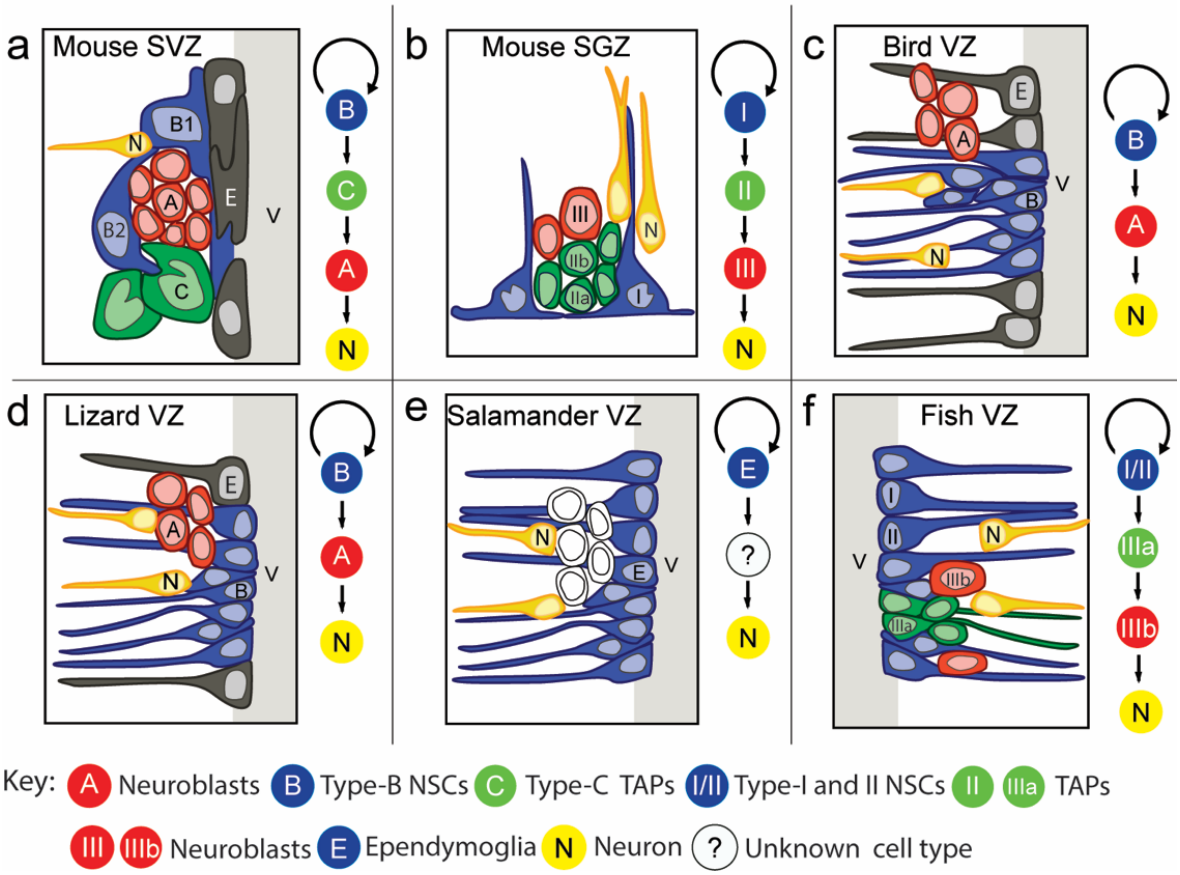

Figure 3: Comparison of neural stem cells (NSCs) across vertebrates.
Adult mammalian neural stem cells are a heterogeneous pool of cell types; for simplicity only major cell types are depicted in the figure.

(a) In the subventricular zone of mice, the type-B (B1, B2) cells are NSCs which give rise to type-C transient amplifying progenitors (TAPs) which produce type-A neuroblasts. (**b**) In the subgranular zone of mice, NSCs are called type-I cells. The type-II (a, b) cells are TAPs and type-III cells are neuroblasts. (**c**, **d**) In birds and lizards, the nomenclature is shared: type-B cells are NSCs, while type-A cells are neuroblasts which give rise to neurons. (**e**) In salamanders, ependymoglial cells are the NSCs and they produce neurons. The existence of TAPs is uncertain here. (**f**) In fish, type-I and type-II cells are stem cells in a quiescent/proliferative state. Type-IIIa cells are the transient amplifying progenitors and type-IIIb cells are neuroblasts.
V- ventricular Zone. The figure was inspired by (Doetsch, 2003), including more recent discoveries found in mammals (Bond et al., 2015), birds and lizards (García-Verdugo et al., 2002), salamanders (Berg et al., 2010) and fish (März et al., 2010).

3.3.2 Fish

In the adult teleost fish, the brain retains numerous neurogenic niches. New neurons are formed in different regions including olfactory bulb, dorsal telencephalon, hypothalamus, optic tectum and cerebellum (Adolf et al., 2006; Ganz and Brand, 2016; Zupanc, 2011). Zebrafish neurogenesis has been extensively studied and there are three major types of stem cells identified (Figure 3f). Type-I cells, which are slowly cycling and label retain with BrdU, express certain glial markers such as S100β, GFAP, BLBP, Nestin, and Sox2 (März et al., 2010). Type-II cells express similar markers as type-I cells in addition to PCNA indicating that they are fast cycling cells. Proliferating cells give rise to transient amplifying progenitors, and express GFAP, BLBP, Nestin, Sox2, PCNA, and PSA-NCAM as makers. These cells are called type-IIIa cells which give rise to neuroblasts expressing Sox-2, PCNA and PSA-NCAM (type-IIIb cells) (März et al., 2010).

From the ventral telencephalon of the teleost brain, the PSA-NCAM$^+$ neuroblasts migrate and differentiate into GABAergic and TH$^+$ neurons in the olfactory bulb (Adolf et al., 2006). Recently, it has been reported that the zebrafish cerebellum retains two kinds of progenitors, one neuroepithelial-like stem cell and the other radial glial-like cells (Kaslin et al., 2013). Similar to aging in the mammalian brain, the zebrafish brain also shows age-related decline in neurogenesis. Comparative studies between a three month and one-year-old zebrafish brain have shown that the latter has reduced proliferation capacity (Kaslin et al., 2009).

3.3.3 Amphibians

In anuran amphibians, both gliogenesis and neurogenesis have been described in several regions of the adult brain, including telencephalon, hypothalamus, optic tectum, torus semicircularis and cerebellum (Raucci et al., 2006; Simmons et al., 2008). Nevertheless, proliferative activity progressively decreases during development in most brain regions, an exception being the preoptic area (Raucci et al., 2006). Cell proliferation in the frog brain has been found to be modulated by seasonality (Margotta, 2012) and social behaviour (Almli and Wilczynski, 2012).

Studies dealing with proliferation and neurogenesis in the brain of adult salamanders are very limited. In red-backed salamanders, proliferation changes seasonally (Dawley et al., 2000). Analysis of axolotl brain has revealed that most of the regions in the brain retain the neurogenic potential. Radial glial-like GFAP$^+$ cells with radial processes are involved in the proliferation and act as NSCs. Dcx$^+$ neuroblasts have also been identified but a detailed molecular heterogeneity of these cell types is currently lacking (Maden et al., 2013). This

study has shown that it takes two weeks to form mature neurons, but in some cases, Dcx^+ cells were present even after four weeks (Maden et al., 2013). In the red-spotted newt, neurogenesis is mostly restricted to regions of the telencephalon. The NSCs located in the ventricular layer are known as ependymoglial cells (Figure 3e), which express the glial marker GFAP (Berg et al., 2010). Interestingly, $GFAP^+$ ventricular ependymoglial cells also present in the midbrain; however, they are in a quiescent state during homeostasis (Berg et al., 2011; Parish et al., 2007).

NSCs in the ependymal layer of the red-spotted newt brain has extensive neurogenic potential. Further characterisation of these cells in terms of molecular regulation and heterogeneity is important to understand their exceptional regenerative ability.

3.4 HETEROGENEITY OF NEURAL STEM CELLS

In the previous section, I discussed the different cell types in the adult brain and their characteristics. Of the various cell types, the NSCs are heterogeneous at the genetic level, and the progeny they produce also differs considerably. Recently two new cell types have been identified in the SGZ, which are called radial glial-like cell-α and radial glial-like cell-β. The alpha subtype accounts for 76% and beta subtype amounts to 24% of radial glial-like NSCs in the SGZ (Gebara et al., 2016). Molecular characterisation of these cells shows that alpha cells have long radial processes, which penetrate into the granule cell layer and retains proliferative potential. Beta cells have short radial processes and express stem cell markers and appear to be quiescent (Gebara et al., 2016). This study confirms the heterogeneous nature of adult NSCs in the mammalian brain.

In addition, their heterogeneity is also manifested in which type of progeny NSCs produce. Even embryonic RGCs are heterogeneous in nature with respect to their neurogenic commitment. In embryonic RGCs, lineage tracing studies indicate that cux-2 expression is restricted to certain RGCs and they predominantly produce upper layer II-IV $cux\text{-}2^+$ neurons with few lower layer neurons. $Cux2^+$ RGCs are restricted in their expression. This study reveals the presence of fate-restricted RGCs even in early development (Franco et al., 2012). Intriguingly, the specification of NSCs in adult SVZ is already defined in the early embryonic stage. Tracing experiments of embryonic NSCs at different time points concluded that certain RGCs slow down their cell cycle before E17.5. Fast cycling RGCs exhaust during development and RGCs which divide slowly are destined to be retained in the adult SVZ (Furutachi et al., 2015). This study confirms the establishment of heterogeneity during the early developmental stage.

Likewise, adult NSCs are also heterogeneous with regard to the progeny they produce. In the adult SVZ, expression of Pax6 is restricted to certain progenitors. Neuroblasts which migrate from SVZ to the olfactory bulb express a variety of factors, indicating the different types of progeny derived from NSCs. Analysis of neuroblasts expressing Pax6 indicates that they are essential for dopaminergic periglomerular neuron differentiation and SP8 transcriptional expression in neuroblast is important for the specification of a subpopulation of $SP8^+$ interneurons specification (Hack et al., 2005; Waclaw et al., 2006). It is possible that the fate of neuroblast is determined already in the NSCs. The heterogeneity of ventricular progenitors was further evaluated by lineage analysis, which indicated that dorsally located NSCs in SVZ produce superficial granule cells and TH^+ peri-glomerular cells. In parallel, the ventrally located NSGs produce deeps granule cells and calbindin-positive peri-glomerular cells (Merkle et al., 2005). This study also showed that NSCs are heterogonous both in terms of gene expression profile and the progeny they produce.

3.4.1 Role of Notch and GS in neural stem cells heterogeneity

3.4.1.1 Notch signaling

NSCs respond differentially depending both on the stimuli they receive as well on their niche environment. A number of signalling pathways have been identified that can influence the NSC, including Shh, WNT, and Notch signalling. Of the many signalling mechanisms, only Notch-mediated signalling requires direct cell-cell contact. Expression of Notch ligand or receptor on NSCs could identify heterogeneity of NSCs (Giachino and Taylor, 2014).

Notch signalling is an evolutionarily conserved pathway. In canonical Notch signalling, the Notch ligand, a transmembrane protein expressed in one cell, interacts with the Notch receptor on another cell to induce downstream signalling (Kopan and Ilagan, 2009). In mammals, four Notch receptors (Notch 1-4), and ligands, Delta-like (Dll1, 3, and 4) and jagged (Jag1 and Jag2) have been identified (D'Souza et al., 2010). Notch signalling involves binding of a ligand to the Notch receptor, which leads to cleavage of the intracellular domain (NICD) by γ-secretase. Cleaved NICD then translocates into the nucleus and binds to CBF-1/RBPJk. This interaction facilitates the recruitment of mastermind/MAML which in turn leads to activation of targets such as the Hes or Hey genes (Fortini, 2009; Kopan and Ilagan, 2009)

Notch signalling plays a crucial role in brain development and NSCs fate. Complete knockout of the Notch signalling leads to embryonic lethality. Null mutant of the Notch downstream target *Hes* gene revealed that Notch signalling is important for RGC maintenance. *Hes* deletion resulted in depletion of RGCs and in turn led to increased differentiation of RGCs to neurons (Hatakeyama et al., 2004). The role of Notch signalling in the maintenance of NSCs was also studied by using a conditional *knockout* of *Rbp-j*. Conditional *knockout* of *Rbp-j* at E9.5 leads to a reduction in brain size and decline in RGCs with an overall decrease in ventricular zone at E15.5 (Imayoshi et al., 2010). These studies indicate that Notch signalling is important for maintaining embryonic NSCs. Moreover, in the adult brain, Notch signalling is known to maintain the quiescent state of NSCs. Ependymal cells from the SVZ are maintained in a quiescent state and upon stroke, down-regulation of Notch signalling leads to increase in cellular proliferation. Blocking Notch signalling is sufficient to induce cell proliferation of ependymal cells (Carlén et al., 2009). In SVZ of adult mice, *Knockout* of *Rbp-j* specifically in $Nestin^{+}$ NSCs led to depletion of progenitor cells (Imayoshi et al., 2010). Similar to SVZ, *Rbp-j* mediated Notch signalling is important for maintenance of adult NSCs in the SGZ (Ehm et al., 2010). Further studies, revealed that specifically Notch-1 is important for maintaining $Nestin^{+}$ NSCs in the hippocampus. *In vitro* analysis further confirmed that the number of neurospheres is drastically reduced in Notch-1 deleted NSCs (Ables et al., 2010). In adult mammals, the Notch receptor dll1 controls the quiescence of NSCs. During asymmetric division, one daughter cell acquires dll1 and promotes other cells to become quiescent (Kawaguchi et al., 2013).

Several lines of evidence show that the role of Notch signalling in NSCs activation is evolutionarily conserved. In zebrafish, activation of Notch signalling leads to conversion of type-II (activated stem cells) to type-I (quiescent stem cells), consistent with findings in mammalian NSCs. Interestingly, activated progenitor gives rise to neurons, which indicates that Notch signalling is important to maintain quiescence in progenitors (Chapouton et al., 2010). Moreover, a recent study from the same group showed that specifically Notch-3, but not Notch-1, is important for maintaining quiescence in progenitors (Alunni et al., 2013).

Thus, it appears that the fate of a particular cell could be manipulated by controlling the Notch pathway. It is therefore important to investigate how the Notch pathway is involved in normal homeostasis and regeneration in the newt.

3.4.1.2 Glutamine synthetase

In Paper II, we found that GS mark a specific population of ependymoglial cells in the newt brain. Therefore, in this section I will discuss the role of GS in the brain of vertebrates.

In the mammalian brain, GS plays a pivotal role in removing excess ammonia and regulating glutamate level (Hertz and Zielke, 2004). GS is predominantly expressed in astrocytes (Anlauf and Derouiche, 2013), however, during early development GS expression appears in RGCs around E14 in rat brain and its expression level increases as development proceeds (Akimoto et al., 1993). Neuronal and astroglial contacts are essential for maintaining GS level in the cells.

In fishes and amphibians, no distinct parenchymal $GFAP^+$ astrocytic cells have been identified. The $GFAP^+$ radial glia-like cells act as progenitors (Berg et al., 2010; Grupp et al., 2010) and some of these cells also express GS (Grupp et al., 2010). Hence, it is possible that GS in the radial glia-like cells may perform a similar function as in mammalian astrocytes to regulate glutamate and ammonia level. GS expression pattern has been studied in zebrafish, where, most ventricular proliferating cells retain GS, but the proliferating cells in tectum and dorsolateral telencephalon do not express GS (Grupp et al., 2010). This indicates a heterogeneity of NSCs in zebrafish that can be determined based on GS expression. The expression pattern and role of GS has been not elucidated in newts and it is of great importance to understand their role in NSCs heterogeneity.

3.5 INJURY-INDUCED NEUROGENESIS

All vertebrates retain moderate neurogenic potential as adults. However, not all the vertebrates retain the ability of CNS regeneration after injury. Especially, mammalian CNS appears to have limited ability for regeneration. However, certain fish and salamanders are able to regenerate the CNS after injury. In the sections below, I discuss how NSCs respond to different injury models.

3.5.1 Mammals

A number of injury models have been developed in rodents. Ischemic injury models established in rodents are known to cause neuronal cell death as well as elicit a neurogenic response. Ischemic injury, which restricts blood circulation, causes diminished oxygen supply to the tissue which in turn leads to tissue damage. Ischemic injury induces increased neurogenesis both in SVZ and SGZ (Jin et al., 2001). Ischemic injury-induced activation of NSCs leads to migration of neuroblast to the infarct area (Parent et al., 2002). Functional lineage studies revealed that NSCs from SVZ migrate toward the infarct area and it takes two-four weeks for neuroblasts to migrate to the injured striatum (Ohab et al., 2006). However, proper integration of these neurons in the injured area is very restricted.

Likewise, stroke induces activation of quiescent ependymal cells (type-E) in the SVZ, but activation of these cells leads to depletion of ependymal cells (Carlén et al., 2009). Recent *in vivo* tracing analysis revealed that reactive astrocytes in the injured areas were predominantly produced from the SVZ. This study also proposed that overexpression of Ascl1, a pro-neural transcription factor, is sufficient to convert reactive astrocytes into neurons (Faiz et al., 2015).

Seizures are also known to induce NSCs activation both in SVZ and SGZ (Jessberger and Parent, 2015). Studies on the role of seizure on NSCs are predominantly focused on the SGZ, as this area is known to play a major role in learning and memory. Kainic acid, an agonist of neuroexcitatory amino acid acts on glutamate receptors, and administration of kainic acid induces neuronal cell death (Pollard et al., 1994). Concomitantly in neonatal rats, kainic acid is also shown to induce hippocampal progenitor cell proliferation, and cell migration (Dong et al., 2003). Electroconvulsive shock in rats leads to activation of quiescent NSCs and induces neurogenesis. Vascular endothelial growth factor-mediated signalling is important for activation of NSCs in electroconvulsive shock-induced lesioning (Segi-Nishida et al., 2008). In a recent study, kainic acid-induced hyperactivation of hippocampal NSCs led to their depletion, which in turn induced the generation of astrocytes (Sierra et al., 2010).

A model for Parkinson's disease has been established in rodents, primarily by administration of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) or 6-OHDA (6-hydroxydopamine) which leads to ablation of dopaminergic neurons (Chen et al., 2001; Zhao et al., 2003). Evidence indicates that a dopaminergic lesion causes activation of resident progenitors in the midbrain (Zhao et al., 2003). However, functional integration of these neurons has not been studied. Another study indicates that dopamine per se is important during normal homeostatic proliferation and lesioning of dopaminergic cells and denervation of dopaminergic projection leads to a reduction in the proliferating progenitors both in SVZ and SGZ (Baker et al., 2004; Höglinger et al., 2004). A recent study showed that lesioning of olfactory bulb dopaminergic neurons by 6-OHDA elicits a regenerative response with increased migration of neuroblasts to the injured area and behavioural deficit recovery after two months (Lazarini et al., 2014). Nevertheless, the long-term functional integration and electrophysiological properties of the newborn neurons were not measured in the study.

Regardless of endogenous NSCs activation, resident astrocytes also appear to be activated upon injury. Reactive astrocytes retain some stem cell features, and have the potential to give rise to neurospheres *in vitro* (Götz et al., 2015). *In vivo*, these reactive astrocytes are

known to induce scar formation and are considered to be the hallmark of mammalian CNS regeneration. However, lineage tracing studies revealed that striatal astrocytes could produce $Ascl1^{+}$ Dcx^{+} neuroblasts after stroke. Inhibiting Notch signalling in striatal astrocytes is sufficient to induce neuroblast production (Magnusson et al., 2014). These findings indicate that local astrocytes could be converted to neurons. Models using both invasive and non-invasive injury methods show a disparity between the two approaches. Non-invasive injury such as transgenic animals with amyloid beta accumulation or p25-mediated neuronal injury produces reactive astrocytes with limited neurosphere-forming capacity *in vitro*, whereas, an invasive injury (stab wound or MCAO injury) elicits reactive astrocytes with maximum neurosphere-forming capacity (Sirko et al., 2013).

Even though neurogenesis and neuronal injury response have been intensively studied for the past two decades, there is still no clear report showing that the mammalian brain is able to functionally regenerate lost neurons. Therefore, it is critical to study animal species that retain unique regenerative ability. Ideally, findings in the regenerative species should be compared with data obtained in mammals in order to find the potential mechanism to induce endogenous NSCs to regenerate lost neurons in species where regeneration does not normally occur.

3.5.2 Birds and Reptiles

Limited neuronal regenerative studies have been performed both in birds and in reptiles. Regeneration in birds has been mostly studied in relation to learning and memory. In ring dove, electrolytic lesioning (lesion induced by electrical shock) of the hypothalamus leads to necrosis and behavioural deficits. BrdU pulse-chase experiments show that new neurons are added to the lesioned area. Within six weeks most of the cells were regenerated, and by eight weeks the neurons matured to produce long projections leading to a behavioural recovery in ring dove (Chen et al., 2006). As discussed earlier, in adult songbird new neurons are added to the HVC. Ablation of neurons in HVC leads to increase in new neuronal addition in HVC. Intriguingly, a study demonstrated that the ablation of projection-neurons from HVC-RA led to complete recovery in two months, however HVC-Area-x projection neurons were not regenerated. HVC projection to RA is important for song production but not Area-x projection (Scharff et al., 2000). This indicates the region-specific regenerative preference based on their functional requirements in this species.

In reptiles, especially in lizards, *Podarcis hispanica*, neuronal injury with 3-acetylpyridine (3-AP) has been extensively studied. 3-AP has been shown to induce neuronal cell death, which in turn leads to an increase in neurogenesis. Autoradiographic analysis showed an

increase in ^{3}H-thymidine-labelled cells by two weeks after neuronal ablation in the ependymal and sub-ependymal region. Furthermore, the medial cortex showed complete morphological recovery seven weeks after neuronal lesioning (Font et al., 2001; Font, 1991). Stab-lesioning of cerebral cortex performed on the lizard, *Gallotia galloti*, and ultrastructural analysis at different time points indicated recovery and complete regeneration (Romero-Alemán et al., 2004). This study indicates, lizards retain the neuro-regenerative ability to a certain extent.

3.5.3 Fish

The brain of adult teleost fish appears to retain widespread neuronal regeneration ability due to the persistence of radial glial-like cells (Becker and Becker, 2015; Kroehne et al., 2011; Zupanc and Clint, 2003). Stab-injury is the most commonly used injury model in the teleost fish brain, and this type of injury in the cerebellum of ghost knife fish (*Apteronotus leptorhynchus*) leads to cell death. BrdU pulse-chase experiments have shown an increase in cell proliferation and regeneration after injury. The radial glial-like cells have been identified as the source which contributes to the regeneration of fish cerebellum (Zupanc, 1999; Zupanc and Zupanc, 2006).

Recently, a stab-lesioning model has been established to study neuronal regeneration in zebrafish. Stab-lesioning of telencephalon leads to neuronal cell death, which followed by activation of proliferation. Genetic lineage tracing analysis with *her4.1-gfp* positive radial glial cells revealed that they respond to injury. These activated radial glial cells were found to contribute to neuronal regeneration in the fish telencephalon (Kroehne et al., 2011). In another study, stab-lesioning of zebrafish telencephalon also led to the activation of ventricular progenitor cells. In this lesioning model, S100b^{+} type-II cells, and type-III neuroblast, were up-regulated in the ventricular zone and contributed to regeneration. Moreover, transient accumulation of microglia and oligodendrocytes occurs at the injury site, but it does not lead to scar formation. It has been hypothesised that absence of scar formation may be one possible reason behind the neuronal regeneration ability in zebrafish (März et al., 2011).

Progenitor cell activation also occurs during cerebellum regeneration in zebrafish. Recently, lineage tracing studies were performed during cerebellum regeneration which indicates distinct progenitor activation after injury. In zebrafish cerebellum, *ptf1a*+ ventricular zone radial glial-like cells and nestin^{+} neuroepithelial-like cells were identified as NSCs. In juvenile fish, radial glial-like cells generate all major cell types, whereas, in an adult fish, the radial glial-like cells become quiescent and do not contribute to neurogenesis

after injury. On the other hand, nestin^{+} neuroepithelial-like cells are active even in the adult stage and contribute to neurogenesis after injury. This indicates heterogeneity among stem cell activation after injury and also the limited regenerative capacity in zebrafish cerebellum (Kaslin et al., 2017).

3.5.4 Amphibians

Amphibians, which include *Xenopus* and salamanders, have been extensively studied in neuronal regeneration research. In *Xenopus*, the regenerative ability declines as development proceeds. Larval *Xenopus* is known to regenerate the CNS, however, its regenerative ability decreases during metamorphosis and is completely lost in adulthood (Endo et al., 2007). On the contrary, the urodele amphibians have extensive regenerative potential even in adulthood. In the great crested newt, regeneration of the optic tectum was evaluated by ^{3}H-thymidine incorporation (Minelli et al., 1987), but a detailed cellular analysis was lacking in this study.

6-OHDA-mediated dopamine-lesioning has been developed in the red-spotted newts. Administration of 6-OHDA selectively ablated the dopaminergic neurons and activated GFAP^{+} quiescent ependymoglial cells, which contributed to the functional regeneration of dopaminergic neurons. In this model, it took approximately 30 days to regenerate the lost neurons (Berg et al., 2011; Parish et al., 2007). Recently, partial regeneration was demonstrated in the axolotl after surgical removal of a segment of the telencephalon. This injury induced a regenerative response that depended upon olfactory cues and involved activation of GFAP^{+} progenitor cells (Maden et al., 2013). In another study in axolotl, major lesioning in pallium led to the regeneration of lost neuronal cell types. The lesioned area was repaired by four weeks, and BrdU labelling showed that a majority of BrdU^{+} cells expressed the neuronal marker NeuN by 11 weeks in the injured area. An interesting finding from this study is that although several neuronal cell types were regenerated, tissue architecture was not fully recovered (Amamoto et al., 2016).

As I discussed earlier, the neurogenic potential is widespread in aquatic vertebrates, and in mammals, only two niches retain this ability. Moreover, NSCs in all these species appears to be heterogeneous in nature. Nevertheless, upon injury, fishes and salamanders are able to regenerate lost neurons. Injury induces progenitor cell activation in all studied species to some extent, but it does not lead to the functional recovery in mammals. Hence, there is a need for further comparative analyses to identify critical differences between regenerative and non-regenerative species.

4 ROLE OF ROS IN REGENERATION

Oxygen is a vital factor for the existence of organisms on this planet and they utilise oxygen for their metabolic activity. Mammals solely use their lungs to uptake oxygen from their environment. However, several aquatic vertebrates predominantly use their gills and skin, in addition to lungs for their oxygen consumption (Johansen, 1971; Piiper, 1982; Shield and Bentley, 1973). Yet, certain species, like aquatic black-bellied salamanders exclusively depend on their skin to consume oxygen, as gills and lungs do not exist in their adulthood (Maginniss and Booth, 1995). Environmental oxygen level is around 21% in the atmosphere and utilization of oxygen by different organs and cells varies with their metabolic requirements. After consumption, only two to nine percent of oxygen is able to reach different organs in mammals (Brahimi-Horn and Pouysségur, 2007; Mohyeldin et al., 2010). The brain, one of the most metabolically active organs, which alone takes up to 20% of the oxygen consumed by humans in resting state (Harris et al., 2012; Mink et al., 1981).

Deprivation of oxygen, even for few minutes leads to deleterious effects on mammals (Michiels, 2004). However, non-mammalian vertebrates, certain fishes, salamanders, and turtles are well known to experience extremely low level of oxygen in their natural habitat (Maginniss and Booth, 1995; Nilsson, 2004; Ultsch, 1985). Most intriguingly, these vertebrates also retain the ability to regenerate lost or damaged organs (Berg et al., 2011; Kroehne et al., 2011). In this section, I discuss organisms that experience fluctuations of oxygen in their natural habitat, and how these fluctuations in oxygen correlate with their regenerative ability. Specifically, I discuss the role of ROS in stem cells and regeneration.

4.1 HYPOXIA TOLERANCE IN VERTEBRATES

Non-mammalian vertebrates, especially the fish and amphibians, experience variations in oxygen in their environment. Depends on their habitat, several factors impact oxygen availability. During winter, lakes and ponds are usually frozen due to extremely low temperature. In these conditions, there is a reduction in oxygen level in lakes and ponds. Availability of insufficient sunlight to produce oxygen through photosynthesis, and rapid utilisation of oxygen by species living under the ice are central to the reduction in oxygen level. Persistence of this condition for a long time can lead to hypoxia or even anoxia (Nagell and Brittain, 1977). There are a number of aquatic species known to live under these extreme environmental conditions. For example, the crucian carp is known to tolerate an extremely low level of oxygen close to anoxia and they survive the winter time for months (Nilsson, 2004). Goldfish are also appeared to survive hypoxic conditions but

compare to crucian carp they have a very low level of tolerance to hypoxia (Bickler and Buck, 2007). Zebrafish, which lives in a tropical habitat, is also known to tolerate extreme hypoxia. During dark hours where there is no photosynthesis and utilisation of oxygen is rapid, the river becomes hypoxic and experimental studies have shown that zebrafish can survive hypoxia for few minutes to days (Braga et al., 2013; Cao et al., 2008).

Apart from fishes, turtles and snakes also tolerate extreme hypoxic conditions. Among turtles, *Chelydra serpentina* is known to survive in hypoxia/anoxia for months at 3°C (Ultsch, 1985). Red-sided garter snakes can live up to two days under anoxic conditions (Hermes-Lima and Zenteno-Savín, 2002). Among aquatic salamanders, the black-bellied salamander copes with mild to moderate level of hypoxia (Maginniss and Booth, 1995). The red-spotted newt appears to live under ice for a prolonged time during winter months. Detailed information on how long they survive under ice and what level of hypoxia/anoxia they experience is not clear. However, researchers have found that they do live and sometimes breed under the ice-covered ponds (George et al., 1977). Therefore, it is likely that red-spotted newt also experiences hypoxia in their natural environment.

4.1.1 Impact of hypoxia and re-oxygenation

Organisms that live in hypoxic or anoxic conditions have adopted different strategies to survive these conditions. Either the organisms adapt to utilise the available oxygen efficiently or reduce their metabolic activity. It has been shown in crucian carp that during hypoxic exposure for seven days, the gill lamella protrudes for efficient uptake of oxygen (Sollid, 2003). Additionally, crucian carp haemoglobin has a high affinity for oxygen (Nilsson, 2004). Nevertheless, crucian carp also reduces its protein synthesis in muscle and liver by 50–80%, which considered being energy costly for the cell (Nilsson, 2004; Smith et al., 1996), and turtles become completely inactive during anoxic condition (Ultsch, 2006). Apart from these adaptations, species also need an efficient way to remove metabolic by-products such as lactate, which accumulates during hypoxia. Turtles and crucian carps have established ways to combat lactate-mediated metabolic acidosis during hypoxia. Turtles convert lactate to calcium lactate, which is transported from the blood to their shell to elude metabolic acidosis. Conversely, crucian carp convert lactate to ethanol which is later excreted through the gills (Bickler and Buck, 2007; Jackson, 2004). Hypoxia followed by re-oxygenation leads to an increase in intracellular reactive oxygen species (ROS) level and organisms have evolved adaptations to minimise it. Studies with red-sided garter snakes and goldfish demonstrated an up-regulation of certain antioxidants during hypoxia/re-oxygenation as a way to deal with ROS (Bickler and Buck, 2007; Hermes-Lima and Zenteno-Savín, 2002; Larson et al., 2014). Similarly, heat shock proteins become up-

regulated during re-oxygenation in turtles, which could reduce the level of ROS (Ramaglia, 2004). Another major factor, neuroglobin is also up-regulated in turtle during hypoxia/re-oxygenation and silencing of neuroglobin RNA leads to further increase in ROS during re-oxygenation (Larson et al., 2014). These findings indicate that neuroglobin increase likely to reduce ROS level in turtles.

Although, different factor contributes to hypoxic adaptation, still a large number of turtles die after extreme hypoxic conditions (Bickler and Buck, 2007; Dinkelacker et al., 2005). Moreover, experiments with zebrafish have shown that hypoxia leads to brain damage and behavioural deficits (Braga et al., 2013). The crucian carp is also prone to brain injury despite their extraordinary adaptations to extreme anoxia. A recent study illustrated that anoxia followed by one day of re-oxygenation leads to a three-fold increase in cell death and deficit in behaviour in the crucian carp (Lefevre et al., 2017). These examples show that although species develop different strategies to adapt and survive hypoxia/anoxia, tissue damage may still occur in these conditions. Interestingly, both zebrafish and crucian carp recover after their injury (Kroehne et al., 2011; Lefevre et al., 2017). Therefore, it is likely that in addition to different adaptations to survive hypoxia/anoxia-induced injury, if the tissue damage occurs then these species also should have developed ability to repair the damaged tissues.

If an organism retains the ability to regenerate, it is intriguing to know how and by which mechanism they do regenerate. It has been shown that there is ROS accumulation during hypoxia/re-oxygenation in several contexts (Granger and Kvietys, 2015; Li and Jackson, 2002). Furthermore, ROS signalling has been implicated in a number of cellular pathways and it is therefore important to study their role in regeneration.

4.2 REACTIVE OXYGEN SPECIES

Oxygen species that are more reactive than free oxygen is collectively called reactive oxygen species (ROS) (Chaudhari et al., 2014). Several forms of ROS are present in an organism, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen (O_2), and hydroxyl-radical ($^{\cdot}HO$) (Apel and Hirt, 2004; Sharma et al., 2012). While ROS are considered to be highly reactive, several antioxidants and enzymes that can eliminate ROS to maintain the redox status of the cell. Superoxide dismutase (SOD) is one such enzyme, which readily converts superoxide to H_2O_2, and H_2O_2 is degraded to water and oxygen molecule by catalase (Fukai and Ushio-Fukai, 2011). Other enzymes such as glutathione peroxidase (GPx), glutathione reductase (GRx) and thioredoxin reductase (Thx-

R) also play a major role in the antioxidant defence in the cell (Valko et al., 2007). Apart from antioxidant enzymes, non-enzymatic compounds including glutathione have a role in maintaining redox status of the cell (Dröge, 2002; Ostrakhovitch and Semenikhin, 2013; Valko et al., 2007). Although antioxidant system regulates ROS level, low to moderate level of ROS is present in all cells and have shown to regulate a number of cellular processes (Zhou et al., 2014).

4.2.1 Source of ROS

In homeostatic condition, the cells produce ROS in several ways but it is the mitochondria that produce the majority of ROS. Apart from mitochondrial-mediated ROS production, NADPH oxidases-mediate the production of non-mitochondrial ROS.

Mitochondria are the major energy source of cells. The mitochondrial electron transport chain is one of the major producers of ROS and is located in the inner membrane of mitochondria. The electron transport chain has four complexes numbered I to IV. During oxidative phosphorylation, electron transfer occurs from complex I till IV and reduce O_2 to form water. Leakage at complex I and III leads to a reduction of an oxygen molecule to produce superoxide anion (Forkink et al., 2010; Murphy, 2009). This superoxide is further converted to more stable H_2O_2. H_2O_2 is more stable than the superoxide anion and known to act as a second messenger in a number of signalling pathways (Sies, 2014).

Another major source of ROS production in the cells is mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) complexes. They are clusters of transmembrane proteins and altogether seven Nox family of enzymes are found in mammals (Nox 1-5 and Duox1-2) (Lambeth, 2004; Prozorovski et al., 2015). NADPH oxidases also utilises oxygen to produce superoxide anion which in turn is readily converted to H_20_2 (Steinbeck et al., 1992). NADPH oxidases are involved in several cellular functions that include defence mechanism against pathogens, and signal transduction (Geiszt and Leto, 2004). Apart from mitochondrial and NADPH oxidase-mediated ROS production, enzymes such as lipooxygenase, xanthine oxidase, cyclooxygenase, and monooxygenase are also capable of producing ROS in the cell (Di Meo et al., 2016; Kuppusamy and Zweier, 1989).

In the context of hypoxia/re-oxygenation, it is essential to recognise the nature of ROS production and how they are implicated in tissue damage and/or regeneration. In cultured mammalian neuronal cells, hypoxia and re-oxygenation studies have identified several sources of ROS production. Mitochondrial-mediated ROS are produced early during

hypoxia, whereas xanthine oxidase is responsible for the second burst of ROS, which lead to neuronal cell death. In addition, NADPH oxidase-mediated ROS production is increased only during the re-oxygenation period (Abramov et al., 2007). This indicates that during re-oxygenation, NADPH oxidase-mediates the ROS synthesis, at least in *in vitro* neuronal culture condition.

Irrespective of their source, ROS play a major role in several signalling pathways. ROS have been implicated in MAPK pathway, and a number of mitogenic signals mediated by FGF2, PDGF and EGF could activate NADPH-mediated ROS production (Heppner and van der Vliet, 2016; Kang et al., 1998; Son et al., 2011). Wnt-mediated signalling is also known to elevate ROS level in the cells (Caliceti et al., 2014; Covarrubias et al., 2008). ROS-mediated signalling pathways are known to regulate quiescence, self-renewal, differentiation, and senescence/apoptosis (Bigarella et al., 2014; Ji et al., 2010). ROS appears to elicit different responses based on the level of ROS present within each cell. Further below I will consider the roles of ROS in stem cells and the importance of ROS signalling in certain regenerative condition.

4.2.2 Role of ROS in stem cells and regeneration

4.2.2.1 ROS in stem cells

Adult stem cells are located in a specific niche, and several factors influence their fate choice. ROS is one such factor that regulates adult stem cell fate. Adult stem cells generally have high metabolic rate and shown to produce ROS during self-renewal and differentiation (Bigarella et al., 2014). Here I will look at few examples of how ROS regulate adult stem cells.

Hematopoietic stem cells (HSCs) occupy two different niches in the bone marrow. Quiescent HSCs located in the osteoplastic niche express very low level of ROS. These cells retain long-term self-renewing capacity and are considered to be bona fide stem cells. HSCs are usually in a quiescent state. Low level of increase in ROS activates quiescent HSCs, while the high level of ROS leads to exhaustion of HSCs (Jang and Sharkis, 2007). Studies with *Foxo knock-out* mice showed that *Foxo* is essential for regulating ROS level and absence of *Foxo* leads to exhaustion of HSCs (Tothova et al., 2007). Therefore, the level of ROS in HSCs is a determining factor, indicating whether HSCs are quiescent or proliferative. ROS also have been shown to regulate mesenchymal stem cells (MSCs). MSCs are multipotent and give rise to adipocytes, chondrocytes, and osteocytes (Ullah et al., 2015). ROS are essential for differentiation of MSCs, and inhibition of Nox-4 leads to

the reduction in adipocyte differentiation. However, once adipocytes differentiated into a mature state, Nox-4 becomes down-regulated in the matured adipocytes. Thus, a Nox-4 mediated ROS increase in MSCs is essential for adipocyte differentiation (Chaudhari et al., 2014; Kanda et al., 2011; Schröder et al., 2009). An interesting finding by Schröder (Schröder et al., 2009) is that Nox-1 does not influence the differentiation of MSCs and only Nox-4 is required for their differentiation. The data taken together assign specific roles for Nox isoforms. Moreover, in spermatogonial stem cells, both *in vivo* and *in vitro* analysis revealed that Nox-1-mediated increase in ROS is essential for self-renewal through the p38-MAPK and JNK pathways in mice (Morimoto et al., 2013).

Similar to other stem cells, ROS have been implicated in the regulation of NSCs located in both SVZ and SGZ. In embryonic NSCs, p53-mediated regulation of ROS level is essential for the inhibition of premature neurogenesis. In p53 *knock-out* mice, elevation of ROS has been observed in NSCs, which in turn leads to neuronal differentiation (Forsberg et al., 2013). In SVZ of an adult mouse, NADPH oxidase-mediated increase in ROS level is important for NSCs self-renewal. Blocking of Nox with apocynin decreases ROS level, which leads to a reduction in NSCs proliferation. Analysis of mutant Nox-2 mice further confirmed that Nox-mediated increase in ROS level is required for PI3K/Akt-mediated cell proliferation (Le Belle et al., 2011). In adult hippocampus, NSCs in SGZ also retain a high level of ROS during proliferation. Nox-2 *knock-out* studies observed that Nox-2-mediated increase in ROS is important for proliferation of hippocampal NSCs (Dickinson et al., 2011). However, apart from NADPH-mediated ROS role in stem cells, mitochondrial-mediated ROS also play a significant role in NSCs specification. Evidence from *in vivo* and *in vitro* studies indicate the presence of high level of ROS in differentiating neurons, both during embryonic development and in the adult stage. Inhibiting ROS during neuronal differentiation by antioxidants did not reduce neuron numbers but increased the ratio of smaller to larger neurons (Tsatmali et al., 2006, 2005). In addition, a recent *in vitro* study showed that mitochondrial-mediated increase in superoxide anion regulates cortical neural progenitor differentiation into neurons (Hou et al., 2013). These studies indicate, distinct roles of ROS depending on whether they are generated by NADPH oxidase or by the mitochondria. However, the precise role of mitochondrial and NADPH oxidase-mediated ROS signalling in NSCs is still unclear and requires further studies.

From the examples discussed above, it is apparent that ROS play a major role in the regulation of adult stem cells. The regulation is also dependent on the level of ROS present within the internal milieu and the cells elicit responses accordingly. One of the major hurdles is to know what determines the precise concentration of ROS present within a particular cell type of a specific species. What should be regarded as a low and high level of

ROS across phylogeny? This question is relevant because the oxygen uptake and the level of ROS may vary largely among organisms. Currently, there is no sufficient data available on these aspects and hence it is essential to carry out wider comparative studies among tetrapods to understand ROS role in depth.

4.2.2.2 ROS in regeneration

In addition to the role of ROS in normal homeostatic processes, a number of recent studies show evidence for their involvement in regeneration in several species.

During zebrafish heart regeneration, induced by hypoxia followed by re-oxygenation, there is an inflammatory response and an increase in ROS signalling, which occurs within six hours after injury. The authors also recorded an increase in cell proliferation, which peaks at seven days after injury (Parente et al., 2013). Although the exact role of ROS has not been discussed in this context, one could speculate that ROS caused both an injury as well as activated regenerative proliferation. Nevertheless, recent transcriptional analysis by Peidong Han (Han et al., 2014) shows that after myocardial injury seven ROS related genes are up-regulated including Duox/Nox-2. Further, they concluded that H_20_2 increased after injury and that H_20_2 was sufficient to induce myocardial regeneration. They also found that down-regulation of Dusp6 mediated by H_20_2 is necessary for zebrafish heart regeneration. This confirms that ROS signalling is essential for zebrafish heart regeneration (Han et al., 2014).

During *Xenopus* larval tail regeneration, amputation of tail leads to activation of ROS signalling within hours and it persists at the injury site during the entire regeneration period. ROS increase is required for the early and intermediate stages of regeneration, since blocking NADPH oxidase, using apocynin, results in a reduction in ROS signalling and counteracts tail regeneration. Elevated ROS level is also correlated with an increase in Wnt signalling, and fgf20 up-regulation. Fgf20 is not only increased but is also essential for regenerative response in *Xenopus* larval tail regeneration (Love et al., 2013).

The source of ROS, and whether it acts in a paracrine way during regeneration have been investigated in several systems. During *Drosophila* imaginal disc regeneration, ROS levels are increased in the wound region. Further *ex vivo* imaging has shown that ROS produced by apoptotic cells at the injury site impact the neighbouring cells, demonstrating a different source of ROS in regeneration (Santabárbara-Ruiz et al., 2015). During gecko tail regeneration, Nox-2 mediated increase in ROS signalling occurs in muscle cells. By transcriptional analysis, and *in viv*o blocking of Nox, the authors concluded that ROS is essential for gecko tail regeneration (Zhang et al., 2016). In Planarians, increase in initial

ROS level establishes the correct anterior/posterior identity during regeneration. Interestingly, low-level of ROS does not alter the proliferation but impacts the differentiation process. In CNS of planaria, reduction in ROS level leads to inhibition of neuronal differentiation, which eventually affects the neural regeneration events (Pirotte, 2015). Overall, ROS appear to play a critical role in regeneration either by directly regulating cellular proliferation or controlling the differentiation process. It is difficult to draw a firm conclusion that ROS is sufficient to drive the regenerative process but it appears to be one of the major factors contributing to regeneration in several contexts.

4.2.3 ROS and Inflammation during regeneration

Previous studies have suggested several roles of inflammatory cells during regeneration. Inflammatory cells also produce ROS (Mittal et al., 2014; Sorci and Faivre, 2009), therefore, it is relevant to examine how inflammatory cells and ROS impact on regeneration.

In the context of inflammation and regeneration, most of the studies have been carried out using zebrafish as a model. A key paper by the Michael Brand group identified that inflammatory cells are essential for CNS regeneration (Kyritsis et al., 2012). They showed that stab-lesioning of CNS leads to an increase in inflammatory cells, such as leukocytes and microglia. By inhibition and activation of inflammatory cells, their study revealed that inflammatory cells are sufficient for induction of cell proliferation and proper CNS regeneration. Remarkably, the study also found that the neural progenitors express receptors for the factors secreted by inflammatory cells (Kyritsis et al., 2012). Moreover, experiments on zebrafish fin regeneration further confirmed the role of inflammatory cells during regeneration. Stage-specific depletion of macrophages during fin regeneration has identified that macrophages are essential for fin regeneration. Interestingly, blastema formation was not affected by the absence of macrophages but lack of macrophages affected the cellular proliferation at day three (Petrie et al., 2014). In axolotl, the recruitment of macrophages to the site of injury is essential for blastema formation and successful regeneration of limbs. Blocking of macrophages at the later stage during regeneration delays, but does not completely inhibit regeneration (Godwin et al., 2013). In neonatal mice, heart regeneration also depends upon the presence of macrophages. At postnatal stage P1, the presence of macrophages facilitates vascularization, which is necessary for regeneration to progress. However, cardiomyocyte proliferation was not altered by lack of macrophages. Intriguingly, neonatal mice lose their regenerative ability after postnatal stage P7. Profiling of macrophages indicates that at stage P7 macrophages

secrete factors which facilitates fibrosis, whereas, they do not produce angiogenic factors as compared to the P1 stage (Aurora et al., 2014).

In *Xenopus* tail regeneration, *in vivo* cell tracking analysis indicates that ROS is up-regulated within an hour after tail amputation and inflammatory cells are recruited to the injury site later. Injection of morpholino against *spib*, a transcription factor required for myeloid cell development, reduced the number of inflammatory cells (Love et al., 2013). However, ROS level was not altered after morpholino injection. This study indicates that ROS up-regulated irrespective of inflammatory cells and they are necessary for regeneration. This study was focused on ROS increase related to inflammatory cells in a narrow time window, however, the role of inflammatory cells as a possible long-term response to ROS was not addressed (Love et al., 2013). Furthermore, this study used larval *Xenopus* as a model, but mature immune cells are known to function differently in the adult stage, and *Xenopus* is largely non-regenerative as an adult (Beck et al., 2009). In contrast, in zebrafish heart regeneration, Pediong Han (Han et al., 2014) showed that H_20_2 is up-regulated within three days after tissue resection, which is essential for repressing Dusp6 that eventually leads to activation of genes responsible for regeneration. However, inhibiting H_20_2 production leads to a reduction in inflammatory cell recruitment but it does not affect Dusp6 expression and inhibition of Dusp6 rescues regeneration. This study suggests that H_20_2 -mediated suppression of Dusp6 is sufficient to induce regeneration and exclude the role of inflammatory cells during zebrafish heart regeneration (Han et al., 2014).

Despite the documented role of ROS in regeneration, both inflammation and ROS influence regenerative outcomes (see above), it is difficult, at present, to link each other in regenerative contexts. Nevertheless, H_20_2 can act as a chemo-attractant and recruit immune cells to the site of injury (Niethammer et al., 2009; Wittmann et al., 2012). Monocytes and macrophages are known to play a central role in the clearance of apoptotic cells and facilitate tissue remodelling (Mantovani et al., 2013). Therefore, it is possible that both ROS and inflammatory cells may have roles in regeneration, but this requires further in-depth studies.

4.3 EVOLUTIONARY CONSIDERATION OF REGENERATION AND ROS

Extensive regeneration occurs among all phyla and there is no correlation between the complexity of an organism and its regenerative ability. Though planarians and hydra show great regenerative capability (Bosch, 2007; Rink, 2013), there are many invertebrates lack any regenerative ability (Brockes, 1997). Among the vertebrates, regenerative capacity is restricted in mammals, whereas, fish and amphibians show widespread ability to regenerate organs and appendages (Berg et al., 2010; Sandoval-Guzmán et al., 2014; Zupanc, 2009).

Why certain species retain the ability to regenerate, while others have not, engaged the field of regeneration biology for decades. There is a debate about whether regeneration is an ancestral trait, an epiphenomenon without selective pressure, or a result of natural selection. One model proposes regeneration as an ancestral trait, where certain species retained the regenerative ability and others have lost it during evolution. According to the other model, regeneration has evolved independently in several species owing to certain environmental stimuli. Some evidence supports the view that regeneration might have evolved partially by evolutionary pressure. A section of my thesis focusses on evolutionary pressure and its role in regeneration, and I will discuss this aspect in detail.

The expression pattern of individual genes in a given species, which could promote or counteract regeneration in a certain species might be a result of evolutionary pressure. For example, retinoic acid mediates patterning of the proximal-distal axis in newt limb regeneration, and one of the isoforms of retinoic acid, retinoic acid-δ expression is a specific feature in newts compared to other tetrapods (Ragsdale et al., 1993). Another important gene, Prod-1 appears to exist only in the salamander genome (Garza-Garcia et al., 2010). Also, even closely related salamanders show differences in the gene expression profile. While Pax-3 and Pax-7 are present in newts, Pax-3 is missing in the axolotl genome (Elewa et al., 2017; Nowoshilow et al., 2018). In limb regeneration, dedifferentiation is essential for newt muscle regeneration, but axolotl limb regeneration is independent of dedifferentiation of muscle (Sandoval-Guzmán et al., 2014). Moreover, adult newts regenerate their lens throughout their lifespan, while axolotl cannot (Tsonis et al., 2004). Among teleost fish, zebrafish is known to regenerate the heart but medaka cannot (Lai et al., 2017; Vivien et al., 2016). Similarly, certain planarian species retain extensive regeneration compared to others (Sikes and Newmark, 2013; Umesono et al., 2011). Together, these examples indicate that local evolutionary pressures may act to create species-specific differences, which could explain both the loss as well as the acquisition of regeneration abilities.

4.3.1 Environmental pressure and regeneration

Environmental oxygen fluctuations are likely a factor which contributes to regeneration abilities. As I discussed earlier, crucian carp survive anoxic condition for several months, but this could lead to blindness for a short time. Dan Johansson (Johansson et al., 1997) analysed light-stimulated electroretinogram and the evoked potential in crucian carp during anoxia. He found that a decrease in electrical activity, up to 90% occurs in one hour after anoxia. In this experiment optic tectum activity shown to be reduced in order to preserve energy. However, it is not known to what extent any damage had occurred to the retina before recovery. Nevertheless, it was shown that the crucian carp brain damages after anoxia and they do recover (Lefevre et al., 2017). Moreover, zebrafish also tolerate a moderate level of hypoxia and can regenerate heart and brain (Jopling et al., 2010; Kroehne et al., 2011). It is unlikely that hypoxia tolerance is the sole cause of their regenerative capacities. However, the necessity to replace lost cells during hypoxia and/or re-oxygenation could be a driving force for regeneration to occur in an essential body part. Evolutionary considerations are often theoretical, but laboratory simulation and manipulation of naturally occurring environmental constraint could be a way forward to test some of these hypotheses. In paper III of this thesis, we made an attempt to address this problem.

5 PRESENT INVESTIGATION

5.1 AIM OF THE THESIS

The overall aim of the thesis is to characterise newt ependymoglial cells and understand the reasons behind their unique regenerative ability.

Paper-I

To evaluate how the newt ependymoglial cells mature and relate this to brain development, and to the acquisition of complex behaviour.

Paper-II

To characterise adult newt ependymoglial cells during normal homeostasis and upon neuronal injury.

Paper-III

To assess the impact of hypoxia followed by re-oxygenation on the newt brain by modelling naturally occurring shifts in environmental oxygen tension.

5.2 PAPER I

5.2.1 Results

The adult newt brain is able to replace lost structures and specific cell types after different types of injury (Berg et al., 2011). In order to provide a framework for future systematic studies of newt brain regeneration, we have analysed the proliferation patterns, ependymoglia maturation and neurogenesis in the developing brain of two newt species, and assessed how the maturation of different brain regions is linked to the acquisition of stereotyped behaviours.

First, we studied the proliferation patterns of ependymoglial cells from early development to adulthood in both *P. waltl* and *N. viridescens*. Proliferation analysis throughout different brain areas revealed that in comparison to *N. viridescens,* the *P. waltl* brain has higher Mcm2 labelling index both during development and in adulthood. In the adult brain, only 4 out 12 brain regions were quiescent in *P. waltl*, whereas in *N. viridescens* 7 out of 12 regions were inactive, indicating a species-specific difference in the distribution of adult neurogenic zones.

Next, we considered ependymoglia maturation. In early larval stages, proliferating $GFAP^+$ cells were devoid of GS expression. With the maturation of ependymoglial cells, the GS expression appeared in some of these cells. This allowed us to distinguish two ependymoglial cell subpopulations, the majority of proliferating cells were GS^-. Label retention analysis with EdU further confirmed that GS^+ cells retain more EdU than GS^- after 30 days, which shows that the GS^+ cells are slowly dividing cells and might have stem cell characteristics.

The appearance of GS in ependymoglial cells correlated with acquisition of quiescence in the different brain areas analyzed. We measured cell cycle length by sequential injection of thymidine analogue, EdU and BrdU. The length of S-phase was estimated to be constant among regions, but the entire cell cycle length varied significantly. Notably, regions that were becoming quiescent retained more slow-cycling population. Overall, there was an increase in cell cycle length during development. Clonal analysis of transgenic newts expressing multicolour *Nucbow* (labels the nucleus) or *Cytbow* (labels the cytoplasm) cassettes further revealed that the clones in proliferating areas were composed of ten-fold more cells than those clones located in the quiescent regions. This suggests an increase in cell cycle length during ontogeny correlated with the acquisition of quiescence.

We next analysed neurogenesis in the forebrain at different larval stages with EdU pulse-chase experiments. Analysis of EdU^+ cells and the dynamics of $NeuN^+$ cells appearance in the parenchyma indicated increased neurogenesis in early-active larvae. However, there

was a decline in neurogenesis during development, and in late-active larvae, most of the EdU^+ cells were restricted to $Sox2^+$ $NeuN^-$ ventricular positions. Further analysis of neuronal maturation in different telencephalic regions showed that the striatum and medial pallium matured first, followed by lateral pallium and ventral pallium with pallidum development occuring last. The sequence of neuronal maturation was correlated with the acquisition of complex feeding and locomotor behaviour.

In the final part of the study, we evaluated the maturation of dopaminergic and cholinergic neuronal subpopulations. Antibodies were used against tyrosine hydroxylase (TH) to label dopamine neurons, and against choline acetyltransferase (ChAT) which identified mature cholinergic neurons. In general, *P. waltl* brain showed a higher number of TH^+ and $ChAT^+$ cells compared to *N. viridescens*. The growth of the analysed subpopulations was found to be region- and species-specific. Moreover, ablation of midbrain dopaminergic neurons by 6-OHDA further identified their involvement in higher cognitive functions, including instrumental learning, fear processing, and decision-making.

5.2.2 Discussion and future experiments

In this study, we showed the appearance of quiescence among the ependymoglial cells in the newt brain. We described how the number of quiescent areas increases during development until adulthood. It is possible that early entrance into quiescence contributes to ependymoglial cells retention in the adult newts. These results are valuable since the existence of ependymoglial cells in the adult newt brain has been previously related to their regenerative potential. Moreover, quiescent progenitors respond to brain insult by re-entering the cell cycle and subsequently contributing to neuronal regeneration. The factors controlling the quiescent state of specific ependymoglial cells are unknown. Further characterisation of ependymoglial cells during ontogeny could identify intrinsic factors important for the dynamics between quiescence and proliferation.

Increase in cell cycle length occurs during vertebrate brain development (Thuret et al., 2015; Watanabe et al., 2015). We confirmed the increase in cell cycle length during development, and moreover, this lengthening in the cell cycle was associated with the emergence of GS^+ ependymoglia. In other words, the maturation of a proliferative region involves longer cell cycles at the population level. The correlation between cell cycle lengthening and differentiation has been reported across diverse model organisms, including mammalian neurogenesis (Hardwick et al., 2015). We showed how an increase in cell cycle length is a characteristic of newt ependymoglia maturation. Cross-species comparisons, together with future studies on newt ependymoglia characterisation at single-cell level could help unravel the factors contributing to quiescence in newts and maintenance of ependymoglial cells.

We also studied developmental neurogenesis in different brain areas and the emergence of stereotyped behaviours in larval development. Our results on this topic provide valuable information, as they represent a reference point for future studies in the comparison between developmental and adult regenerative neurogenesis. To understand how adult neurons are specified after injury, it is essential to know their ontogeny during development. Conversely, the behaviours linked to specific brain areas allow for future systematic studies on neuronal regeneration and the examination of behavioural recovery. These assays will be able to complement previous tests to assess recovery of locomotor behaviour (Parish et al., 2007). We described the timing of the specific neurogenic programs for several dopaminergic and cholinergic subpopulations. This is relevant because newts are able to regenerate both dopaminergic and cholinergic neurons after chemical ablation through proliferation and differentiation of ependymoglial cells (Berg et al., 2011; Berg et al., 2010). However, cell-intrinsic potential, the developmental origin, and retention of cells during regeneration in the adults necessitates further scrutiny. With the availability of genetic tools, it is now possible to develop transgenic newts that will help answer these questions. For example, a transgenic line expressing a dopaminergic progenitor determinant *lmx1a* will be helpful to track the origin of dopaminergic cells.

5.3 PAPER II

5.3.1 Results

Previous studies showed that ependymoglial cells give rise to neurons both in normal homeostasis and after neuronal injury in the adult newt brain (Berg et al., 2010; Parish et al., 2007). However, the heterogeneity of ependymoglial cells, if any, was not explored in these studies. In this paper, we evaluated the heterogeneity of ependymoglial cells in normal homeostasis and upon neuronal injury.

First, we tested whether ependymoglial cells possess stem cell characteristics. Formation of neurospheres in *in vitro* is a hallmark of NSCs. We found that newt brain cells formed neurospheres in primary cell cultures with $GFAP^+$ cells in the centre of the spheres. When growth factors were removed, neurospheres differentiated. We found that the majority of differentiated cells expressed Tuj-1, in addition to the appearance of $GFAP^+$ cells in the periphery of the neurosphere. These data indicats that $GFAP^+$ ependymoglial cells have stem properties.

Next, we analysed $GFAP^+$ ependymoglial cells in *in vivo*. We noticed that NSC markers such as Sox-2 and GLAST were expressed in all ependymoglial cells. However, glutamine synthetase (GS) expression was detected in a subset of ependymoglial cells. The GS^+ subpopulation (type-1) represents the majority of cells in the quiescent regions that we denoted as non-hot spots. GS^- cells (type-2) were, on the other hand abundant in proliferating hot spots. We analysed proliferation and concluded that in the hotspots the majority of proliferating cells are type-2 ependymoglia, while in the non-hotspots type-1 cells are the predominant proliferating cells in normal homeostasis.

Notch signalling is implicated in NSCs regulation, and therefore we assessed Notch-1 expression in ependymoglial cells. Similar to GS expression, we detected Notch-1 in the majority of $GFAP^+$ ependymoglial cells. In hotspots, the majority of $GFAP^+$ cells were $Notch\text{-}1^+$ but the proliferating $GFAP^+$ cells were essentially devoid of Notch-1 expression. In non-hotspots, most of the proliferating $GFAP^+$ cells were $Notch\text{-}1^+$. We also found proliferating $PSA\text{-}NCAM^+$ $GFAP^-$ cells (neuroblasts) only in the hotspots, which indicated that hotspots were constitutively active neurogenic regions.

We further examined the stemness of these two cell types by long-term BrdU label retention and insensitivity to Ara-C treatment, which are considered as stem cell characteristics. BrdU pulse-chase experiments revealed that the majority of type-1 cells retained BrdU after 90 days, unlike type-2 cells which hardly retained any BrdU. Ara-C

treatment is known to selectively eliminate rapidly dividing cells. Ara-C treatment led to a massive reduction in type-2 cells, whereas type-1 cells were not affected by the treatment. These data indicated that type-1 ependymoglia have stem cell properties, and type-2 have transient amplifying characteristics.

Next, we analysed ependymoglial response to neuronal injury. Injection of a specific neurotoxin (AF64A) led to the ablation and subsequent regeneration of cholinergic ($ChAT^+$) neurons. Upon injury, the proliferation of both type-1 and type-2 cells was increased. Interestingly, type-2 cells and PSA-$NCAM^+$ cells appeared in the non-hotspots after injury. These data indicated the conversion of the non-hotspots into neurogenic niches after neuronal ablation. We evaluated the implication of Notch signalling in ependymoglia regulation using N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) which inhibits γ-secretase, thereby Notch signalling. In homeostasis, we found that the proliferation of both type-2 cells and PSA-$NCAM^+$ cells were increased in the hotspots, whereas type-1 cells were not affected. However, upon injury, DAPT treatment did not alter the proliferation of any cell type in the hotspots. Nonetheless, an injury-mediated increase in type-1 cell proliferation was inhibited by DAPT treatment in the non-hotspots.

5.3.2 Discussion and future experiments

The results from the current study indicate that ependymoglial cells are heterogeneous in the newt telencephalon. Type-1 cells are predominately in a quiescent state, whereas the type-2 cells are dynamic and proliferating. In the newt telencephalon, the vast majority of the ventricular cells are type-1 and possess stem cell characteristics. The intriguing question is how type-1 cells are maintained in quiescence. We know that in the midbrain, ependymoglial cells are re-activated by the absence of dopamine to produce new dopaminergic cells after selective ablation (Berg et al., 2010). However, we do not know whether a similar mechanism is active in the telencephalon. Further studies need to be done using a variety of neurotransmitters and exploring the response to injury in different brain areas (Berg et al., 2013). In this paper, we observed a *de novo* appearance of a neurogenic niche in non-hotspots after cholinergic ablation, but the mechanism involved in the possible transformation of type-1, quiescent cells into type-2, proliferating cells remains unknown.

We have evaluated the Notch-1 expression pattern in telencephalic ependymoglial cells. We found different effects of Notch signalling in normal homeostasis and upon injury. Concretely, our data based on DAPT treatment suggest that Notch signalling is involved in the maintenance of quiescence in homeostasis. In the injury context, Notch signalling could increase proliferation of type-1 cells. Due to the limitation of specific technical tools we

could not dissect out the exact role of Notch receptor-mediated signalling. As discussed in Chapter 3 (Section 3.4.1.1), there are different types of Notch receptors identified in vertebrates (Lasky and Wu, 2005). In zebrafish, Notch-3 mediates the quiescence; however, Notch-1b expressed in type-II and type-III cells controls neurogenic differentiation (Alunni et al., 2013). This suggests the presence of different Notch receptors in different cells with various functions. Hence, it is necessary to characterise different Notch receptors and their function in different cell types in newt ependymoglial cells. Moreover, specific knockout of Notch-1 by genetic methods could address whether specifically Notch-1 is implicated in ependymoglial cells regulation or other Notch-mediated signalling is involved. Finally, an age-related decline in NSCs has been shown in mammals (Shook et al., 2012). Hyperactivation of NSCs also leads to depletion of NSCs (Sierra et al., 2015). A testable hypothesis is whether maintaining the vast majority of cells in a quiescent state in the newt brain is a prerequisite for the retention of ependymoglial cells in the adult. If so, could hyperactivating these cells lead to their depletion? One could address this question using transgenic approaches, by manipulating ependymoglial cells with specific cell cycle regulators. This could help us to understand how the newt ependymoglial cells are maintained in a quiescent state.

5.4 PAPER III

5.4.1 Results

In this paper, we aimed to assess the impact of hypoxia followed by re-oxygenation on the newt brain by modelling naturally occurring shifts in environmental oxygen tension.

First, we looked at whether the newt*, Notophthalmus viridescens* could cope with a low level of oxygen. Remarkably, we found that newts could survive under low oxygen conditions, up to 10% for five days. However, we found signs of tissue damage, as hypoxia followed by re-oxygenation led to an increase in neuronal cell death. Moreover, microglial activation was noticed by an increase of proliferating microglia ($IBA1^+$ $PCNA^+$). Neuronal cell death and activation of microglia are hallmarks of injury response in CNS.

We next questioned whether neuronal injury leads to a regenerative response. Five days of hypoxia followed by three days of re-oxygenation led to an increase in proliferation ($PCNA^+$) among $GFAP^+$ ependymoglial cells. Consistently, pulse-chase experiments with EdU indicated an increase in neurogenesis after re-oxygenation. These data revealed that neuronal injury induced activation of ependymoglial cells, which led to neuronal regeneration (EdU^+ HuC/D^+). We evaluated the ROS expression in ependymoglial cells by the superoxide sensitive dye, hydroehtidine (HEt), in order to test whether re-oxygenation leads to increased production of ROS. Hypoxia followed by re-oxygenation increased the HEt signal in the ependymoglial cells. Apocynin, which inhibits NADPH oxidase-mediated ROS production, reduced ROS level in ependymoglial cells. Reduction in ROS led to a decrease in proliferating ependymoglial cells and reduced neurogenesis. These findings indicate that ROS-mediated increase in cell proliferation is crucial for activation of ependymoglial cell proliferation, and thereby neuronal repair.

We next inhibited microglia activation with dexamethasone, to evaluate whether these inflammatory cells are implicated in newt ependymoglia activation after re-oxygenation. Dexamethasone treatment reduced the number of activated microglia after re-oxygenation. However, inhibition of microglia did not alter ependymoglial cell proliferation. Conversely, apocynin decreased ependymoglial cell proliferation but did not affect the number of activated microglia. Taken together, these results indicate that the activation of ependymoglial cells is independent of microglial activation.

Next, we asked whether ROS production was important for neuronal regeneration also during normoxic conditions by lesioning of dopaminergic neurons with 6-OHDA. The degeneration of dopaminergic neurons evoked an accumulation of ROS in ependymoglial

cells. Treatment of newts with apocynin after dopaminergic neuronal ablation led to a reduction of ROS in ependymoglial cells. ROS reduction was accompanied by a decrease in the proliferating ependymoglial cells and to reduced dopaminergic neuronal regeneration. We conclude that injury-induced ependymoglial cell proliferation depends on ROS production during normoxia.

5.4.2 Discussion and future experiments

Our results indicate that hypoxia followed by re-oxygenation leads to brain damage and inflammation. Currently, it is not clear how, but both low oxygen concentration as well as increased ROS could be involved in neuronal death (Banasiak et al., 2000; Poh Loh et al., 2006; Valencia and Morán, 2004). Nevertheless, ROS-mediated activation of ependymoglia proliferation leads to neuronal regeneration in the newt. A detailed study of the signalling molecules impacted by ROS is essential to identify their role in the activation of ependymoglial cells.

In the present study, we demonstrated that ROS is essential for ependymoglia activation. On the contrary, in zebrafish, the activation of inflammatory cells upon injury is important for NSCs activation and functional regeneration (Kyritsis et al., 2012), whereas activated newt microglial cells did not have any impact on ependymoglial cells. Generally, inflammatory cells do play a major role in the clearance of dead cells and tissue remodelling in vertebrates (Koh and Dipietro, 2005) but this aspect is unexplored in the context of newt brain regeneration. Therefore, future studies could be directed towards identifying the potential role of microglia during differentiation and functional integration of neurons during regeneration.

In addition to the brain, the heart is another organ which has high metabolic activity. Given that hypoxia followed by re-oxygenation has an impact on the brain, it is likely to have an impact on the heart as well. The newt heart also regenerates after mechanical injury. Hence, it will be interesting to analyse whether the heart tissues are also vulnerable to hypoxia, and if so, does ROS play a role in heart regeneration. ROS-mediated increase in proliferation and regeneration has been studied only in the brain of *N. viridescens*. Another newt, such as the Iberian newt (*P. waltl)* retains widespread regenerative ability. Future comparative assessments across closely related species could give evolutionary insights in relation to ROS-induced brain regeneration.

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