REGIONAL DIFFERENCES IN THE RESPONSE OF NEURAL STEM CELLS AND THEIR MICROENVIRONMENT TO IONIZING RADIATION

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Cover illustration: Radiation beams directed toward the neurogenic niche; blood vessel (red), astrocytes (orange), radial glia-like stem cell (green), rapidly proliferating progenitor cells (blue), migrating neuroblast (yellow), microglia (grey). Illustration by Charlotta Lindwall.

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ABSTRACT

Radiation therapy is one of the most effective tools for treating malignant tumors; however, cranial irradiation often results in intellectual impairment and cognitive deficits, such as impaired learning and memory. Ionizing radiation generates DNA damage, causing proliferative cells to undergo apoptosis. In most brain regions, the generation of neurons is complete at birth. However, in two discrete regions, the granule cell layer of the hippocampus and the subventricular zone (SVZ) of the lateral ventricle, stem cell continuously proliferate and generate new neurons throughout life. Due to their high proliferative capacity, these cells are particularly vulnerable to ionizing radiation.

The studies in this thesis focused on the immediate and late effects of ionizing radiation on neural stem cells and their microenvironment. We found that a single dose of 6 Gy at postnatal day 9 leads to long-lasting decreases in both stem cell proliferation, as well as neurogenesis, in the adult rat. Even though the two stem cell regions were equally affected by the initial radiation, there was a differential response in stem cell recovery. While hippocampal stem cells were long-term affected; SVZ stem cells seemed to recover with time. In addition, the radiation injury caused an immediate inflammatory response in the postnatal brain, which was not sustained into adulthood. Interestingly, irradiated microglia in the SVZ, but not hippocampus, upregulated several genes coding for growth factors known to promote stem cell maintenance, proliferation and survival. The specific upregulation of these stem cell-related genes in irradiated SVZ microglia could potentially contribute to the recovery of the stem cell population seen in the SVZ, which was lacking in the hippocampus. Taken together, these data demonstrate the pronounced susceptibility of hippocampal stem cells to ionizing radiation, and highlight the importance of shielding this structure from irradiation to minimize functional consequences.

Key words: ionizing radiation, neurogenesis, neural stem cells, inflammation, microglia, stem cell niche, trophic support

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Joniserande strålning är en av de mest effektiva behandlingsformerna för elakartade cancertumörer. Tyvärr kan strålterapi riktad mot hjärnan ge upphov till biverkningar i form av försämrat minne och inlärningssvårigheter. Joniserande strålning skapar skador på DNA, vilket gör att celler som delar sig dör. I de flesta områden i hjärnan föds inga nya nervceller efter födseln, men i två områden, den laterala ventrikelväggen och hippocampus, finns det stamceller som producerar nya nervceller genom hela livet. Stamceller i hippocampus tros bidra till bildandet av nya minnen, och dessa stamceller är extra känsliga för skador orsakade av joniserande strålning.

Målsättningen med avhandlingen har varit att förstå de tidiga och sena biverkningar som orsakas av joniserande strålning, och hur de påverkar stamcellerna och deras närmiljö i hjärnan. Våra studier visar att en medelhög dos (6 Gy; vilket motsvarar den dos barn kan få vid strålbehandling) orsakar en dramatisk minskning i andelen nybildade nervceller. Trots att de två stamcellsregionerna fick lika mycket strålning, återhämtade sig stamcellerna olika bra. Stamceller i den laterala ventrikelväggen återhämtade sig med tiden, men det lyckades aldrig stamcellerna i hippocampus att göra. Vi såg också att den joniserande strålningen initialt, men inte permanent, orsakade en inflammation i hjärnan. Mikroglia (hjärnans egna immunförsvarsceller) i den laterala ventrikelväggen aktiverades av strålningen, och ökade uttrycket av gener som stimulerar tillväxt och överlevnad hos stamcellerna. Att just mikroglia från laterala ventrikelväggen uppreglerar dessa gener kan vara en förklaring till varför stamcellerna i denna region återhämtar sig bättre än stamcellerna i hippocampus. Sammanfattningsvis poängterar denna avhandling hur känslig hippocampus är för joniserande strålning, och visar på vikten av att skydda detta område under strålterapi.

LIST OF ORIGINAL PAPERS

The thesis is based on the following papers:

I. Hellström NA, Zachrisson O, Kuhn HG and Patrone C.
Rapid quantification of neurons and stem/progenitor cells in the adult mouse brain by flow cytometry
Letters in Drug Design and Discovery (2007) 4:532-39

II. Hellström NA, Björk-Eriksson T, Blomgren K and Kuhn HG.
Differential recovery of neural stem cells in the subventricular zone and dentate gyrus after ionizing radiation
Stem Cells (2009) 27:634-41

III. Hellström NA, Ståhlberg A, Swanpalmer J, Björk-Eriksson T, Blomgren K and Kuhn HG.
Unique gene expression patterns indicate microglial contribution to neural stem cell recovery following irradiation
Manuscript

Additional papers not included in the thesis:

Åberg ND, Johansson UE, Åberg MA, Hellström NA, Lind J, Bull C, Isgaard J, Anderson MF, Oscarsson J and Eriksson PS.
Peripheral infusion of insulin-like growth factor-1 increases the number of newborn oligodendrocytes in the cerebral cortex of adult hypophysectomized rats
Endocrinology (2007) 8:3765-72

Diederich K, Schäbitz WR, Kuhnert K, Hellström NA, Sachser N, Schneider A, Kuhn HG and Kneckt S
Synergetic effects of granulocyte-colony stimulating factor and cognitive training on spatial learning and survival of newborn hippocampal neurons
PLoSONE, accepted

ABBREVIATIONS

ATM – ataxia telangiectasia-mutated
ATR – ATM/Rad3-related
BLBP – brain lipid binding protein
Brca1 - breast cancer 1
BrdU – bromodeoxyuridine
Cdc2 – cell division cycle 2
Cdk – cyclin-dependent kinase
Chk – checkpoint kinase
CNTF – ciliary neurotrophic factor
CSF – cerebrospinal fluid
Ct – cycle of threshold
DCX – doublecortin
DG – dentate gyrus
DSB – double strand break
ECM – extracellular matrix
EGF – epidermal growth factor
ES – embryonic stem (cells)
FACS – fluorescent-activated cell sorting
FGF2 – fibroblast growth factor 2
FSC – forward scatter
Gadd45 – growth arrest and DNA damage-inducible 45
GCL – granule cell layer
GFAP – glial fibrillary acidic protein
IGF-1 – insulin-like growth factor 1
IL – interleukin
IMRT – intensity-modulated radiation therapy
LeX – lewis X
LIF – leukemia inhibitory factor
LTP – long-term potentiation
MAP2 – microtubule-associated protein 2
Mdm2 – mouse double minute-2
NeuN – neuronal nuclei
NSC – neural stem cell
OB – olfactory bulb
PCNA – proliferating cell nuclear antigen
PDGF-BB – platelet derived growth factor, dimeric B polypeptides
PGE2 – prostaglandin E2
RT-qPCR – reverse transcription quantitative PCR
SSC – side scatter
SSEA-1 – stage specific embryonic antigen 1
SVZ – subventricular zone
TGFβ – transforming growth factor beta
TNF – tumor necrosis factor
VEGF – vascular endothelial growth factor

For additional gene lists, see supplementary table 1 and 2 in paper III

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INTRODUCTION

Cancer in children younger than fifteen years of age is rare, but corresponds to 0.5% of all cancers in Sweden, approximately 300 new cases per year. The most common forms of childhood cancers are brain tumors and leukemia (Gustafsson et al., 2007). Treatment paradigms are the same for children and adults, consisting of surgical removal, chemotherapy and/or radiotherapy. Although radiotherapy is one of the most powerful tools available in fighting malignant cancer cells, cranial irradiation is often associated with side effects later in life such as intellectual impairment and decreased learning and memory skills (Crossen et al., 1994; Abayomi, 1996; Surma-aho et al., 2001). Radiation results in DNA damage, and in rapidly dividing cells, such as cancer cells, the DNA damage will cause the cell to die. Nerve cells in the brain do not divide and are therefore less affected by radiation. Until recently, the dogma has prevailed that if neurons die for one reason or another, they are lost forever and cannot be replaced. However, pioneering studies from the mid-1960s by Joseph Altman (Altman and Das, 1965) showed that this was clearly not the case and that, indeed, cell renewal takes place in the brains of adult rodents. In the early 1990s, with the discovery of adult neural stem cells, the field of adult neurogenesis was launched. Today, we know that adult neurogenesis occurs throughout life (Kuhn et al., 1996) and in all vertebrate species investigated, including humans (Eriksson et al., 1998; Curtis et al., 2007). The first step in the process of adult neurogenesis is neural stem cell division. Therefore, radiation not only damages tumor cells, but also the brain's stem cells, with direct consequences for the production of new neurons! The studies in this thesis focused on the effects of ionizing radiation on stem cells and neurogenesis. The overall aim was to contribute to a greater understanding of the primary and late effects of ionizing radiation, with the ultimate goal of designing superior treatment modalities for brain cancers.

Adult neurogenesis

Even though the phenomenon of neurogenesis in the adult brain is now widely accepted, it only occurs in two distinct locations: the subventricular zone (SVZ) of the lateral ventricle and the granule cell layer (GCL) of the hippocampal dentate gyrus (DG). The generation of new neurons is a multistep process, including stem cell proliferation, migration to the proper site, differentiation, survival and finally integration (figure 1). Each of these steps is regulated by various factors.

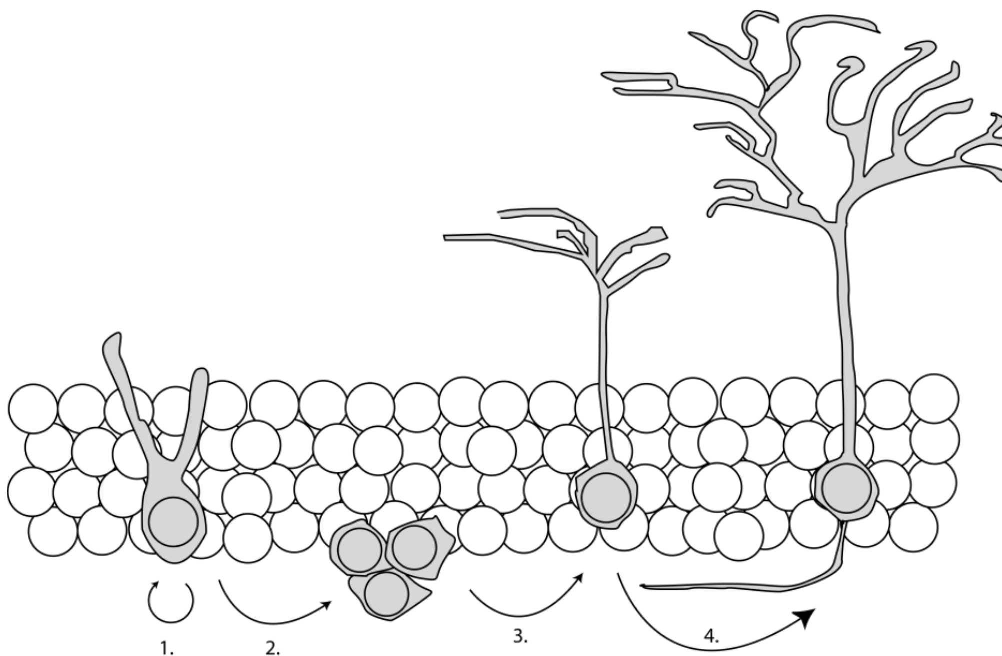

Figure 1. The generation of new neurons is a multistep process. Neural stem cells self-renew (1) and expand (2) the progenitor cell pool by generating rapidly proliferating progenitor cells. Young neuroblasts migrate (3) a short distance into the GCL where they integrate (4) as mature neurons.

Proliferation

For a cell to divide, it must pass through all phases of the cell cycle, starting with the G1-phase, which is the normal, diploid (2N) stage. The cell then synthesizes new DNA during the S-phase, and becomes tetraploid (4N). In

the G2-phase, the cell cycle is briefly arrested, and then subsequently proceeds through division, or M-phase. This results in two daughter cells, each with identical genomes. Each daughter cell either exits the cell cycle to become quiescent or differentiate, or reenters the cell cycle (figure 2).

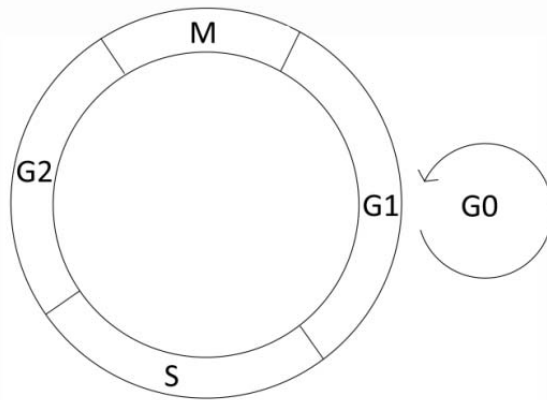

Figure 2. The cell cycle. The start of the cell cycle is the G1-phase, the cell then synthesizes new DNA during the S-phase, arrests for a brief pause in the G2-phase, and then subsequently proceeds through division, or M-phase. This results in two daughter cells, each with identical genomes. Each daughter cell either exits the cell cycle and become quiescent or differentiates (G0), or continues in the cell cycle for another round of division.

During different phases of the cell cycle, various endogenous proteins are expressed that can be used as cell cycle markers. For instance, the proliferating cell nuclear antigen (PCNA) is expressed in all phases of cell cycle except G0/G1 (Kurki et al., 1986). This protein acts as a co-factor for DNA polymerase delta (Prelich et al., 1987). It becomes ubiquinated in response to DNA damage, and participates in the Rad6-dependent DNA-repair pathway (Hoege et al., 2002). Another commonly used protein for detecting proliferation is Ki67, which is expressed during all active phases of the cell cycle, yet absent in quiescent cells. The phosphorylated form of histone H3 is an M-phase-specific marker, which labels cells that are progressing through the final stage of division (mitosis). The use of endogenous markers for proliferation provides a researcher with an instantaneous view of the cell's proliferative state at the time of sacrifice. In contrast, more permanent labels of cell division, used for birthdating and lineage analysis, are achieved through the use of various thymidine analogs. The thymidine analog Bromodeoxyuridine (BrdU) is permanently incorporated into newly synthesized DNA during the S-phase. It is retained within the postmitotic cell, allowing for simultaneous detection of mature cell markers, through the use of specific antibodies, in conjunction with BrdU.

However, even though BrdU provides an opportunity to birthdate a cell and study the phenotype at a later stage, the use of BrdU has limitations in terms of biological availability, as well as detection. BrdU is rapidly degraded in biological tissue, and has a half-life of 15 minutes (Mandyam et al., 2007). Furthermore, incorporated BrdU is eventually diluted below detection level if the cell continues to proliferate. Therefore, researchers frequently utilize multiple BrdU injections over several days to increase the number of labeled cells.

Migration

For a newly generated cell to properly integrate into a neuronal network, it must migrate to the site of integration. In the adult dentate gyrus, newly formed granule cells migrate a short distance into the lower third of the granule cell layer. This migration takes place in close association with radial glia fibers. Stem cells born in the SVZ, however, have a different destination – the olfactory bulb (OB). The distance to the OB is fairly long, *i.e.* several millimeters in rodents (Doetsch et al., 1997). Neuroblasts utilize another mode of migration in this system, the so-called chain-migration. Once the immature neurons reach the olfactory bulb, the chains are dissolved, and the neuroblasts disperse into the granule cell layer or periglomerular layer.

Lineage determination and differentiation

A key feature of stem cells is multipotency – the ability to generate progeny that will differentiate into multiple types of specialized cells. In the case of the brain, the three cell lineages generated from neural stem cells are astrocytes, oligodendrocytes and neurons. Astrocytes are among the most numerous cell types in the brain, serving a wide range of functions in the CNS. They interact with neurons, and provide structural, metabolic and trophic support (Markiewicz and Lukomska, 2006). Astrocytes can be identified by markers such as glial fibrillary acidic protein (GFAP), S-100β and glutamine synthetase (Tanaka et al., 1992). Oligodendrocytes are also a type of glial cell, which function to facilitate nerve transduction by wrapping myelin processes around axons. Oligodendrocytes are identified with markers such as CNPase and myelin basic protein (MBP). There is a wide range of antibodies available to label mature neurons: neuronal nuclei (NeuN) and microtubule-associated protein 2 (MAP2) are two of them.

Integration of newly generated cells

Granule cells of the dentate gyrus receive synaptic input through the perforant path, which originates in the entorhinal cortex. The granule cells, in turn, project to CA3 neurons *via* the mossy fibers. The CA3 neurons relay signals through Schaffer collaterals to cells in the CA1 region. Newborn granule cells migrate into the granule cell layer and integrate into the above-described microcircuit (figure 3).

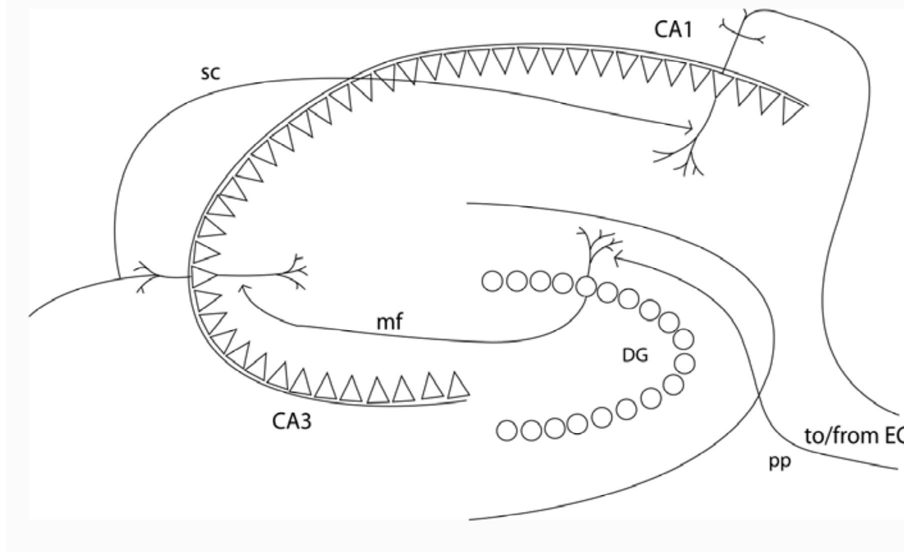

Figure 3. The hippocampal tri-circuit. The synaptic input to the hippocampus is received through the perforant path (pp), projecting onto the granule cells of the dentate gyrus. The granule cells, in turn, project to CA3 neurons via the mossy fibers (mf), and the signal from the CA3 to the CA1 area is relayed through the schaffer collaterals (sc). The output of the hippocampus is relayed back to the entorhinal cortex (EC).

In the olfactory bulb, olfactory epithelium receptor neurons make synaptic connections to tufted and mitral cells, forming clustered structures called glomeruli. The mitral and tufted cells are prenatally generated neurons and project to the rhinencephalon. Adult-generated cells of the olfactory bulb are interneurons, and contribute to OB regulation by modulating the output (figure 4).

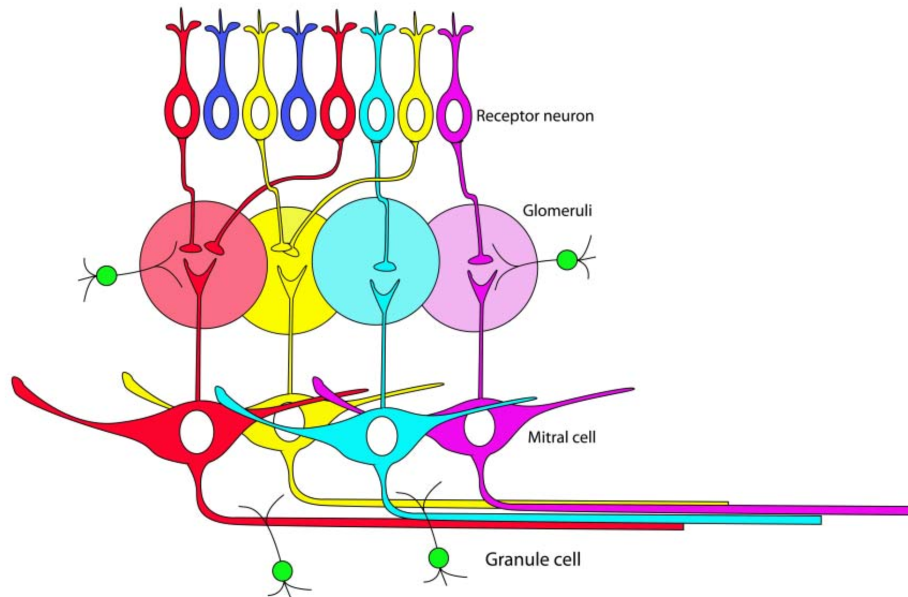

Figure 4. Wiring of the olfactory bulb. Axons from receptor neurons expressing the same odorant receptor gene synapse in specific glomeruli. The glomeruli also contain the apical dendrites of the main output neurons of the OB; the mitral cells. The adult-generated cells (in green) are inhibitory granule cells, situated either in the granule cell layer or the periglomerular layer.

Functional significance of neurogenesis

Even though adult neurogenesis is an accepted phenomenon in the field, the functional significance of these newly generated cells remains under debate. In the dentate gyrus, new neurons are added to the network, rather than replacing lost cells. The newly generated hippocampal granule cells have a lower threshold for evoking long-term potentiation (LTP), which is considered the electrophysiological correlate of learning. This high degree of synaptic plasticity, and the ability to respond to stimuli that older neurons do not react to, might be crucial for formation of new memories (Wang et al., 2000; Schmidt-Hieber et al., 2004). Interestingly, if irradiation is applied to the brain prior to electrophysiological examination, the ability to induce LTP through stimulation of the perforant path is abolished (Snyder et al., 2001). In the olfactory bulb, continuous neurogenesis is required to replace, reorganize and maintain the interneuron system of the OB, but is not crucial for olfactory-related behavior (Imayoshi et al., 2008b). However, results are conflicting, and other studies have indicated that olfactory neurogenesis is indeed necessary for certain behaviors, such as maternal behavior, mate

selection and odor discrimination (Shingo et al., 2003; Mak et al., 2007; Mouret et al., 2008).

The neurogenic niche

In order for neurogenesis to occur *in vivo*, not only must stem cells be present, but the surrounding microenvironment must also be permissive for neuronal development. This microenvironment is often termed “the niche”, and is not a new concept in stem cell biology. Rather, in the 1970s, transplantation studies within the hematopoietic system suggested that somatic stem cells are harbored in specific anatomical locations that also control development *in vivo* (Schofield, 1978). The stem cell niche, with all its components, is the smallest unit of interaction between stem cells and their environment. Apart from neural stem cells, the neurogenic niche contains endothelial cells, ependymal cells (in the SVZ), astrocytes, microglia and an extracellular matrix, all of which contribute to the niche *via* cell-cell contact or through the release of factors (figure 5).

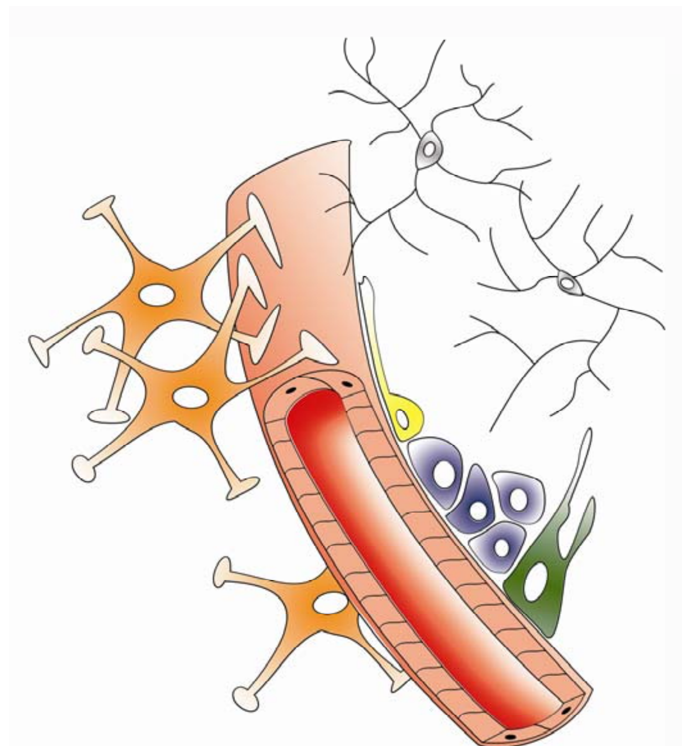

Figure 5. The neurogenic niche. Apart from neural stem cells (green), the neurogenic niche contains progenitor cells (blue), migrating neuroblasts (yellow), endothelial cells (red), astrocytes (orange) and microglia (grey), all of which contribute to the niche *via* cell-cell contacts or through blood stream-released factors.

Endothelial cells

Endothelial cells are key components of the blood-brain barrier, protecting the brain from potential toxic agents circulating in the blood. They form the inner lining of blood vessels, and regulate the exchange of hormones, metabolites and small molecules between capillaries and the surrounding parenchyma. Theo Palmer and colleagues (Palmer et al., 2000) showed that in the hippocampal dentate gyrus, clusters of proliferating neural stem cells are often found in close proximity to the vasculature, especially capillaries. Similarly, a close relationship between precursor cells in the SVZ and vasculature has also been established (Shen et al., 2008). Another link between neurogenesis and angiogenesis is through vascular endothelial growth factor (VEGF), which has been shown to have strong effects on adult neurogenesis (Jin et al., 2002; Schanzer et al., 2004).

Ependymal cells

In the subventricular zone, cells are exposed to yet another source of regulatory factors – the cerebrospinal fluid (CSF). Ependymal cells line the ventricular wall, shielding the parenchyma from the CSF. Ependymal cells have multiple cilia projecting into the ventricular lumen, employed to circulate the CSF through the ventricular system. Similar to the endothelial cells lining the blood vessels, ependymal cells also serve as sentinels, allowing for certain metabolites and hormones to pass through to the underlying neurogenic niche.

Astrocytes

Astrocytes are one of the most abundant cell types of the brain, and in many regions outnumber the neurons by at least two to one (Markiewicz and Lukomska, 2006). Astrocytes have traditionally been considered supporting cells for neurons, giving structural and functional support, and modulating neurotransmitter reuptake. However, increasing evidence points to a much wider range of functions than previously appreciated, among them the role of stem cells (Buffo et al., 2008). Currently, there is no single marker to distinguish an astrocytic-like stem cell from non-neurogenic astrocytes. In the subventricular zone, cells expressing GFAP, nestin and Sox-2, collectively called “B-cells”, are thought to be the quiescent stem cells (Doetsch et al., 1997). Similarly, in the subgranular zone of the dentate gyrus, radial glia-like GFAP+/nestin+/Sox-2+ cells are also believed to be the quiescent neural

stem cells (NSC) (Seri et al., 2004). In contrast, some researchers suggest an ependymal origin of the stem cell (Johansson et al., 1999; Coskun et al., 2008).

Microglia

Microglia are the immune-competent cells of the brain, currently believed to be derived from hematopoietic cells invading the brain during late embryonic/early postnatal life (Navascues et al., 2000). In their resting state, they are constantly surveying the brain, looking for potential damage. Even though little is known about the role of microglia in the normal brain, under pathological conditions, microglia are activated and start secreting pro-inflammatory molecules. These factors, *e.g.* interleukins and tumor necrosis factors (TNFs) appear to be detrimental to normal neurogenesis (Ekdahl et al., 2003; Monje et al., 2003). On the other hand, accumulating evidence highlights the role for microglia in stimulating neurogenesis (Walton et al., 2006; Ekdahl et al., 2009) and in contributing to spatial learning (Ziv et al., 2006). Microglia have different morphological phenotypes depending on their activation state. Normal resting microglia have ramified processes and express markers such as CD11b and Iba-1. In their activated state, the cells round up and take on an amoeboid shape concomitant with expression of markers such as ED-1 (CD68).

The extracellular matrix

The extracellular matrix (ECM) provides the scaffold in which all above-mentioned cells reside. In addition, it sequesters a wide range of growth factors and acts as a local depot for them. The adult neurogenic niches contain several ECM molecules, such as tenascin-C, laminin, fibronectin and different types of proteoglycans (Gates et al., 1995; Mercier et al., 2002). Integrins serve as the binding partner for laminin, and NSCs express several types of integrins.

An interesting observation is that *in vivo*, neural stem cells often take on a neuronal fate during maturation, whereas *in vitro*, the default differentiation path is an astrocytic lineage. This could indicate that the niche is much more important in the regulation of adult neurogenesis and neuronal fate determination than has been assumed so far.

Regulation

As with most complex biological processes, there are numerous ways to regulate adult neurogenesis, both positively and negatively. Positive regulators of neurogenesis include enriched environment and exercise/physical activity, as well as seizures. Among the negative regulators are stress, aging and irradiation.

Enriched environment

Laboratory rodents are normally kept in standard plastic cages containing fine wood shavings, nesting material, chewing sticks, water and food. By improving ("enriching") this environment with toys, running wheels, fresh fruit, *etc*., and replacing toys a few times per week, Kempermann and colleagues (Kempermann et al., 1997) found a large increase in the number of newly formed granule cells in the hippocampus of enriched animals compared to mice housed in standard cages. This effect was mediated by an increased survival of the newly generated cells, rather than an increased rate of proliferation *per se*. Similar effects of enriched environment have also been demonstrated in laboratory rats (Nilsson et al., 1999). Furthermore, the positive effects on adult neurogenesis could be induced even in older ages (Kempermann et al., 1998b).

Physical activity

Of the many factors contributing to enriched environment, the largest increase in neurogenesis is mediated through wheel running (van Praag et al., 1999). Rodents in their natural habitats exhibit continued physical activity, which can be mimicked by introducing a running wheel to the home cage. Mice with access to running wheels will cover distances of up to 8 km per night. Increased neurogenesis due to running is mainly a stimulatory effect on progenitor cell proliferation. This increase is accompanied by increased LTP levels, as well as improved performance in the Morris Water Maze task (van Praag et al., 1999). As with enriched environment, physical activity evokes an increased neurogenesis response also later in life (van Praag et al., 2005; Kronenberg et al., 2006). The beneficial effects of running, however, seem to be transient, as extended running for 24 days results in a down-regulation of hippocampal neurogenesis by 50%. This reduction could be normalized back to control levels if the rats were restricted in their usage of the running

wheels (Naylor et al., 2005). Interestingly, running does not influence SVZ/olfactory bulb neurogenesis (Brown et al., 2003).

Aging

Aging is one of the strongest negative regulators of neurogenesis. Neurogenesis levels are highest in early postnatal life and up to puberty. Thereafter, there is an exponential decline, which has been shown for mice (Kempermann et al., 1998a), rats (Kuhn et al., 1996) and primates (Leuner et al., 2007). However, it is not likely that aging *per se* is the regulator of neurogenesis, but might be secondary effects of other physiological changes that accompany aging.

Stress

When an individual is challenged with stress, the initial reaction is coupled with release of the “fight or flight” hormones – epinephrine (adrenalin) and norepinephrine (noradrenalin). If the stress is prolonged, the hypothalamus-pituitary-adrenal (HPA) axis is activated, which releases glucocorticoids as effector molecules. In the initial phase, glucocorticoids primarily mobilize energy resources by raising glucose levels and affecting carbohydrate and lipid metabolism. However, if the stress becomes chronic, glucocorticoids can cause deleterious effects, such as muscle weakening, hyperglycemia, gastrointestinal ulceration and atrophy of the immune system. In the hippocampus, prolonged exposure to elevated levels of glucocorticoids has been described to reduce hippocampal excitability, long-term potentiation and hippocampal-related memory tasks (reviewed in Kim et al., 2006). Interestingly, stress-induced reductions in neurogenesis can be alleviated by antidepressant treatment (Dranovsky and Hen, 2006; Warner-Schmidt and Duman, 2006) and, in addition, neurogenesis is required for the behavioral effects of antidepressants to work (Santarelli et al., 2003).

Neural stem cells and neurosphere cultures

Self-renewal and multipotency

Even though evidence of adult neurogenesis was presented to the scientific community in the 1960s (Altman and Das, 1965), it was not until the early 1990s that neural stem cells were first described (Reynolds and Weiss, 1992). The best-known example of a stem cell system is the hematopoietic system,

originating from a single stem cell residing in the bone marrow, and differentiating to all lineages of blood cells – lymphocytes, granulocytes, monocytes, erythrocytes and platelets. There are two requirements for a cell to be considered a stem cell: (a) self-renewal and (b) multipotency. The term self-renewal refers to the ability to generate at least one daughter cell that is identical to the original cell. If the division yields two identical copies, it is called "symmetric" division. If the division generates one stem cell and one cell continuing along a differentiation pathway, the division is called "asymmetric". As a cell starts to differentiate, it loses some of its stem cell properties and is, thereafter, termed a "progenitor cell". In reality, it is often difficult to distinguish a stem cell from a progenitor cell, hence the term "precursor cell", which comprises both stem and progenitor cells, can be used instead (Kempermann, 2006). Multipotency, the second feature of a stem cell, indicates the ability to generate cells of multiple lineages – in the case of the brain, neurons, astrocytes and oligodendrocytes (figure 6).

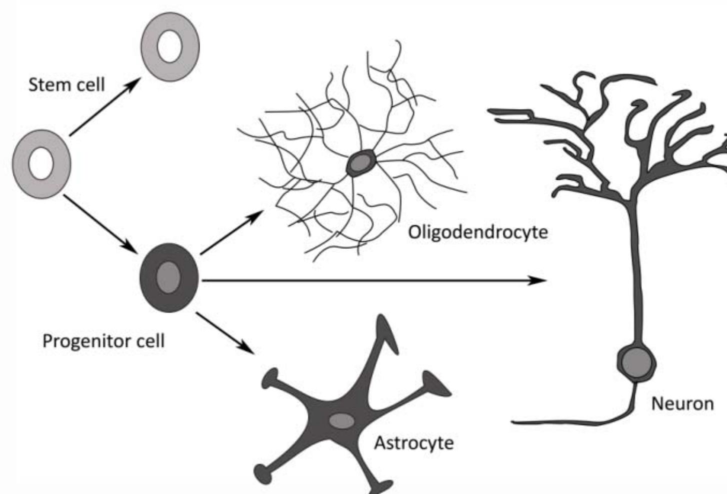

Figure 6. Self-renewal and multipotency. A key criterion for stem cells is the ability to self-renew. Through asymmetric divisions, two different daughter cells are generated, one which is identical to the stem cell, and one which will proceed to generate one of the three lineage-specific cell types; an astrocyte, an oligodendrocyte or a neuron, fulfilling the multipotentiality criterion of the stem cell.

The radial glia-like stem cell

In the developing brain, radial glia are of key importance in guiding newly formed neurons to their target sites. The radial glial cell body is situated on the ventricular border, extending fibers to the pial surface along which the

neurons can migrate. Radial glial cells express proteins such as glial fibrillary acidic protein (GFAP), nestin and brain lipid binding protein (BLBP). In many brain regions, the radial glia transform into astrocytes at the end of embryonic neurogenesis (Rakic, 1971); however, in the regions where adult neurogenesis exists, radial glia-like cells persist into adulthood. Evidence suggests that these radial glia-like cells are the true stem cells of the adult brain (Malatesta et al., 2000; Alvarez-Buylla et al., 2002). So far, no single positive marker for neural stem cells has been described. Given the astrocytic origin of stem cells, the usage of GFAP as a marker is insufficient, as many mature astrocytes also express this protein. Hence, a combination of markers (*e.g.*, GFAP/nestin double-positive cells), or the exclusion of certain markers, has been utilized to identify the stem cell. The failure to identify a single stem cell marker might indicate that neural stem cells are not a single, distinguishable population (Kempermann, 2006).

Neural stem cell markers

Several different strategies have been applied to identify and isolate neural stem/progenitor cells. Using fluorescent-activated cell sorting (FACS), Rodney Rietze and colleagues showed in 2001 that multipotent cells could be isolated based on size and low expression of heat-stable antigen and peanut agglutinin. These cells express nestin, but lack expression of GFAP (Rietze et al., 2001). In 2003, Kim and Morshead (Kim and Morshead, 2003) identified prospective neural stem cells through their ability to rapidly efflux a DNA-binding dye, yielding a so-called “side population” in the flow cytometric analysis. Both the main and side population of cells contained GFAP-positive cells. However, the majority of sphere-forming cells were found within the side population. Capela and Temple (Capela and Temple, 2002) further developed this paradigm by combining side population analysis with expression of Lewis X (LeX) antigen, and by doing so, further enriched the number of sphere-forming cells. The LeX antigen is identical to CD15 and stage-specific embryonic antigen (SSEA-1), a marker commonly used to identify human embryonic cells. Another surface marker, CD133, or its mouse homolog prominin-1, has also been utilized to isolate prospective neural precursor cells from the fetal brain (Uchida et al., 2000). However, CD133 is also expressed by ependymal cells (Coskun et al., 2008).

Sox-2 is a transcription factor that is expressed by self-renewing and multipotent stem cells of the embryonic neuroepithelium (Avilion et al.,

2003). Even though Sox-2 is widely expressed by mature astrocytes throughout the parenchyma of the adult brain (Komitova and Eriksson, 2004), there is also evidence suggesting that Sox-2 is expressed by the neural stem/progenitor cells (Suh et al., 2007). Another commonly used intracellular marker is the intermediate filament nestin. However, *in vivo*, nestin antibodies often cross-react with blood vessels (Palmer et al., 2000). Transgenic reporter mice that use the second intron regulatory region of the nestin gene as the promoter (Yamaguchi et al., 2000; Sawamoto et al., 2001), seem to be much more specific for the progenitor cell phenotype.

Culturing neural stem cells

In the absence of specific stem cell markers, the question remains of how to identify these cells *in vivo*. *In vitro*, both "stemness" and multipotency are somewhat easier to determine using functional assays. Neural stem cells can be propagated *in vitro* in one of two ways: as floating aggregates called "neurospheres" or as an adherent monolayer. Reynolds and Weiss were the first to describe the neurosphere culture system in 1992 (Reynolds and Weiss, 1992), and due to its relative ease and good survival rate of neurospheres, this is currently the most widely used technique. To prove the self-renewal properties of NSCs, single cells are plated in individual wells and their ability to generate neurospheres is analyzed over time. Multipotentiality can then be assessed by plating clonally-derived neurospheres and examining the potential to generate cells of all three lineages. The second culturing technique was initially described by Palmer and co-workers in 1995 (Palmer et al., 1995) and resembles more traditional cell cultures. The isolated cells are grown as monolayers on surfaces coated with adhesive substrates, such as laminin or fibronectin. The two methods display both similarities and differences. For instance, both conditions use completely serum-free media with certain growth factors, such as epidermal growth factor (EGF) and/or fibroblast growth factor 2 (FGF2). In adherent cultures, growth factors and mitogens are homogenously distributed throughout the culture vessel. Neurospheres, on the other hand, form three-dimensional clusters, yielding a gradient of nutrient supply from the outer surface and inwards. Cell-cell contact is much greater in neurospheres, providing better survival for individual cells. Regardless of the cell culture system, extracting a cell from its natural environment inflicts many changes in a cell, and data acquired in culture systems should therefore always be interpreted with care.

DNA damage and repair

Cells are constantly under threat from both extrinsic and intrinsic DNA-damaging agents. Some well-known examples of external DNA-damaging mechanisms are UV light and ionizing radiation. Within the cell, many processes generate metabolites that can act as alkylating agents, and during cellular respiration, reactive oxygen species are formed (Hoeijmakers, 2001). DNA repair enzymes continuously monitor chromosomes to correct damaged nucleotides generated by these exogenous and endogenous agents. If damage is detected, cells activate several DNA repair pathways, allowing for cell cycle arrest and sufficient time for DNA repair. Failure to complete repair before chromosomal replication can lead to fixation of mutations in the genome and might enhance the rate of cancer development. Alternatively, if the damage remains unrepaired, or if the extent of damage is too large, the cell is eliminated via apoptotic pathways (Khanna and Jackson, 2001; Norbury and Hickson, 2001). In addition to DNA repair mechanisms, several free radical scavengers are present in cells to ameliorate the potential DNA-damaging effects.

Ionizing radiation

Ionizing radiation consists of either electromagnetic waves (photons) or particles (neutrons, protons, alpha particles and beta particles) that are energetic enough to detach electrons from atoms or biomolecules. This leads to ionization of biomolecules and free-radical formation. Ionizing radiation generates a variety of damages, such as single-strand breaks, double-strand breaks and cross-linking of DNA and proteins. Double-strand breaks (DSBs) are the most serious form of DNA damage, and the amount of DSBs is correlated to cell death. DSBs affect both strands of the DNA duplex and, therefore, prevent the use of the complementary strand as a template for repair (Hoeijmakers, 2001). Unrepaired DSBs can be lethal for a cell, whereas misrepaired DSBs can cause chromosomal fragmentation, translocations and deletions. The cell cycle exhibits three possible checkpoints for control and arrest (figure 7), where the G1- and S-phase checkpoints protects against DNA replication errors, *e.g.*, mutations and replicative gaps, whereas the G2 checkpoint protects against mitotic errors, such as chromosomal aberrations (Hoeijmakers, 2001).

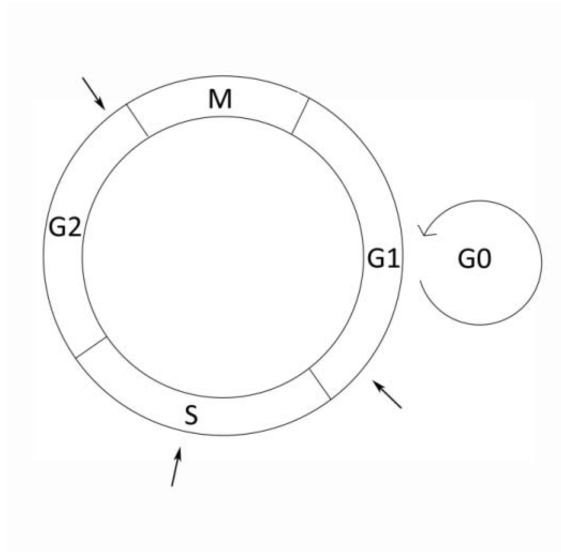

Figure 7. Cell cycle checkpoints. The three cell cycle checkpoints are present in late G1-phase, mid-S-phase and late G2-phase.

DNA repair pathways

In response to DNA damage, the cell activates several checkpoint proteins in a lesion-specific fashion. Whereas Ataxia Telangiectasia-Mutated (ATM) is specific for agents that induce double-strand breaks, ATM/Rad3-related (ATR) most likely responds to UV-induced damage (Helt et al., 2005). Activation of ATM triggers a cascade of downstream events and phosphorylations in which the tumor suppressor protein p53 is central. With a short half-life, p53 is normally maintained at low levels in unstressed cells. The mouse double minute-2 (Mdm2) acts as a major regulator of p53 by targeting p53 for proteolysis by the ubiquitin/proteasome pathway (Francoz et al., 2006). However, in response to DNA damage, p53 is phosphorylated, which interrupts its degradation and p53 accumulates in the nucleus. p53 can be phosphorylated either directly by ATM or ATR, or through the checkpoint kinases (Chk) Chk1 and Chk2. After p53 is activated, it is involved in cell-cycle inhibition, apoptosis and repair. Cell cycle inhibition is achieved by p53-dependent stimulation of the cyclin-dependent kinase inhibitor p21 (p21/Waf1/Cip1). This protein binds to and inhibits interactions between cyclin-dependent kinases (Cdks) and their partner cyclin, which prevents progression from G1 to S-phase and G2 to M-phase (Helt et al., 2005). The growth arrest and DNA damage-inducible gene (Gadd45) is one of several known p53 target genes, which is involved in a variety of growth regulatory mechanisms, including DNA replication and repair, G2/M checkpoint control and apoptosis. It binds to several proteins involved in these processes, including PCNA, p21 and cell division cycle-2 (Cdc2) (Yang et al., 2000; Maeda et al., 2005). ATM also activates the tumor suppressor gene breast

cancer-1 (Brca1) and histone-coding H2afx, both of which are implicated in DNA repair (Khanna and Jackson, 2001).

Ionizing radiation and neurogenesis

Since highly proliferating cells are very sensitive to irradiation, different radiation paradigms have been used to block neurogenesis for experimental purposes, both *in vitro* and *in vivo* (Parent et al., 1999; Tada et al., 1999; Tada et al., 2000; Santarelli et al., 2003; Raber et al., 2004a). The effect on progenitor cell proliferation has been shown to be dose-dependent, age-dependent and long lasting (Mizumatsu et al., 2003; Fukuda et al., 2004; Rola et al., 2004b; Fukuda et al., 2005). Because adult hippocampal neurogenesis has been associated with learning and memory, irradiation-induced loss of newborn cells could contribute to the long-term cognitive impairments observed following cranial irradiation (Crossen et al., 1994; Abayomi, 1996; Raber et al., 2004b; Rola et al., 2004a). Several mechanisms of radiation-induced dysfunction have been proposed, ranging from cellular to behavioral level. Limoli and colleagues (Limoli et al., 2004) proposed an increased sensitivity to oxidative stress, and apoptosis mediated through the p53-dependent pathway, as Trp53-null mice exhibited less apoptosis than wildtype mice after irradiation. Also, treating neural stem cell cultures with antioxidants protected against radiation-induced cell death *in vitro* in one study (Ishii et al., 2007), but failed to protect proliferating cells *in vivo* (Limoli et al., 2006). The long-term effects on diminished neurogenesis could also be mediated through changes in the microenvironment, and irradiation has been shown to cause gliosis, diminished angiogenesis, increased inflammation and increased numbers of microglia (Monje et al., 2002). In addition, radiation has also been shown to cause white matter damage (Corn et al., 1994). Furthermore, a cause-effect relationship between adult hippocampal neurogenesis and antidepressants has been shown. In 2003, Santarelli and colleagues showed that mice treated with the selective serotonin reuptake inhibitor fluoxetine exhibited reduced anxiety in a behavioral test, where mice had to cross a brightly lit area to reach food. Treatment with fluoxetine and reduction in anxiety were associated with increased hippocampal neurogenesis. However, if the progenitor cell proliferation and neurogenesis were abolished using irradiation, there was no longer any positive effect from antidepressant treatment (Santarelli et al., 2003).

AIMS

The overall aim was to study how neural stem/progenitor cells react in response to radiation injury.

The specific aims were:

To investigate the long-term effects of early postnatal irradiation on stem cells and neurogenesis in the adult rodent brain

To establish a rapid quantification method for brain cells using flow cytometry

To isolate specific cell populations of the brain using flow cytometry for downstream experiments

To investigate intrinsic and extrinsic pathways regulating the response to ionizing radiation *in vitro* and *in vivo*

METHODS

As scientists we utilize different models to increase our understanding of the world around us. These studies include experiments on isolated cells grown in culture (*in vitro* experiments), different (behavioral) effects studied in animal experiments (*in vivo* experiments) and sometimes clinical trials in humans. The work in this thesis involves both *in vitro* and *in vivo* experiments. Isolated cells grown in a culture dish can aid in interpreting biological pathways that are regulated by specific experimental conditions, but can never fully replace studies in the complex environment of a living organ. As highlighted in this thesis, the brain microenvironment contains different specialized cell types, which can have a profound impact on any other given cell studied in that system, hence the complete picture can only be achieved when all these interactions are kept intact.

The individual papers give detailed descriptions on the material and methods used in the respective study. Below follows a description of some methods that have been of particular importance for this thesis.

Ethical permissions

All animal experiments were approved by the local animal welfare committees of Stockholm and Gothenburg. The application numbers were 251/03 (Stockholm) and 184/03, 165/06 and 26/08 (Gothenburg).

Perfusion fixation

For many purposes adequate fixation is obtained by immersing the tissue into the fixative solution. However, the fixative will normally penetrate the tissue better if the fixative is distributed via the heart and vascular system. The initial perfusion with physiological saline or PBS clears the microvasculature from blood cells that otherwise would clog the arteries. In addition, due to their high antibody-binding capacity, the blood cells could otherwise interfere with the interpretation of the true immunostaining. For histological studies in this thesis, the animals were deeply anesthetized and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (for adult animals) or 6% buffered formaldehyde (for postnatal rats). Brains were removed with olfactory bulbs intact and transferred to the same fixative over night. To

reduce the amount freezing artifacts during sectioning, the tissue was transferred to and stored in 30% sucrose until sectioning.

Sectioning

For sectioning, the brains were frozen with dry ice and cut with a sliding microtome. Every single section throughout one hemisphere of the brain was collected and stored. From this, series of different sampling frequencies could be generated, *e.g.* by taking every 3rd, 4th, 6th or 12th section depending on the specific histological staining. All tissue analyzed in this thesis was processed as free-floating sections and the thickness of each section was either 25 or 40 μm.

Stereology

The outcome of many adult neurogenesis studies is often total number of newly generated neurons. However, in the structures analyzed, in particular in the olfactory bulb, the total number of newly generated cells has been estimated to be up to 10.000 new cells each day, making manual counts of all cells an almost impossible task. Therefore, researchers often use stereological methods to sample a representative fraction of the cells of interest, from which estimates of the total cell number within a structure can be calculated. The cell counts are often performed with computer-aided systems connected to a microscope with a motorized stage. The borders of the structure of interest, for example the granule cell layer of the olfactory bulb, can be drawn. The computer will overlay a grid system with counting frames at places defined by the grid. Cells within the counting frames are counted, however, cells touching two out of four pre-determined sides of the counting box are excluded, to avoid oversampling. However, as the tissue is rather thick (see above), cells are also counted in the z-axis. In the end, the counting volume can be calculated from multiplying the area of all counting boxes with the section thickness. The cell density can be calculated from the sum of all cell counts divided by the counting volume. To calculate the total number of cells, the density is multiplied with the reference volume, which is calculated as the sum of all traced areas multiplied by the section thickness and the sampling frequency of the sections, *e.g.* if every twelfth section was analyzed, the reference volume was multiplied by 12.

Immunofluorescence

A key method for this thesis has been the detection of specific antibodies bound to unique epitopes in the tissue. Regardless of the method of analysis (*e.g.* confocal microscopy or flow cytometry) the same basic principles apply. A primary antibody directed toward an antigen is incubated with the tissue/cells for a specified amount of time, excess and unbound antibody is washed out, and a fluorescence-coupled secondary antibody that will specifically recognize the first antibody is applied. The fluorescence from the secondary antibody can be visualized by exciting the fluorophore with either a halogen UV light source, or with photons of specific wavelengths generated by a confocal laser microscope. The fluorophore can only stay in its excited state for a short period, then it falls back to its normal state and the difference in energy is emitted as fluorescent light of a lower wavelength, which can be recorded as a digital image in the microscope. Table 1 summarizes the primary antibodies and their concentrations used in the different studies.

Table 1. Summary of primary antibodies used and their concentrations.

Microscopy	**Conc.**	**Flow cytometry**	**Conc.**
rt α BrdU	1:250	ms α BrdU:FITC	1:50
ms α NeuN	1:250	gt α Dcx	1:100
ms α CD68 (ED-1)	1:500	ms α NeuN	1:100
ms α rt nestin	1:500	ms α S-100β	1:500
rb α Phospho-H3	1:500	ms α MAP2	1:1000
rb α GFAP	1:1000	rb α Iba-1	1:1000
rb α Iba-1	1:1000		

Confocal microscopy

An important issue in the field of adult neurogenesis has been to prove that a newborn cell has differentiated into a mature neuron. When using BrdU as a birthdating label, it is necessary to detect a BrdU-positive nucleus contained within a cell expressing a mature neuronal marker. However, the thicker the section is, the higher the chance that two cells, each expressing only one of the markers, are aligned in the axis of the light, which would give the impression that both markers are expressed in a single cell. For detection of

new neurons this would mean that one cell is positive for BrdU, thus newly generated, and the other is a neuron, thus expression NeuN, but with conventional fluorescence microscopy these cells would be detected as one single cell expressing both markers. To overcome this problem, confocal microscopy has been a key technique, since it collects light information from an extremely thin “optical slice” in the z-axis, disregarding all other fluorescence that is not emitted from this particular focal plane. Information from each fluorescent channel can be gathered in a sequential mode, reducing the chances of cross-talk between fluorescent channels. By focusing (optically) throughout the z-axis, multiple images containing the fluorescent content from each channel can be generated and reconstructed into a 3-D image.

Irradiation

Both *in vivo* and *in vitro* irradiation experiments were carried out at the Department of radiotherapy at the Sahlgrenska University Hospital in Gothenburg. A linear accelerator (Varian Clinac 600CD) of 4 MV nominal photon energy was used in both instances. To ensure a relatively homogenous distribution throughout the brain of the animal, the head was covered with a one cm thick tissue equivalent. For *in vitro* irradiations, the neurosphere cultures were irradiated from upside down, i.e. with the gantry rotated 180°. The flask was placed inside a polystyrene phantom to obtain a relatively uniform radiation dose to the cell culture media. The polystyrene phantom was positioned on a holder without any material between the radiation source and the phantom.

Flow cytometry

Flow cytometry is designed for quantification and sorting of microscopic particles flowing in a stream of fluid, allowing simultaneous detection of physical and fluorescent characteristics of individual cells. A laser light is aimed at a stream of fluid and a number of detectors are situated at various angles to the laser. One of them is placed at 180° to the laser, the so-called Forward Scatter (FSC), detecting a “shadow” of a cell as it passes through the laser light, thereby determining the size of the cell. Other detectors are arranged perpendicular to the stream. The Side Scatter (SSC) detector distinguishes the granular content of any given cell. For the detection of

emitted fluorescent light, different photomultiplying tubes (PMTs) are equipped with specialized filters. Data acquired during a flow cytometric experiment can be plotted in one-dimension, producing a histogram, or in two-dimensions, creating dot plots or density plots. Cells that cluster into different regions on these plots can subsequently be separated by creating a series of electronic "gates" where different subpopulations can be studied in more detail. As the cells are aspirated from the test tube, the cell suspension is forced into a narrow stream of single cells. At a certain point, a vibrator causes the stream of cells to break into droplets. Just before the stream breaks up into droplets, the cells are characterized by the fluorescence detectors. An electrical charge can be placed on the droplet based on the fluorescence measure, which allows sorting of positively and negatively charged cells as they pass by two deflection plates. The charged cells can be collected into separate tubes, and used for subsequent experiments.

Reverse transcription quantitative PCR (RT-qPCR)

Quantitative PCR combines the exponential amplification of DNA during the polymerase chain reaction with optical detection using fluorescent dyes or fluorescent probes, which makes it possible to measure the amount of product after each amplification cycle. Based on this information, the initial amount of template can be estimated. The fluorescent probes used for qPCR analyses can be divided into two categories: non-specific dyes and sequence specific probes. An example of a sequence-specific dye is the TaqMan probe. The TaqMan probes utilize a reporter fluorophore at the 5' end of the oligonucleotide, and a quencher fluorophore at the 3' end. The TaqMan probe is complementary to a sequence within the PCR product recognized by the forward and reverse primer. As the PCR reaction starts, the polymerase initiates the extension of the primer and replicates the template to which the TaqMan probe is hybridized. The 5' exonuclease activity of the polymerase cleaves the probe, releasing the reporter fluorophore from the quencher, thus emitting light. As the starting material of our RT-qPCR experiment was treated in a harsh way (cell isolation from the brain, fixation, staining and FACS sorting) we speculated that the RNA could potentially be degraded. Hence, we chose to use a sequence specific dye to facilitate detection of our genes of interest in the material.

At a certain point during the amplification cycles, the amount of product has accumulated to increase the fluorescent signal above background. This point is normally referred to as the cycle of threshold (Ct), and is defined as an arbitrary set fluorescence threshold line. For an accurate quantification, the Ct must be determined in the exponential phase of the curve. Most quantitative gene expression studies measure the relative expression between genes in different samples. One is the reference gene, often a housekeeping gene, whose expression is estimated to be unchanged between the experimental conditions, and the other is the gene of interest.

Cell isolations

Due to the high number of synaptic connections between neurons, the isolation of viable cells from the adult brain has been very difficult (Brewer, 1997). Therefore, two different protocols for cell isolation have been used in this thesis, one for the isolation of mature neurons from the adult mouse brain (paper I) and another for the isolation of stem cells and microglia from the postnatal rat brain (paper III). To isolate mature neuronal cells, we used the proteinase papain and a longer incubation time (30 min) at a lower temperature (30°C), whereas for the stem cell preparation from postnatal brain, we used an enzyme mix consisting of papain, dispase and DNase and a shorter incubation time (20 min) at a higher temperature (37°C). Both systems were optimized to find the best combination of enzymes/temperature for each experimental condition.

RESULTS AND DISCUSSION

Isolation and quantification of stem/progenitor cells and mature neurons using flow cytometry (paper I)

The possibility to specifically isolate a particular population of cells from the brain and study them in further detail can be extremely valuable. Due to multiple dendritic processes and synaptic connections, the isolation of viable, mature neurons from the adult brain has been very difficult (Brewer, 1997). We aimed to develop specific protocols that would allow for isolation of viable cells from the adult brain. Using these cells for cell culture experiments was not the goal of the current study, but rather the qualitative and quantitative assessment of the cell composition in specific brain regions using flow cytometry. We were able to successfully isolate cells with high viability from the adult mouse hippocampus and olfactory bulb. We profiled these cells using immunofluorescence markers for stem/progenitor cells and neurons. The accuracy of the flow cytometric method was validated with stereological counts. The flow cytometric results showed that 53% of the olfactory bulb cells were positive for the neuronal marker NeuN (paper I, figure 1D), which was in accordance with stereological cell counts of 58% NeuN-positive cells (paper I, table 1). Hippocampal quantification yielded 38% NeuN-positive cells, as assessed by flow cytometry (paper I, figure 1F), and 52% NeuN-positive cells, as determined with stereological counts (paper I, table 1). The lower yield in the flow cytometric analysis of the hippocampus is most likely due to a higher degree of complexity of hippocampal neurons compared to the olfactory bulb, which contains, to a large extent, amacrine (axon-less) granule neurons. To investigate whether we could also detect a smaller population of immature cells, we stained cells with doublecortin (DCX) and BrdU. For this purpose, 5-week-old male mice received five daily injections of BrdU (50 mg/kg) and were sacrificed three days after the last injection. The flow cytometric analysis yielded 22% olfactory bulb cells positive for DCX and 6.6% positive for BrdU (paper I, figure 2A-B). In the hippocampal cell population, the corresponding flow cytometric values were 2.7% DCX and 2.8% BrdU-positive cells (paper I, figure 2C-D). The stereological counts of BrdU-positive cells in the OB and hippocampus were 3.4% and 1.9%, respectively (paper I, table 1). These results demonstrated that we could readily isolate and quantify neurons and

stem/progenitor cells in a very reproducible manner, in fractions that were in accordance with numbers obtained from stereological quantification.

Long-term reduction in proliferation and neurogenesis in the dentate gyrus and olfactory bulb following early postnatal irradiation (paper II)

Ionizing radiation is one of the most powerful tools for treating malignant tumors, because radiation results in apoptosis of proliferating cells due to DNA damage. Stem/progenitor cells of the brain have a high proliferative capacity. Therefore, this population of cells exhibits a pronounced susceptibility to DNA-damaging effects of ionizing radiation. Our aim was to investigate how a moderate dose of radiation would affect stem cells and neurogenesis in the adult brain.

To study the late effects of radiation, young rats received a single dose of ionizing radiation (6 Gy) to the brain. The long-term impact of radiation on the number of newly generated neurons was investigated when the animals were 2.5 months old. Four weeks prior to sacrifice the animals received three daily injections of BrdU (50 mg/kg) to label dividing cells. When we examined the hippocampus, we found a dramatic reduction in the number of BrdU/NeuN double-labeled neurons in the dentate gyrus of irradiated animals compared to controls; irradiated animals had less than 5% of the neurogenesis levels compared to sham-irradiated animals. For the olfactory bulb GCL, the number of BrdU/NeuN double-positive cells in irradiated animals was 40% of control levels (paper II, figure 3A-B). Similarly, the number of proliferating (phospho-histone H3-positive) cells in the irradiated dentate gyrus was reduced to 10% of control levels, and the corresponding population in the irradiated SVZ was reduced by 50% compared to controls (paper II, figure 4D-E). These results confirmed the pronounced susceptibility of proliferating stem/progenitor cells to ionizing radiation and indicated that the effects on proliferation and neurogenesis were sustained long-term.

Differential effects of ionizing radiation on the number of stem cells at nine weeks after irradiation (paper II)

Because the number of phospho-histone H3-positive cells reflects the rapidly dividing progenitor cell pool, we next wanted to investigate how the slowly dividing, immature stem cells were affected by radiation. We used nestin/GFAP double-positive cells as an indication for immature stem cells

and quantified the number of double-positive cells in the dentate gyrus subgranular zone and SVZ. In the hippocampus, the number of nestin/GFAP double-positive cells in the irradiated animals was only 15% of control levels; however, we could not detect any significant differences in the number of stem cells residing in the SVZ (paper II, figure 5A). This large difference in stem cell numbers between the two neurogenic regions at a time point late after radiation triggered the question as to whether this was an effect due to differences in initial sensitivity to irradiation between the regions, or whether this difference was due to differential repair and recovery potentials.

Equal reduction in the number of proliferating cells in the dentate gyrus and SVZ one day post-irradiation (paper II)

As shown by Shinohara and colleagues (Shinohara et al., 1997), there is a rapid elimination of proliferating cells within the first 24-48 hours after radiation. To investigate whether there was a difference in the initial sensitivity to radiation in SVZ and subgranular zone stem cells, animals were irradiated with 6 Gy and sacrificed after 24 hours. We quantified the number of phospho-histone H3-positive cells in both neurogenic regions, but no difference in the number of proliferating cells was detected (paper II, figure 6D). Therefore, we concluded that both regions are equally sensitive to the initial radiation injury.

Neurosphere cultures from hippocampus and SVZ express different levels of stem cell markers (paper III)

It has been proposed that the SVZ harbors “true” stem cells whereas the hippocampus contains more restricted progenitor cells (Seaberg and van der Kooy, 2002). To explore whether intrinsic genetic differences exist between the two stem cell populations, which could explain the observed differential recovery following irradiation, we isolated stem cells from the hippocampus and SVZ of untreated animals and compared gene expression profiles of certain genes known to be involved in stem cell regulation. To study the intrinsic differences without the influence of the surrounding microenvironment (Hitoshi et al., 2002; Armando et al., 2007), we cultured the stem cells as neurospheres for one week prior to genetic profiling. Relative gene expression of SVZ and hippocampal neurosphere cultures was compared using RT-qPCR. In contrast to our hypothesis, and to what has previously been shown, we found that the hippocampus expressed high levels

of mRNA for genes known to be stem cell markers (paper III, figure 2A-E). However, the hippocampal neurosphere cultures also exhibited relatively lower expression level of two genes involved in DNA repair and the S/G2 checkpoint complex (paper III, figure 2F-G).

Similar responses to ionizing radiation by SVZ and hippocampal neurospheres (paper III)

A differential ability of stem cells to cope with radiation injury could be a possible explanation for the differential recovery seen in our *in vivo* experiments. Radiation activates DNA repair mechanisms and free radical scavenging systems, both of which could provide a significant survival advantage if present in one region but not the other. We challenged the neurosphere cultures with irradiation, and isolated RNA sixteen hours later to address this hypothesis. A select panel of genes known to be involved in apoptosis, DNA repair, cell cycle regulation and stem cell maintenance was investigated using RT-qPCR. We found that stem cells isolated from both regions responded very similar to ionizing radiation. Both regions exhibited a significant downregulation of several genes involved in DNA damage response pathways. The up- or downregulation of most genes analyzed in one region was paralleled by an identical regulation in the other region (paper III, figure 5). From these data, we concluded that the regional differences seen in stem cell recovery after irradiation *in vivo* were not likely due to differences in the intrinsic capacity of cells to cope with irradiation injury.

No sustained inflammatory response after irradiation (paper II)

Because we did not detect differences *in vivo* in the initial response to irradiation, the regional differences seen in the long-term response could be due to an altered microenvironment. For instance, the hippocampus could contain a more hostile environment for stem cell survival than the SVZ. As shown by Monje and colleagues (Monje et al., 2002), irradiation causes major changes in the microenvironment, with inflammation being a key component of the detrimental effects. To investigate whether the hippocampus displayed a sustained inflammatory response, while the olfactory bulb did not, we analyzed both tissues for microglia markers. At one day post-irradiation, we found a marked increase in the number of cells expressing the marker for activated microglia, ED-1, in both regions (paper II, figure 7B), which was consistent with previous findings (Monje et al.,

2003; Kalm et al., 2009). However, there were no signs of activated microglia nine weeks after irradiation, and we could not detect any differences between control and irradiated animals when we quantified the number of resident (Iba-1 positive) microglia at this time point (paper II, figure 7C). These results led us to conclude that a persistent inflammatory environment could not explain the differences. However, even though we could not detect any long-term inflammation, the early response of the inflammatory cells could be different and, thus, provide a superior recovery situation for SVZ stem cells.

Irradiated microglia from the subventricular zone, but not hippocampus, upregulate genes important for stem cell maintenance, proliferation and survival (paper III)

Microglial cells are key mediators of CNS inflammatory responses. However, microglia have also been shown to release anti-inflammatory signals, as well as trophic support (Walton et al., 2006; Ekdahl et al., 2009). To specifically study the immediate response of microglial cells to ionizing radiation, we irradiated animals with 6 Gy and isolated cells from the hippocampus and SVZ. The cells were labeled with antibodies to Iba-1 and selectively sorted using the flow cytometric method described in paper I. Gene expression was investigated in control and irradiated microglia from both the hippocampus and SVZ, with a focus on genes involved in apoptosis, inflammation and trophic support. The most prominent difference between irradiated microglia from the SVZ and hippocampus was that SVZ microglia upregulated several genes coding for stem cell maintenance, proliferation and survival. For instance, the SVZ microglia significantly upregulated the gene encoding fibroblast growth factor 2 (Fgf2), whereas this same gene was downregulated in hippocampal microglia (paper III, figure 7A). Similarly, the gene encoding leukemia inhibitory factor (Lif) showed a three-fold higher upregulation in SVZ microglia compared to hippocampal microglia (paper III, figure 7B). Furthermore, irradiated SVZ microglia also upregulated their platelet-derived growth factor beta (Pdgfb) expression, whereas expression levels in irradiated microglia from the hippocampus remained unaltered (paper III, figure 7C). We conclude that factors released from the local microenvironment in general, and microglia in particular, could be of key importance in the differential recovery potential seen after ionizing radiation *in vivo*.

Due to the paucity of specific markers for neural stem cells, it remains difficult to study this cell population *in vivo*. Currently, neural stem cells are believed to be radial glia-like cells, termed "B cells" in the SVZ (Doetsch et al., 1999) and "type-1 cells" in the hippocampus (Filippov et al., 2003). These cells express nestin, GFAP and Sox2. However, mature astrocytes and astrocytes activated by injury, *e.g.* ionizing radiation, exhibit many similarities to the radial glia-like stem cells with regard to marker expression. Yet, if the cells quantified in our *in vivo* studies were mainly reactive astrocytes, one would expect the region with the greater and more sustained damage, *i.e.* the hippocampus, to have a greater number of reactive astrocytes. Because our data rather indicate the opposite, *i.e.* very few nestin+/GFAP+ cells in the hippocampus, this cell population should reflect neural stem cells and not activated astrocytes.

Seaberg and van der Kooy showed in 2002 that cells isolated from the hippocampus failed to initiate secondary neurospheres, indicating a loss of self-renewal. These cells also exhibited difficulty in generating cells of all three lineages, hence, they proposed that the hippocampus contains more restricted progenitor cells, and only the SVZ contains true stem cells (Seaberg and van der Kooy, 2002). We were therefore initially surprised to find that our hippocampal neurosphere cultures expressed relatively higher levels of genes associated with a stem cell profile (LeX, CD133 and Hes genes), compared to neurospheres established from the SVZ. However, LeX is known to bind both Wnt molecules as well as FGFs (Capela and Temple, 2006). In 2005, Lie and colleagues showed that Wnt signaling promotes adult hippocampal neurogenesis, while the absence of Wnt inhibits neurogenesis (Lie et al., 2005). One could therefore speculate that the high gene expression levels of LeX, which was observed in hippocampal stem cell cultures, might not necessarily support neural stem cell proliferation and neurogenesis, but rather the opposite through regulation of the Wnt pathway. Another interesting aspect was presented by Jirmanova and colleagues, who showed that low levels of LeX promotes FGF2-mediated proliferation of stem cells, whereas higher concentrations resulted in a reduction of mitogenic activity (Jirmanova et al., 1999). Whether this holds true for adult neural stem cells remains to be investigated, but it might explain why hippocampal cells frequently exhibit a slower proliferation rate *in vitro* (personal observation). On the contrary, high levels of Hes genes, as is the case for hippocampal neurosphere cultures, have been shown to maintain stem cells in an

undifferentiated state, at least during development. Yet, during later stages of development, high levels of Hes genes promote gliogenesis (Ross et al., 2003; Imayoshi et al., 2008a; Kageyama et al., 2008). If this is linked to the relatively higher expression of GFAP that was observed in hippocampal neurospheres, it might be a sign of gliogenesis. On the other hand, since GFAP is one of the markers used to identify neural stem cells, the solution might be more complex.

As indicated by our results, we did not detect reactive microglia nine weeks after the initial radiation injury, but observed a very strong inflammatory response by the microglial cells immediately after irradiation. However, the microglia are just one component of the stem cell niche, and other cells, such as astrocytes and endothelial cells, also exhibit cell-cell interactions and release factors that can modulate inflammation, stem cell proliferation and differentiation. For example, endothelial cells release soluble factors that stimulate self-renewal of NSCs through the upregulation of Hes1 (Shen et al., 2004). Cell-cell contact between an astrocyte monolayer and NSCs, or release of factors such as interleukin-1β (IL-1β) and IL-6, promote neuronal differentiation of NSCs (Lim and Alvarez-Buylla, 1999; Song et al., 2002; Barkho et al., 2006), whereas leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) have been shown to induce astrocytic differentiation of NSCs (Faijerson et al., 2006; Nakanishi et al., 2007). Interestingly, even though IL-1β and IL-6 are classically known pro-inflammatory cytokines, their ability to mediate neuronal differentiation of NSCs (Barkho et al., 2006) has challenged the view that inflammation is detrimental for neurogenesis. In addition, astrocytes are activated by cytokines released from microglia. These cytokine-activated astrocytes can act as free radical scavengers, remove excess glutamate and restore ionic homeostasis of the CNS (Liberto et al., 2004).

Microglia seem to have an important role in modulating adult neurogenesis, as indicated by several studies (Ekdahl et al., 2003; Monje et al., 2003; Butovsky et al., 2006; Ziv et al., 2006). The effects can be both detrimental and beneficial. The beneficial effects have been documented both *in vivo* after status epilepticus or adrenalectomy (Battista et al., 2006; Bonde et al., 2006), as well as *in vitro* (Walton et al., 2006; Cacci et al., 2008). Activated microglia release factors that positively regulate neurogenesis, including insulin-like growth factor-1 (IGF-1), transforming growth factor β (TGFβ),

prostaglandin E2 (PGE2) and interleukins such as IL-6 and IL-10 (Battista et al., 2006; Nakanishi et al., 2007; Cacci et al., 2008; Thored et al., 2009). In our study, we observed that irradiated microglia from the SVZ upregulated mRNA levels of Fgf2, Lif and Pdgfb.

Basic fibroblast growth factor (bFGF, FGF2). FGF2 is a pleiotrophic growth factor, which promotes the survival and proliferation of a large number of cell types (Weksler et al., 1999; Sulpice et al., 2002; Krejci et al., 2004; Lafont et al., 2005; Guilarducci-Ferraz et al., 2008). For the CNS, it was long known as a neurotrophic and neuroprotective factor; however, more recently it became apparent that FGF2 is also essential for the maintenance and proliferation of neural stem cells *in vitro* (Palmer et al., 1995; Tropepe et al., 1999). When FGF2 is infused into the lateral ventricles of adult rats, it results in increased numbers of proliferating cells in the SVZ and striatum, and a subsequent increase in neuronal progenitors migrating from the SVZ to the OB (Kuhn et al., 1997). Endogenous FGF2 released from apoptotic cortical explants has been shown to play a major role in stimulating lesion-induced proliferation of SVZ stem cells (Agasse et al., 2007). FGF2 has also been shown to increase proliferation and neuroprotection after transient focal cerebral ischemia (Ma et al., 2008).

Leukemia inhibitory factor (LIF). Many types of injuries to the central nervous system are accompanied by a rapid and transient increase in expression of LIF (Banner et al., 1997; Suzuki et al., 2000). LIF is well-known for its ability to promote embryonic stem (ES) cell self-renewal (Williams et al., 1988). It belongs to a family of cytokines that act through the heterodimer complex of LIFR and the gp130 receptor. LIFR/gp130-mediated signaling is necessary for maintaining embryonic neural stem cells *in vivo* (Shimazaki et al., 2001). LIF has also been shown to act directly on adult NSCs *in vivo* by promoting symmetric self-renewing divisions and preventing the formation of more differentiated progeny (Bauer and Patterson, 2006).

Platelet-derived growth factor (PDGF). PDGF is a dimeric glycoprotein composed of either two A (-AA), two B (-BB) chains, or a combination of the two (-AB). PDGF-BB exhibits both paracrine and autocrine effects on proliferation and early differentiation of neural stem/progenitor cells. If the PDGF receptor is inhibited, neural stem cells differentiates more rapidly, indicating that PDGF-BB participates in maintaining stem cells in a

proliferative state (Erlandsson et al., 2006). PDGF-BB has also been implicated in neuroprotection (Peng et al., 2008), and conditional deletion of the PDGF beta-receptor led to increased cell death in the SVZ (Ishii et al., 2008), which suggests a role for PDGF in NSC survival.

Since radiation-induced DNA damage causes apoptosis mainly in dividing cells, a successful recovery of the neurogenic stem cell niches after radiation injury may require that NSCs are maintained in a quiescent state. Ideally, the pool of NSCs should undergo DNA repair prior to expansion and replacement of eliminated cells, and reconstitution of neurogenesis. We propose that the three molecules identified in the irradiated SVZ microglia exhibit these features – LIF reduces the number of transiently amplifying cells, retaining cells in a more stem cell-like state, while FGF2 and PDGF-BB stimulate proliferation and survival of the expanded stem cell pool and promote their neuronal fate. The upregulation in mRNA levels of Lif, Fgf2 and Pdgfb provides a potential explanation for the recovery of the SVZ stem cell pool seen after ionizing radiation, where the hippocampal stem cell pool is permanently defective. However, the expression data are correlative and additional experiments are required to link the activity of these growth factors to the stem cell pool recovery.

CONCLUSIONS AND OUTLOOK:

From the data presented in this thesis, we can conclude that stem/progenitor cells residing in the brain are indeed very vulnerable to ionizing radiation, and that a single dose of 6 Gy at postnatal day 9 leads to long-lasting decreases in both stem cell proliferation, as well as neurogenesis, in the adult rat. Furthermore, even though the two stem cell regions were equally affected by the initial radiation, there was a differential response in the stem cell pool. While hippocampal stem cells were long-term affected, SVZ stem cells seemed to recover with time. The specific upregulation of several stem cell-related genes in the irradiated SVZ microglia could potentially contribute to the recovery of the stem cell population seen in the SVZ, which was lacking in the hippocampus.

Future directions

For future experiments, it would be interesting to challenge microglial cells with irradiation (either *in vitro* or *in vivo*) and identify the secreted proteins, which could confirm or dismiss the gene expression studies. In addition, the stimulation of irradiated neurosphere cultures with FGF2, LIF and PDGF either alone or in combination, and analysis of neural stem cell survival, would provide insight into whether these proteins could indeed rescue stem cells after irradiation. As an intervention study, the delivery of these growth factors into the irradiated hippocampus *via* osmotic minipumps could provide a way to improve the recovery of hippocampal stem cells and neurogenesis. This would be an important experiment for further understanding stem cell regulation in the adult brain, as well as for future therapeutical interventions to reduce the cognitive defects due to the hippocampal damage following radiation.

Clinical correlations

Radiation therapy in children and juvenile cancer patients involving the head almost invariably leads to cognitive impairment later in life (Mulhern et al., 2004; Palmer et al., 2007). There are currently no therapies available that would eliminate or counteract these severe side effects. The prospective future application of FGF2 in a clinical setting might possess properties of a double-edged sword. On one hand, FGF2 has several qualities important for

stem cell maintenance, proliferation and radiation protection (Fuks et al., 1994; Pena et al., 2000), but on the other hand, given FGF2's angiogenic properties (reviewed in Presta et al., 2005), the potential prospect of stimulating neovascularization in tissue from which a tumor has recently been removed is perhaps undesirable. Enriched environment, physical activity and chronic stimulation with antidepressant drugs have all been shown to positively regulate neurogenesis. Hence, investigation of these paradigms in an irradiation-context has been done by several research groups. Interestingly, both running and chronic treatment with antidepressants could ameliorate the hippocampal-dependent behavioral deficits seen after irradiation (Santarelli et al., 2003; Naylor et al., 2008). However, contradicting evidence exists regarding whether enriched environment could alleviate the irradiation-induced hippocampal dysfunctions at a behavioral level (Meshi et al., 2006; Fan et al., 2007). Yet, these two studies differ in which species was investigated (mice *vs.* gerbils) as well as the gender analyzed (female *vs.* male). At least between mice and rats, there seems to be a species difference with regard to irradiation sensitivity, where mice are much more radio-resistant, and require higher doses of IR to achieve a similar reduction in the number of proliferating stem/progenitor cells. For example, Fukuda and colleagues irradiated both rats and mice with the same dose, but the effect on progenitor cells was much larger in rats (Fukuda et al., 2004).

Rodents rely heavily on their olfactory system to search for food, mate selection and to avoid predators. Consequently, the SVZ supplies the rodent OB with a much larger cohort of new cells each day, compared to the hippocampus. For rodents, which are very dependent on their sense of smell, the recovery of stem/progenitor cells in the SVZ/OB system might be evolutionarily advantageous. Although adult neurogenesis has been described for the human SVZ/OB system (Curtis et al., 2007), humans do not rely as heavily on smell for daily survival. It remains to be investigated if a recovery of SVZ stem/progenitor cells after irradiation also exists in humans. Nevertheless, if the results from this thesis could be extrapolated to humans, it further highlights the importance of shielding the hippocampus whenever possible during radiation therapy. This could be done, for instance, by intensity-modulated radiation therapy. Survival after childhood cancer has improved dramatically over the past decades, and today 1 in 900 young adults are childhood cancer survivors. Approximately 40% of these patients received radiation therapy at some point in their life. If radiation therapy

could be tailored to avoid damaging stem/progenitor cells while targeting tumor cells, the quality of life for an increasing number of patients surviving childhood cancer, but struggling with the late cognitive side effects of early radiation treatment, could be significantly improved.

CONCLUDING REMARKS:

Responses to stated aims:

Protocols for the isolation and quantification of different types of brain cells (*i.e.* stem cells, neurons and microglia) using flow cytometry were successfully established and applied to downstream experiments

Early postnatal irradiation was found to be detrimental for hippocampal neurogenesis and stem cell proliferation, both which remained suppressed into adulthood. The olfactory bulb neurogenesis was also reduced by early postnatal irradiation. However, the stem cell population residing in the SVZ recovered with time.

We found some differences in gene expression of untreated neurosphere cultures isolated from postnatal hippocampus and SVZ; hippocampal cells exhibited higher gene expression in genes described as stem cell markers. However, when neurosphere cultures were subjected to irradiation, the gene expression profiles in response to irradiation were almost identical. In contrast, when microglia were isolated from irradiated hippocampus and SVZ, and their expression profile with regard to inflammation and trophic support was investigated, we found that only SVZ microglia upregulated genes encoding growth factors and trophic support, and thereby contributed to a more permissive microenvironment for stem cell recovery post-irradiation.

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