THE NEUROVASCULAR NICHE AFTER IRRADIATION TO THE DEVELOPING BRAIN

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To my family!

This thesis would not exist without

your endless love and support ♥

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ABSTRACT

Radiotherapy is commonly used in the treatment of pediatric brain tumors but is unfortunately associated with debilitating negative effects, such as impaired memory and learning. Historically, vascular damage following radiotherapy was considered the primary injury which in turn caused ischemia and necrosis. This hypothesis was supported by studies reporting structural changes to blood vessels, such as thickening of the vessel walls, vessel dilation and enlargement of the endothelial cell nucleus. Furthermore, quantitative studies observed time- and dose-dependent loss of endothelial cells, vessel length and density after irradiation. Most experimental studies have, however, focused on the mature brain and used high doses of irradiation. Moreover, the adult brain is capable of generating new neurons throughout life in discrete areas of the brain, and these regions are consistently affected by irradiation. Hence, much research has focused on the neural stem and progenitor cells, since a loss of these cell types has been coupled to cognitive decline after irradiation. Other important cell types have therefore been neglected and need to be examined in order to see the full picture.

In this thesis we investigated the effects after a single moderate dose of cranial irradiation (8 - 10 Gy) to the juvenile brain and focused on the vasculature in different areas. Analysis of vascular structure and complexity up to 1 year after irradiation indicated that the vasculature adjusted to the needs of the surrounding tissue. This was observed in both the hippocampus (gray matter) and the corpus callosum (white matter). We did not observe any apparent endothelial cell death, nor any upregulation of genes involved in endothelial cell death acutely after irradiation. The reduction of neural progenitor cells in the hippocampus was however irreversible and we demonstrated that irradiation in fact accelerated the natural decline in neurogenesis with age. We also investigated the neurovascular niche and found a disruption early after irradiation that however seemed to normalize with time. This hence demonstrated dissociation between the morphological patency of the neurovascular niche and hippocampal neurogenesis.

Using flow cytometry we isolated endothelial cells and investigated gene expression after irradiation. We then surprisingly observed that endothelial cells upregulated proinflammatory genes acutely after irradiation. This has previously not been observed in endothelial cells after *in vivo* irradiation, but indicates that although endothelial cells seem to be less sensitive to radiation, they are involved in the inflammatory response after irradiation.

Populärvetenskaplig sammanfattning

Strålbehandling är en vida använd metod för att behandla tumörer i hjärnan och många patienter med primära eller sekundära tumörer får strålning mot hela eller delar av hjärnan. Förbättrade behandlingsprotokoll har lett till en ökad överlevnad hos barn med hjärntumörer, men även resulterat i allt fler biverkningar som drastiskt sänker deras livskvalitet. Mycket talar för att även låga doser av strålning kan leda till svårigheter med minne och inlärning, en effekt som visat sig vara mer uttalad ju yngre patienterna är vid behandlingen och som förvärras med tiden.

Vi använder oss av en modell där unga möss och råttor strålas för att efterlikna de skador och biverkningar som uppkommer hos barn efter strålbehandling. De flesta celler i hjärnan delar sig väldigt sällan och nervcellerna delar sig inte alls. Därför tål hjärnan, som organ betraktat, strålning bättre än många andra organ. Tills nyligen har man trott att hjärnan påverkas av strålning främst genom att små blodkärl skadas och att störd blodtillförsel skulle skada omgivande vävnad. Då strålning framförallt påverkar celler som delar sig, ser vi i denna modell tydliga skador i hjärnans två områden med neurala stamceller. I denna avhandling har vi fokuserat på ett av dessa områden som kallas hippocampus och som anses viktigt för minne och inlärning. Det tros även finnas ett samband mellan minskad nybildning av nervceller i hippocampus och de problem med minne och inlärning som dessa barn uppvisar efter strålbehandling. Förhoppningen är att hitta strategier som skyddar dessa celler och därmed bidra till ökad livskvalitet för barn som överlever sin cancer. Stamceller i dessa områden är inte jämnt utspridda utan är koncentrerade runt blodkärl, sannolikt för att ha konstant tillgång till näring och de signalämnen som transporteras med blodet. Direkt fysisk kontakt mellan stamceller och endotelceller verkar vara

viktigt för stamcellernas förmåga att bilda nya nervceller. Tidigare forskning har föreslagit att stamcellernas närhet till blodkärl störs efter strålning mot den mogna hjärnan. En central fråga i denna avhandling har därför varit att undersöka om detta sker även i den unga, ännu växande hjärnan.

Studierna i denna avhandling visar att en relativt måttlig stråldos mot den unga hjärnan leder till en bestående minskning av neurala progenitorceller i hippocampus, och att den normala åldersrelaterade minskningen i nybildning av nervceller skedde snabbare i strålade djur. Förutom detta studerade vi även potentiella effekter på blodkärl, och fann att kärldensiteten anpassade sig till den omgivande vävnaden. Detta står i stark motsats mot de minskningar i kärldensitet som tidigare observerats efter strålning, dock efter mycket högre doser än i denna avhandling. Dessutom visar våra resultat att endotelcellerna inte dör direkt efter strålning. Om strålning resulterar i andra effekter än celldöd återstår dock att undersöka. I motsats till tidigare forskning såg vi enbart en akut störning av närheten mellan kärl och celler i hippocampus och att störningen återhämtade sig med tid efter strålning. Detta tyder på att den omogna och mogna hjärnan reagerar olika på strålning, vilket vi redan har sett i andra avseenden. Ett oväntat fynd var att endotelceller verkar vara involverade i det akuta inflammatoriska svaret som sker efter strålning och som har visat sig ha negativa effekter på nybildning av stamceller. Sammantaget tyder våra resultat på att blodkärlens endotelceller är mindre känsliga för strålning än omgivande celler, åtminstone vid dessa måttliga stråldoser, men att de kan spela en viktig roll i regleringen av den omgivande miljön efter strålning.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers/manuscripts, which will be referred to in the text by their Roman numerals:

I. **Irradiation to the young mouse brain caused long-term, progressive depletion of neurogenesis but did not disrupt the neurovascular niche**
Boström M, Kalm M, Karlsson N, Hellström Erkenstam N and Blomgren K.
*J Cereb Blood Flow Metab **33**(6): 935-943 (2013)*

II. **The hippocampal neurovascular niche during normal development and after irradiation to the juvenile mouse brain**
Boström M, Hellström Erkenstam N, Kaluza D, Jakobsson L, Kalm M and Blomgren K.
Submitted

III. **Irradiation to the young mouse brain impaired white matter growth more in females than in males**
Roughton K, Boström M, Kalm M and Blomgren K.
Cell Death and Disease, In press (2013)

IV. **Gene expression in endothelial cells isolated by flow cytometry after irradiation to the young mouse brain**
Boström M, Kalm M, Hellström Erkenstam N and Blomgren K.
Manuscript

LIST OF ABBREVIATIONS

+	Positive
BBB	Blood brain barrier
BLBP	Brain lipid-binding protein
BrdU	Bromodeoxyuridine
CB	Cerebellum
CC	Corpus callosum
CD13	Cluster of Differentiation 13
CD31	Cluster of Differentiation 31
CNS	Central nervous system
DCX	Doublecortin
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cell
GCL	Granule cell layer
Gy	Gray
IHC	Immunohistochemistry
MBP	Myelin basic protein
ML	Molecular layer
NPC	Neural progenitor cell
NSC	Neural stem cell
NSPC	Neural stem/progenitor cell
OB	Olfactory bulb
Olig2	Oligodendrocyte lineage transcription factor 2
P	Postnatal day
RMS	Rostral migratory stream
RNA	Ribonucleic acid
SGZ	Subgranular zone
SVZ	Subventricular zone

TABLE OF CONTENTS

INTRODUCTION

The large number of postmitotic neurons and other cell types with limited proliferation capacity makes the brain relatively radioresistant compared to other tissues with more rapid cell turnover. This view encouraged the use of high radiation doses within the central nervous system (CNS) during the previous century (Russell et al., 1949). However, shortly after the discovery of the X-rays (end of the 19th century) it was noticed that high radiation doses resulted in necrosis and gliosis of the brain some time after radiation. Major blood vessel abnormalities were a consistent finding within the damaged tissue and it was therefore hypothesized that the damage to normal tissue after irradiation was related to insufficient vascular supply (Hopewell et al., 1993). The validity of this hypothesis has been widely challenged but for long there was ample amount of evidence that the vasculature was the primary target that subsequently caused ischemia and necrosis (McDonald and Hayes, 1967).

The survival rates of children with childhood cancers have increased significantly during the last decades (~6) (Steliarova-Foucher et al., 2004, Gustafsson et al., 2013). As a consequence, a population of long term survivors which previously did not exist is now emerging. Individuals treated with cranial radiotherapy gradually develop negative so called late effects, such as hormonal imbalance, perturbed growth as well as impaired learning and memory (Lannering et al., 1990a). During the last decades (~3) treatment protocols have therefore been adjusted to reduce both radiation volumes and doses in the hope of reducing the negative side effects of radiotherapy. In line with this, many animal models have been redesigned, using lower radiation doses in order to more closely mimic the clinical settings. In the current thesis we have therefore exclusively used moderate

doses of radiation. This explains, at least partly, why we were unable to see the kind of vascular damage reported in previous studies following irradiation. However, it is remarkable that these relatively moderate doses nearly ablated hippocampal neurogenesis and that the natural, age-related decline in neurogenesis progressed more rapidly after irradiation. The link between cognitive decline and reduced hippocampal neurogenesis demonstrates the importance of finding ways to prevent or ameliorate this chronic ablation of neurogenesis after radiotherapy.

VASCULAR AND NERVOUS SYSTEM

On the macroscopic level, anatomical similarities between the vascular and the nervous system were observed in the 16th century by the Belgian anatomist Andreas Vesalius. Furthermore, when methods such as histology evolved, a mutual relationship between the two systems was found also on the microscopic level. In fact, the vascular and the nervous system rely on reciprocal communication in order for correct development and functional integration to occur (Mukouyama et al., 2002, Larrivee et al., 2009, Tam and Watts, 2010). Today, it is generally accepted that the modern nervous system arose earlier in evolution than the vascular system. The question regarding which of the systems that regulates or coordinates the other is however under debate. In the developing mouse embryo, outgrowth of the vasculature has been observed to precede neural axon outgrowth (Carmeliet and Tessier-Lavigne, 2005, Tam and Watts, 2010). However, at the same time is has been suggested that peripheral nerves in the skin establish branching patterns of blood vessels as well as arterial differentiation (Mukouyama et al., 2002).

A model has emerged where endothelial tip cells in vessels sense and navigate to their target in a similar fashion that axonal growth cones sense the surroundings and find their way (De Smet et al., 2009). In addition, multiple molecules and signal pathways are used by both the neural and vascular system. Several of these molecules were initially discovered as important for axonal pathfinding (e.g. ephrins, netrins, slit and sematophorins) but have also been identified as involved in vascular remodeling and vessel guidance (Autiero et al., 2005, Carmeliet and Tessier-Lavigne, 2005, Eichmann et al., 2005). Likewise, some well-known factors involved in angiogenesis (e.g. vascular endothelial growth factor; VEGF and angiopoietin 1; Ang-1) are also regulators during development and normal function of the nervous system (Lee et al., 2009). VEGF is in fact a potent stimulator of neurogenesis

both *in vivo* and *in vitro* (Jin et al., 2002). Hence, there is a close connection between the vascular and the nervous system.

Vasculature

During the evolution of multicellular organisms, the delivery of oxygen to all tissues by simple diffusion became insufficient. The need for a circulatory system increased as multicellular organisms progressively evolved (Fisher and Burggren, 2007). In aerobic animals, the vascular system exhibits crucial functions in all tissues of the body through the delivery of oxygen and nutrients, as well as the removal of waste products. In fact, since the vasculature is essential for the development of all other organs, it is the first system to develop during embryogenesis (Hirschi et al., 2002). The human brain has an extensive vascular network consisting of approximately 400 miles of blood vessels. Through these, the brain receives and uses around 20% of the energy consumed within the body. This is remarkable in the sense that the brain only constitutes 2% of the total body mass. A continuous blood supply to the brain is crucial given that the brain has no local energy reserve and damage to neurons can therefore occur within minutes if cerebral blood flow stops or decreases. Vessel diameter and cerebral blood flow are dependent on the local demand for oxygen and nutrients and is consequently not fixed for a certain area or region. Increased neuronal activity is therefore intimately coupled to increased cerebral blood flow (Lok et al., 2007, Zlokovic, 2008, Tam and Watts, 2010).

Vasculogenesis and angiogenesis

The formation of blood vessels involves two different processes; vasculogenesis and angiogenesis. In the embryo, new vessels are formed from endothelial progenitor cells (EPCs, angioblasts), which together create a vascular plexus consisting of a meshwork of endothelial cells (ECs). This

16

process of *de novo* production of blood vessels is known as vasculogenesis and is established before blood flow begins. The vascular plexus then serves as template for further growth and modifications into a mature vascular system. As the embryo grows, expansion of the vascular system is instead dependent on angiogenesis. The term angiogenesis is generally used to describe the growth of vessels, but in its strictest sense only refers to vessel sprouting and elongation from preexisting ECs. Angiogenesis does hence not include *de novo* generation of vessels (Hirschi et al., 2002, Swift and Weinstein, 2009, Tam and Watts, 2010, Potente et al., 2011).

Vasculogenesis was originally believed to occur exclusively during embryogenesis and instead it was believed that all postnatal formation of new blood vessels occurred through angiogenesis. However, during recent years it has been suggested that EPCs from the bone marrow can participate in postnatal neovasculogenesis (Khakoo and Finkel, 2005, Ribatti, 2007). EPCs were first characterized by Asahara et al. when they isolated CD34$^+$ cells from human peripheral blood and showed that these cells were capable of differentiation into ECs *in vitro* (Asahara et al., 1997). The EPCs have been shown to play a critical role in both vascular homeostasis and postnatal vasculogenesis (Khakoo and Finkel, 2005). Furthermore, the EPCs can incorporate into sites of angiogenesis in models of ischemia, hence proposing that enhancing EPC-dependent vasculogenesis could be used for therapeutic purposes in pathological situations (Asahara et al., 1997). However, these cells have also been proposed to play an essential role in the abnormal vascular growth occurring in tumors and it has been shown that ablation of EPCs can reduce tumor growth (Mellick et al., 2010). In addition, compared to healthy controls, increased numbers of circulating EPCs have been found in lung cancer patients (Nowak et al., 2010). The potential therapeutic usefulness of EPCs therefore demonstrates opposite purposes in different

pathological situations: (i) In ischemic tissue EPCs could be used to increase vessel growth and (ii) in tissues with abnormal vessel growth (such as tumors), EPCs could be targeted in order to inhibit further vessel expansion.

Adult neurogenesis

Concept of neurogenesis

The term stem cell means that a cell is capable of self-renewal and to give rise to a clonal progeny capable of differentiation. To be named neural stem cell (NSC), the cell needs to be capable of producing the three major cell types of the brain: neurons, oligodendrocytes and astrocytes (Figure 1). NSCs can give rise to neural progenitor cells (NPCs) that are restricted to the neuronal lineage (Zitnik and Martin, 2002).
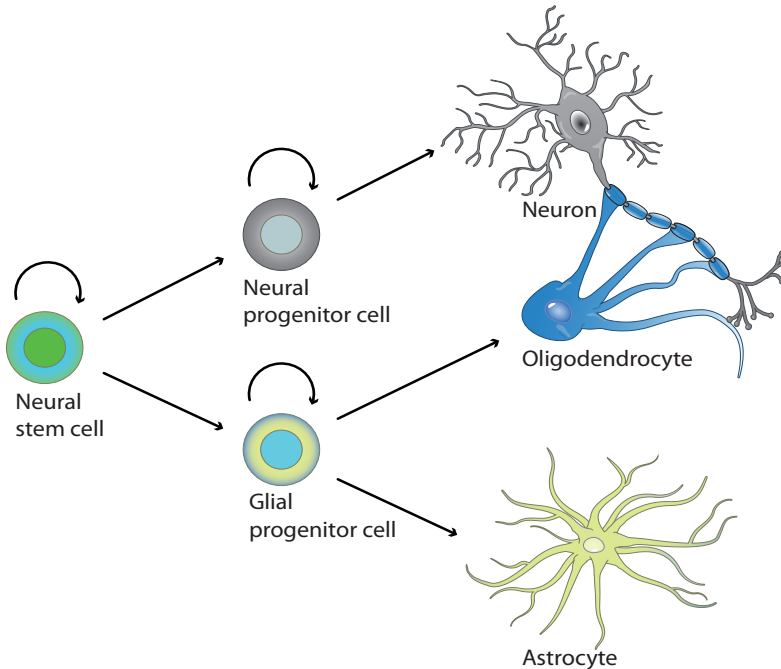
Figure 1. Schematic illustration of the neuronal stem cell lineage in the brain where neural stem cells divide to give rise to new neurons, oligodendrocytes and astrocytes (illustration made by Simon Lundholm @ 300Kelvin).

Neurogenesis is the mechanism by which new neurons are generated from neural stem/progenitor cells (NSPCs). This is most pronounced during embryonic/prenatal development when the newly born neurons populate different areas in the continuously growing brain. The existence of neurogenesis in the adult brain has however historically been extensively questioned.

In the beginning of the 20th century, most neuroscientist including Ramon y Cajal, believed that the adult brain had a definite number of cells without regenerative capacity (Stahnisch and Nitsch, 2002). However, this general consensus was later challenged by Joseph Altman when he discovered that the adult rat brain actually did contain newborn neurons (Altman and Das, 1965). The existence of adult neurogenesis has been observed in many mammals but also in other non-mammalian species, e.g. in insects, reptiles and birds (Goldman and Nottebohm, 1983, Font et al., 1991, Cayre et al., 1996). This indicates that adult neurogenesis could represent an evolutionary ancient phenomenon. Compared to neurogenesis in the embryo, neurogenesis in the adult brain is however considerably limited.

Adult neurogenic areas

Today, neurogenesis is accepted to exist in two areas of the adult brain: (i) adult neurogenesis occurs is the subventricular zone (SVZ) located in the walls of the lateral ventricles. Progenitor cells born in the SVZ migrate long distances via the rostral migratory stream (RMS) to the olfactory bulb (OB) where they differentiate into new interneurons (Alvarez-Buylla et al., 2008). In the current thesis we have however not studied the SVZ but instead focused on (ii) the subgranular zone (SGZ) located at the border between the hilus and the granule cell layer (GCL) of the hippocampal dentate gyrus (DG). Neurogenesis in the SGZ gives rise to new granule cells which make

dendritic connections within the molecular layer (ML), followed by axonal connections with the hippocampal CA3 region (Kempermann et al., 2004a, Frielingsdorf and Kuhn, 2007, Grote and Hannan, 2007, Ming and Song, 2011). As much as 80-85% of the granule cells are generated after birth in the rodent DG, (Bayer, 1980). Kempermann et al. have shown that the majority of new granule cells born in the adult rodent brain stay within the inner third of the GCL (Kempermann et al., 2003). This was further supported by the finding that stem cells and their progeny only give rise to about 1% of the granule cells in the outer adult GCL (Lagace et al., 2007). Furthermore, it has been shown that hippocampal neural stem cells have vascular endfeet, a feature normally attributed to astrocytes. It was therefore proposed that these radial glia-like stem cells arise from astrocytes (Filippov et al., 2003), commonly referred to as type-1 cells (Figure 2) (Kempermann et al., 2004a). This model has assumed that only one type of radial glia-like stem cell exists. However, it was recently shown that the adult DG has a population of antigenically heterogeneous radial glia-like stem cells (DeCarolis et al., 2013). In 1998, Peter Eriksson discovered that neurogenesis in the SGZ of the DG also occurs in the adult human brain and that neurogenesis is a life-long process (Eriksson et al., 1998). The adult human hippocampus is estimated to generate 700 new neurons daily in each hemisphere. Furthermore, it was recently shown that the turnover rate of hippocampal neurons does not differ between females and males (Spalding et al., 2013).
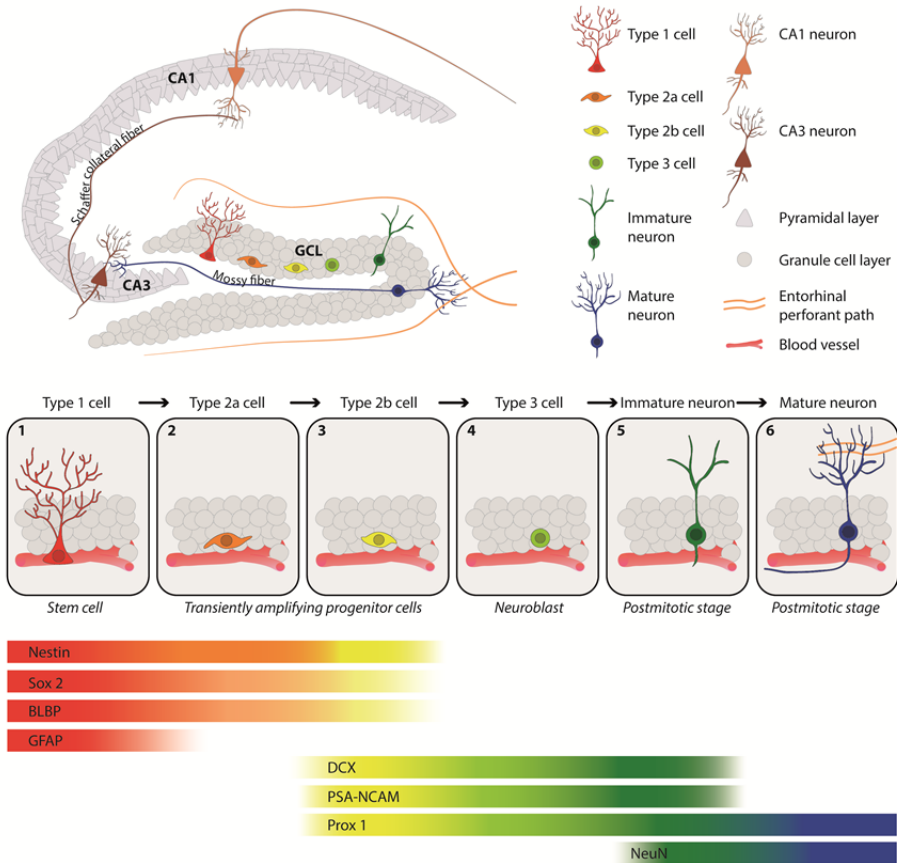
Figure 2. *Development of neural stem cell to mature neuron in the hippocampal dentate gyrus. A model has proposed that new granule cells in the hippocampus are produced by six developmental milestones (starting with the type 1 cell and ending with a mature neuron) (Kempermann et al., 2004a). The cells express various markers during differentiation and maturation (some of them are outlined in the figure). The hippocampal circuit is usually described as trisynaptic. First, the mature granule cells in the GCL receive input from the entorhinal cortex via the perforant path. The granule cells, in turn, projects axons to CA3 neurons, where the signal is relayed through the Schaffer collateral fibers to the CA1 region. Finally the output of the hippocampus is relayed back to the entorhinal cortex (illustration made by Simon Lundholm @ 300Kelvin).*

Apoptosis

Apoptosis or programmed cell death during mammalian development of the brain is an important process to eliminate superfluous or incorrectly functioning neurons (Kuhn et al., 2005). Every day as many as 9 000 new cells are produced through neurogenesis in the SGZ of the DG in a young adult male rat. But if all of these newly generated cells would survive, the number of cells in the GCL of the DG would be twice as great in only a few months. To prevent this, cells must be eliminated. Approximately half of the newly generated cells die during the first weeks of their life, independent of how old the animal is. The majority of dying cells in the GCL are immature neurons undergoing apoptosis when they shift from transiently amplifying progenitor cells to neuroblasts. Consequently, neurogenesis in the DG is probably not a process of replacement but rather refinement of the amount of new cells (Frielingsdorf and Kuhn, 2007, Sierra et al., 2010).

Prolonged postnatal neurogenesis in the cerebellum

Besides the two neurogenic regions, generation of new neurons is restricted to fetal development in most areas of the brain. However, similar to the DG, most interneurons in the GCL of the rodent cerebellum are formed during the first two postnatal weeks (Miale and Sidman, 1961). Furthermore, proliferation in the human cerebellum continues until the 11th postnatal month (Abraham et al., 2001). However, although significant postnatal neurogenesis occurs in the cerebellum it is not considered an adult neurogenic region. The cerebellum was first identified as important for motor functions but has also been linked to higher motor and cognitive functions such as language production and motor planning (Rapoport et al., 2000, Konczak and Timmann, 2007).

NEUROVASCULAR NICHE

The concept of a specific vascular niche within the neurogenic areas was first described by Palmer et al. when they showed that newly generated neurons in the hippocampus were associated with dividing ECs (Palmer et al., 2000). This suggested that NPCs and ECs either respond to the same mitogenic signals or that the cell division of one cell type generates mitogens that in turn triggers the mitotic expansion of the other (Goldman and Chen, 2011). Hence, further studies were needed in order to verify the functional implication of such a specialized vascular niche. Accordingly, *in vitro* experiments showed that ECs release soluble factors that are involved in maintaining the multipotency of stem cells, as well as stimulating self-renewal and increasing neuronal production (Shen et al., 2004). When angiogenesis is pharmacologically inhibited by endostatin, decreased angiogenesis is accompanied by reduced neurogenesis (Nih et al., 2012). Furthermore, stem cells are in close contact with the basal membrane of ECs by extending vascular end feet (Filippov et al., 2003).

When the vascular systems of two mice of different ages were surgically joined together by parabiosis, neurogenesis in the young mouse decreased while neurogenesis in the old mouse increased. A link between age-related reduction in neurogenesis and intrinsic factors in the blood was hence proposed (Villeda et al., 2011). Moreover, the importance of the microenvironment was demonstrated when cerebellar precursors were ectopically transplanted into the DG of neonatal rats, where they integrated into the hippocampal GCL. The transplanted neurons were morphologically similar to hippocampal neurons and expressed the same cell-type specific proteins (Vicario-Abejon et al., 1995). In addition, when striatal progenitor cells were transplanted into cortical environment they adopted features specific for cortical neurons (Fishell, 1995). The microenvironment has

consequently been proposed to determine the regional fate of neural precursors.

Blood brain barrier

The blood brain barrier (BBB) is a physical and metabolic barrier that separates the CNS from the systemic circulation. Physiologically, the BBB is mainly composed of three different cellular elements: ECs, astrocytic endfeet and pericytes (Figure 3). However, it is believed that other cell types such as microglia are also involved in the most important and crucial feature of the BBB which is to maintain homeostasis and limit the penetration of pathogens and toxins into the brain. This is maintained by restricted and regulated exchange of different molecules between the blood stream and the brain (Correale and Villa, 2009, Goldberg and Hirschi, 2009, Tam and Watts, 2010). The BBB is established during fetal development and is well functional by birth (Abbott et al., 2010). Vulnerability of the BBB has been proposed to be age-dependent with increased susceptibility to hypoxia ischemia and inflammation in younger animals (Semple et al., 2013).
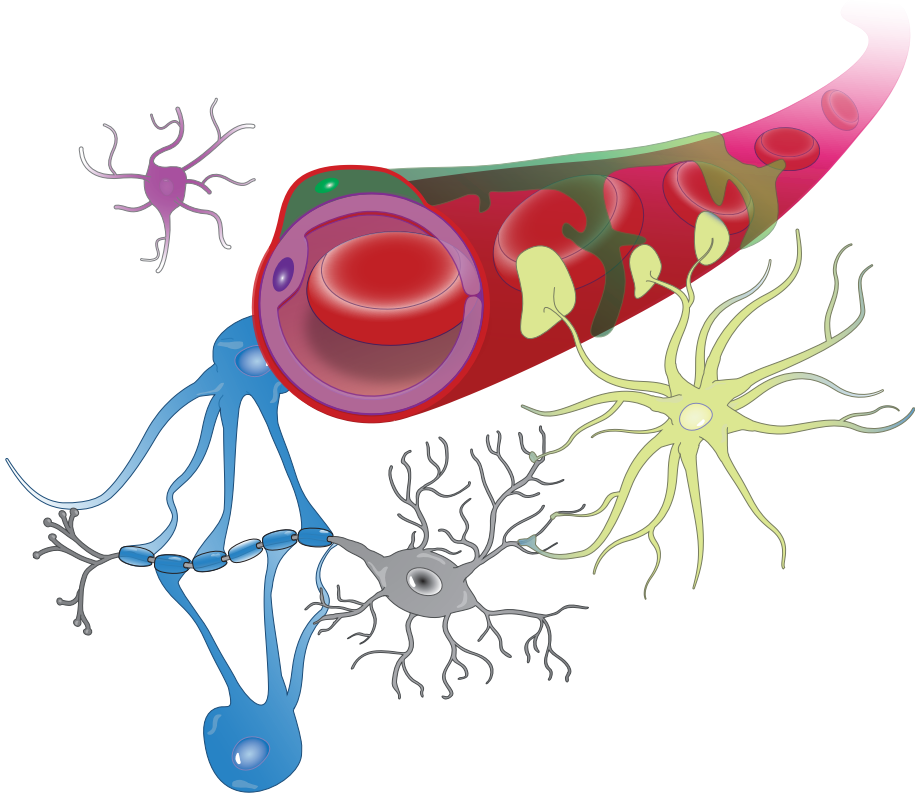
Figure 3. *Illustration of the different cell types involved in maintaining the blood BBB together with other important cell types in this thesis. The BBB physically consists of microvessels (red) with inner lining of endothelial cells (purple), surrounding pericytes (green) and astrocytic endfeets (yellow). Microglia (purple) has also been proposed to play an important role in the BBB by the defense against pathogens. Finally, oligodendrocytes (blue) generate myelin sheaths that wrap around neuronal axons (grey). The astrocyte has contact with both vessels and neurons, thereby acting as a key mediator between these two cell types (illustration made by Simon Lundholm @ 300Kelvin).*

Endothelial cells

The ECs constitute the inner lining of blood vessels and are therefore considered the key component of the BBB. Vascular endothelium is so called semipermeable, which means that the transport into and out of the blood is regulated. However, ECs display an enormous heterogeneity in both morphology and permeability between different organs and a number of physiological properties make the endothelium in the CNS distinct from the vasculature found in the periphery. CNS-derived ECs are connected by tight junctions, lack fenestrations and have low numbers of pinocytotic vesicles (Rubin and Staddon, 1999, Goldberg and Hirschi, 2009, Segura et al., 2009), thereby ensuring a highly regulated exchange between the brain and the blood circulation. Interestingly, transplantation experiments have revealed that BBB properties are not intrinsic to ECs but rather a consequence of signals from the environment within the brain (Stewart and Wiley, 1981). However, DNA microarrays have revealed different transcriptional profiles of ECs from different tissues and from large vessels and microvessels, thereby suggesting that maintaining a specific endothelial phenotype is not exclusively dependent on environmental signals (Chi et al., 2003).

In humans, ECs have an average lifespan of around 1 year and proliferation of ECs in the mature vascular system is generally considered stable with infrequent cell turnover. However, ECs are not an inert cell type but rather highly metabolic and involved in many crucial processes such as: leukocyte infiltration, permeability, regulating the proliferation and survival of surrounding cells, maintaining homeostatic balance and regulating vasomotor tone (Hirschi et al., 2002, Aird, 2007, Goldberg and Hirschi, 2009). Originally, the ECs were considered the ultimate compartment of the BBB but nowadays other cell types have also been identified as key components.

Pericytes

All vessels have an inner lining of ECs surrounded by perivascular mural cells. Microvessels are associated with solitary pericytes while larger vessels instead are associated with several layers of vascular smooth muscle cells. During development, ECs of immature vessels secrete growth factors to attract pericytes. The pericytes extend processes that wrap around the wall of microvessels and they are considered important for microvessel stability. Importantly, ECs and pericytes share the same basement membrane which enables them to communicate directly. Maturation of vessels is associated with attachment of pericytes to the vessel wall (Lindahl et al., 1997, Ramsauer et al., 2002, Bergers and Song, 2005, Lee et al., 2009). The role of pericytes can be compared to that of oligodendrocytes, which are responsible for the myelination of neurons in the CNS; both oligodendrocytes and pericytes support and enhance the function of the cell type that they enclose (Tam and Watts, 2010).

Pericytes are an important compartment of both the neurovascular niche and the BBB. It has been proposed that a mature vascular network consisting of both ECs and smooth muscle cells is crucial for the survival of neuroblasts after cerebral ischemia (Nih et al., 2012). The vasculature in the CNS harbors the highest pericyte coverage compared to other investigated organs. It is interesting to note that increased pericyte density and/or coverage appear to correlate well with barrier properties of the endothelium. In addition, slow endothelial turnover is correlated with high pericyte coverage, and vice versa (Allt and Lawrenson, 2001, Lee et al., 2009, Armulik et al., 2011). Furthermore, pericytes exhibit contractile properties and can regulate the blood flow at the capillary level by modulating capillary diameter (Peppiatt et al., 2006). Pericyte loss is associated with pathological BBB breakdown, resulting in increased BBB permeability (Armulik et al., 2010, Bell et al.,

2010). Interestingly, pericytes have been implicated to act as brain phagocytes at sites with BBB properties (van Deurs, 1976), a role that is normally ascribed to perivascular microglia.

Microglia

Microglia are the main cells of the immune system in the CNS and in the brain represent approximately 10% of the adult cell population. They arise from myeloid cells that during early embryonic development migrate from the yolk sac to the brain. In the brain, microglia progenitors give rise to mature microglia that sustain a population in the adult brain by *in situ* proliferation. In mice, the majority of microglia are produced during the first two postnatal weeks (Alliot et al., 1999, Ajami et al., 2007). Depending on their activation state, microglia can exhibit both beneficial and detrimental effects on adult neurogenesis (Kohman and Rhodes, 2013). In the so called resting state, microglia have a small cell body with many fine processes and survey the surrounding for signs of damage or infection as well as maintaining homeostasis. However, in response to harmful stimuli microglia undergo several important characteristic changes such as increased proliferation, retraction of processes and swelling of the cell body. In addition, they start releasing several proinflammatory molecules which initiates the inflammatory response (Monje et al., 2002, Kohman and Rhodes, 2013). Furthermore, cocultures of brain ECs and human blood-derived macrophages decrease paracellular permeability, thereby proposing that these cells play an active role in BBB maintenance and physiology (Zenker et al., 2003).

Microglia also play a very important role during development by quickly eliminating excessive newborn cells in the SGZ through phagocytosis. Importantly, this phagocytosis by microglia does not involve activation of

microglia, proposing that phagocytosis is possible without complete microglia activation (Sierra et al., 2010). Cocultures of NPCs and microglia have shown that microglia can enhance postnatal neurogenesis in the SVZ (Walton et al., 2006). Furthermore, a cross-talk between microglia and newly formed neurons has been proposed as beneficial for neurogenesis (Ekdahl et al., 2009).

Astrocytes

Although glial cells are the most abundant cell type in the brain they were originally considered as supportive and passive cell types. Astrocytes account for around 50% of all glial cells in the brain and outnumber neurons by four times in higher mammals. Typically, astrocytes have a stellate shape, thereby the name, with multiple processes extended towards neurons and blood vessels. One single astrocyte can contact several vessels and synapses and it is therefore believed that astrocytes function as key mediators of neurovascular coordination. In fact, it has been proposed that the anatomical position of astrocytes could affect blood flow regulation by delivering signals between neurons and vessels. In addition, astrocytes are involved in the regulation and maintenance of the BBB (Allen and Barres, 2009, Lee et al., 2009, Greene-Schloesser et al., 2012). Astrocytes aid in the guidance of neurons in order for them to make the proper connections during fetal development of the brain (Virgintino et al., 1998). Furthermore, migrating neuroblasts in the RMS are surrounded by a network of astrocytes, referred to as the glial tube and bi-directional signals between the two cell types have been shown to regulate proliferation and migration of the neuroblasts (Cleary et al., 2006).

WHITE MATTER

The CNS is divided into two main components: white matter, which consists mostly of glial cells and myelinated axons, and gray matter, which consists mostly of neuronal cell bodies. White matter was historically viewed as a relatively passive tissue but recent research has shown that an intact white matter is important to maintain cognitive functions such as information processing (Palmer et al., 2012). Interestingly, MRI studies have revealed sexual differences in the density of gray and white matter. Men had a higher density of white matter and women had a higher density of gray matter. Moreover, men had a higher density of gray matter in the left hemisphere. For white matter no asymmetry was detected for men, and women showed no asymmetry for neither white nor gray matter (Gur et al., 1999). Furthermore, the general consensus is that the male brain is around 8-10% larger than the female brain. The brain continues to grow after birth and it has been shown that females reach maximum volume earlier than males (10.5 years in females and 14.5 years in males) (Lenroot et al., 2007).

Corpus callosum

The corpus callosum forms an axonal bridge that connects cortical neurons from the two cerebral hemispheres (Figure 4). Functions related to the unification of the hemispheres include e.g. memory retrieval and storage, enhancing language and auditory function. The corpus callosum is the largest white matter commissure in the brain with approximately 200 million fibers, a number that is already set around birth. However, important structural changes such as myelination, pruning and redirection occur during postnatal development (Luders et al., 2010). The corpus callosum continues to grow during childhood and adolescence with the highest growth peak during the first years in life (Lenroot and Giedd, 2006).
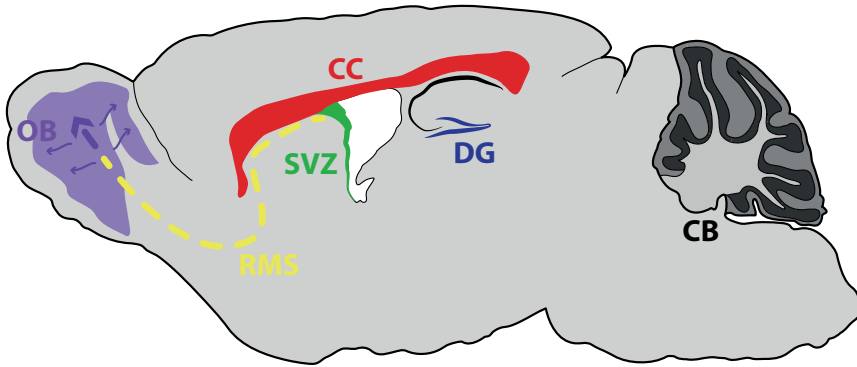
Figure 4. *Sagittal illustration of a mouse brain with specific regions of interest for the current thesis outlined: cerebellum; CB, corpus callosum; CC and dentate gyrus; DG. The illustration also shows the olfactory bulb; OB, rostral migratory stream; RMS and subventricular zone; SVZ (illustration made by Simon Lundholm @ 300Kelvin).*

Oligodendrocytes

Oligodendrocytes generate myelin sheaths that wrap around neuronal axons to increase the neurotransmission within the CNS. The density of oligodendrocytes is remarkably similar between humans and rodents, although of course humans have significantly superior total numbers (Bradl and Lassmann, 2010). However, the density of oligodendrocytes differs between males and females, with the highest densities observed in males. In this aspect it is surprising that the proliferation of new glial cells (predominantly oligodendrocytes and astrocytes) in females is twice as high as the proliferation in males. This could however be explained by the fact that females also have twice the number of apoptotic glial cells. It was therefore proposed that oligodendrocytes in females have a more rapid cell turnover than oligodendrocytes in males (Cerghet et al., 2006, Cerghet et al., 2009). Historically, myelination in humans was considered to be completed during the first years of life but it is nowadays recognized that myelination continues

into the second and third decades of life (Semple et al., 2013). Interestingly, females have been proposed to have more unmyelinated fibers than males. However it has been shown that this ratio changes with age. The volume of white matter, myelinated fibers and myelin sheaths is significantly larger in young males compared to young female rodents, followed by the reversed ratio in middle-aged rodents (Cerghet et al., 2009).

CHILDHOOD CANCERS

In Sweden around 50 000 persons per year are diagnosed with cancer. This number is immense compared to the approximately 300 children and adolescents every year diagnosed with malignancies. Although pediatric cancers represent only a fraction of all diagnoses, the increasing number of childhood cancer survivors is creating a public health issue as they transition into adulthood since they today constitute a population which did not exist before. Pediatric cancers are slightly more frequent in boys than girls and are most common in children between 2 and 6 years. (Barncancerrapporten, 2013). After the leukemias, brain tumors are the most common type of pediatric cancers and account for approximately 20-30% of all childhood malignancies. However, it should be noted that malignant neoplasms before the age of 20 are rare (Parkin et al., 1988, Steliarova-Foucher et al., 2004). In both children and adolescents diagnosed with brain tumors, the survival rates have improved significantly during the last decades and the 5-year survival is today more than 80% (Steliarova-Foucher et al., 2004, Gustafsson et al., 2013). However, with the emergence of increased survival rates it became progressively clear that many of the long-term survivors exhibit multiple so called late effects. In fact, a study proposed that as much as 96% of pediatric brain tumor survivors suffer from late effects (Han et al., 2009).

Radiotherapy

Cancer in both children and adults is treated with a combination of chemotherapy, radiotherapy and surgery, however the treatment protocols differ significantly. This is due to the fact that children generally tolerate chemotherapy better than adults but are at the same time extremely susceptive to radiotherapy (Barncancerrapporten, 2013), mostly because they are still growing and developing. Cranial radiotherapy is usually not even considered in children younger than 4 years of age. Even very small doses of radiation can therefore cause significant damage in children and may influence cognitive function into adulthood (Hall et al., 2004). Radiotherapy is historically the treatment strategy associated with the most complications, but improved protocols during the last decades have significantly reduced these problems. However, the complications still exhibit a major limitation in the treatment. Since radiotherapy commonly is combined with other treatment modalities it complicates the discrimination of what negative effects are attributed to radiation alone and what effects that are due to chemotherapy, surgery and the disease itself.

Cranial radiotherapy is associated with multiple late effects that last into adulthood such as psychological-emotional dysfunction, intellectual and memory impairments and perturbed growth an puberty (Lannering et al., 1990a, Lannering et al., 1990b, Kuhn and Blomgren, 2011). Young age and female gender are associated with greater negative effects, such as cognitive decline (Ris et al., 2001, Fouladi et al., 2005, Lahteenmaki et al., 2007). Furthermore, some of the observed effects could be due to white matter damage as younger age at the time of irradiation significantly reduces white matter volume (Reddick et al., 2000, Mulhern et al., 2001, Palmer et al., 2012).

Radiobiological effects

During radiotherapy there is a delicate balance between the goal of killing all malignant cells but at the same time sparing normal cells from damage. Ionizing radiation means that the radiation has enough energy to create ions in its passage through matter. The major effect of ionizing radiation is DNA damage caused either directly or indirectly. In the direct pathway the DNA molecule is directly damaged by irradiation while in the indirect pathway free radicals are created, which in turn cause DNA damage. Both pathways can create single or double strand breaks of the DNA molecule (Gudkov and Komarova, 2003, Lieberman, 2008, Magnander and Elmroth, 2012). Cells are more vulnerable when they are dividing, and since cancer cells divide more rapidly they have higher susceptibility to radiation than normal cells. Normal cells can therefore generally recover from the effects of radiation more easily than cancer cells can. Radiotherapy exploits the small difference in radiosensitivity between tumor cells and normal cells, referred to as the therapeutic index or therapeutic ratio (Dunne-Daly, 1999, Thoms and Bristow, 2010). However, irradiation targets all cells and it is therefore impossible to completely spare the normal tissue. The normal tissue hence becomes the dose-limiting factor during radiotherapy with the goal to damage as few normal and healthy cells as possible.

It is primarily differences in proliferation that account for the different radiosensitivity between different cell types since the biological effects of radiation is usually not visible until the cell tries to divide. Consequently, in high proliferation cells such as tumor cells and normal rapidly dividing cells (e.g. neural progenitor cells); the effects are observed early after irradiation. However, in non-dividing cells such as postmitotic neurons and rarely diving cells the effects will be observed much later. Following irradiation, a cell can undergo apoptosis, mitotic catastrophe, and permanent or temporary growth

arrest while trying to repair the damage. Frequently dividing cells often undergo apoptosis or mitotic catastrophe after irradiation, while less frequently dividing cells to a greater extent either complete DNA repair or enter a stage of growth arrest. Tumor cells undergo mitotic catastrophe to a greater extent than other cell types since they have an unstable genome, lost their regulation of growth, have a dysfunctional repair system and often also lost their capability to undergo apoptosis (Gudkov and Komarova, 2003).

GENERAL AIM

The aim of this thesis was to investigate how the neurovascular niche is affected by irradiation to the developing brain. This was done in order to increase the knowledge about how neurogenesis could be restored after irradiation and hopefully also result in beneficial effects on behavior.

SPECIFIC AIMS

I.   To investigate structural effects on the vasculature after irradiation in both neurogenic and non-neurogenic areas

II.   To investigate the radiation-induced effects on neurogenesis, both short-term and long-term

III.   To investigate if and how the neurovascular niche is altered after irradiation

IV.   To investigate the molecular response of endothelial cells to irradiation

METHODOLOGICAL CONSIDERATIONS

The following section describes the most important methods used in this thesis. I have tried to highlight pros and cons of each individual method, as well as problems that may occur. For detailed descriptions of the specific experimental procedures, please read the material and methods section for each individual paper/manuscript.

ANIMALS

In the current thesis we only utilized animal experiments (*in vivo)* as a model of irradiation-induced damaged to the developing brain. Another possibility would have been to use cell culture models (*in vitro*) which are very useful and sometimes can replace *in vivo* models partly or completely. However, the main focus on the current thesis was the neurovascular niche that contains many different cell types. Currently used *in vitro* models of brain vasculature do not recapitulate the *in vivo* complexity of the neurovascular niche. We therefore chose to use *in vivo* models only in order to study a complete system where all cell types and physiological responses are included.

Ethical permission

Before conducting animal experiments, all parts of the experimental design need to be approved, previously by the Swedish Animal Welfare Agency (Djurskyddsmyndigheten), currently by the Swedish Board of Agriculture (Jordbruksverket). This procedure ensures that the well-being of the animals is always prioritized. Animals were housed with *ad libitum* access to food and water and carefully monitored. All experimental designs in this paper were thoroughly reviewed and approved by the ethical committee (applications no. 6/2007, 30/2008, 423/2008, 326/2009 and 361/11).

C57BL/6 mice delivered from Charles River Breeding Laboratories in Germany were used in all four papers. In papers I, II and IV, only male mice were used but in paper III we used mice of both sexes to investigate different responses to irradiation between the sexes. If possible, the animals were delivered several days before the irradiation procedure to acclimatize and avoid unnecessary stress that could influence the outcome of the experiment.

Irradiation model

In our model of irradiation we use a linear accelerator with 4 megavoltage nominal photon energy (Varian Clinac 600 CD) located at the Sahlgrenska University Hospital in Gothenburg to irradiate the developing mouse brain. This accelerator is normally used for radiotherapy of patients (Figure 5). The advantage of using the same irradiation source as that used for radiotherapy of patients is that we come closer to mimicking the clinical settings. The downside is that this adds an extra stress to the animals since they need to be transported by car between the hospital and the animal facility.

## General irradiation procedure

In order for the animals to stay completely still during the irradiation procedure, all animals (both control and irradiated) were anesthetized with an intraperitoneal (i.p.) injection of tribromoethanol (Avertin®). Tribromoethanol is an injectable anesthetic agent that is used during short experimental procedures such as surgery. Repeated use of tribromoethanol is associated with increased morbidity and mortality, which is why tribromoethanol is recommended for single use only. This is one reason why the animals in our studies do not receive fractionated irradiation.

The animals were kept on a warm bed (36°C) both before and after irradiation to maintain the body temperature and physiological parameters when separated from the mother. This step is very important, not only to aid survival, but also since hypothermia decreases the radiosensitivity in the tissue and thereby may mask some of the radiation-induced effects. Control animals were anesthetized and kept on a warm bed but did not receive any irradiation.
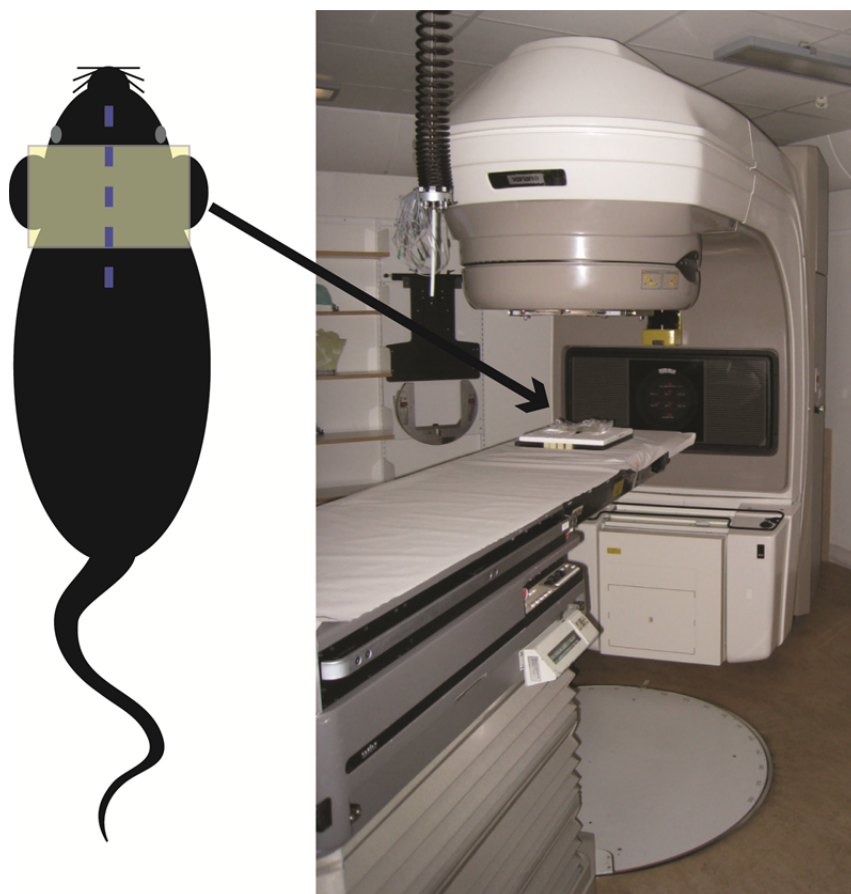
Figure 5. The irradiation model used in this thesis where the mice were irradiated with a linear accelerator normally used for patients (illustration made by Simon Lundholm @ 300Kelvin).

Different radiation qualities (e.g. photons, protons, particles) deposit their energy at different depths during their journey through matter (in our case the brain). In external radiation therapy, a variety of energy sources/radiation qualities can be utilized but the most commonly used for patients are megavoltage photons. For radiation with megavoltage photons, the deposited dose of radiation is low at the skin and the maximum dose is in fact not achieved until several cm into the tissue. This type of irradiation therefore exhibits skin-sparing properties and is very useful in the treatment of many human cancers. However, it is problematic for treatment of superficial lesions in close relation to the skin.

Irradiation with megavoltage photons was used in all papers of this thesis. This type of irradiation is however problematic for our experimental model of whole brain irradiation (rats and mice) since we want the whole brain to be irradiated with the same dose. Given that maximal dose is deposited several centimeters into the tissue, in our case brains not bigger than a kidney bean, most of the brain would not receive the desired irradiation dose. Therefore, the head is covered with a tissue equivalent material (bolus) to obtain an even radiation dose in the recipient tissue. The bolus material builds up the radiation dose prior to entry of the beams into the brain, and thereby increases the radiation dose deposited at the surface. As a consequence, the maximum dose of radiation is delivered throughout the brain.

Clinical perspective

In all papers/manuscripts in the current thesis, mice were irradiated on postnatal day 14 (P14). Our ultimate aim is to correlate human brain development with the mouse so that the experimental effects we observe could be directly transferred into the clinic. However, there are major

differences between human and rodent brain development which significantly complicates this translation (for review see Semple et al., 2013).

When radiation is used for therapeutic purposes, the total dose is divided into different fractions (usually 2 Gy/fraction). This treatment strategy is used to kill cancer cells but at the same time spare normal tissue as much as possible (Kuhn and Blomgren, 2011). It has previously been reported that fractions above 2 Gy should be avoided due to severe tissue damage as a result (Soussain et al., 2009). The optimal scenario would have been to use fractionated doses also in our experimental studies. However, the mice in this thesis were only exposed to single doses of irradiation. This is due to several practical reasons such as the previously mentioned anesthesia issue, stress during transportation and the potential risk of infections when bringing animals back and forth between the animal facility and the hospital. For those reasons, we use the LQ formula to estimate the equivalent dose of 8 and 10 Gy, respectively, if it instead had been delivered in 2 Gy fractions (Fowler, 1989). A single dose of 8 Gy then corresponds to approximately 18 Gy for late effects in normal brain tissue, which is the dose that is delivered for example to the brains and spinal cords of children with relapse leukemia. In addition, a single dose of 10 Gy corresponds to 26 Gy, which is similar to the dose that is delivered to children with medulloblastoma. Compared to the doses that are delivered to the tumor bed of solid brain tumors (up to 60 Gy), the doses we used are therefore considered as relatively moderate doses of irradiation. However, we should always keep in mind that single and fractionated irradiation will have slightly different radiobiological responses. For example, it has been shown that the molecular response to fractionated irradiation is slower compared to irradiation with a single dose which results in a rapid response (Gaber et al., 2003, Yuan et al., 2006).

TISSUE PREPARATION

Tissue preparation is one of the most important steps to consider for the optimizing of downstream analysis. The aim is to preserve the 3D structure of the tissue and allow further processing without change. Important objectives to consider when choosing your fixative are therefore preservation of cellular structure, rapid penetration of the fixative into the tissue and avoidance of autofluorescence.

In papers I-III in the current thesis, tissue for histological studies was fixated by transcardial perfusion with physiological saline (0.9%) or PBS followed by perfusion with formaldehyde solution. The rinsing with saline is important since it expels red blood cells from the vasculature, thereby reducing autofluorescence. Furthermore, the perfusion with formaldehyde enables a more rapid fixation of the tissue and thereby increased preservation. To ensure complete fixation, the tissue is also post-fixed in formaldehyde for 24 hours. The advantage of perfusion fixation is apparent when considering that the penetration rate of formaldehyde is around 24 hours to penetrate the center of a 10 mm thick specimen, i.e. approximately 5 mm per day. However, one major drawback of perfusion with formalin is that fixed tissue does not allow for protein analysis such as ELISA and western blot.

Formaldehyde is the most common fixative for histology studies. In papers I and II, formalin fixation was performed with 4% paraformaldehyde (PFA) solution freshly prepared in the lab. However, due to the hazard risk when preparing and using paraformaldehyde solutions, many laboratories now seek safer alternatives. As a consequence, a solution called Histofix has emerged on the market. Histofix is a buffered 6% formaldehyde solution that eliminates problems associated with toxicity, since it is ready to use without any preparation. In addition, high quality and stability of Histofix enable high

reproducibility between different studies. In paper III, we therefore chose to use Histofix instead of PFA.

STAINING PROCEDURES

General procedure of immunohistochemistry

Immunohistochemistry (IHC) is a method that utilizes the binding of antibodies to detect antigens in a tissue. The binding of an antibody (primary antibody) to its antigen is visualized through different detection systems that can be either direct or indirect and that is visualized by either a fluorescent dye or a chromogenic signal. The direct procedure is quick since the primary antibody is directly conjugated to a label. In comparison, the indirect method utilizes a two-step reaction where the primary antibody is visualized by a labeled secondary antibody (raised against the host species of the primary antibody). The direct method is faster, easily used for multicolor labeling and eliminates the potential nonspecific binding of the secondary antibody arising during indirect labeling. However, the indirect method generally has higher sensitivity than the direct method since the signal is amplified through the use of a secondary antibody that enables the binding of more than one secondary antibody to each primary antibody. Indirect labeling by IHC has been the key method throughout this thesis due to its increased signal amplification.

There are different kinds of primary antibodies. Polyclonal antibodies are usually isolated from serum and are a mixture of antibodies that recognize several epitopes of an antigen. Since the antibody can bind to more than one epitope, polyclonal antibodies can yield increased signal of the target protein. In addition, due to quick and large quantity production, these antibodies are generally cheap. The other class of antibodies is so called monoclonal antibodies. These antibodies only recognize one epitope and therefore

decrease background and potential cross-reactivity. The monoclonal antibodies are produced in smaller quantities and are therefore generally more expensive.

Possible amplification steps

The signal of the staining can be further amplified by utilizing the strong binding between avidin or streptavidin to biotin. In fact, each avidin/strepavidin molecule can bind as many as 4 biotin molecules. When using a biotinylated antibody (primary or secondary), the signal can be amplified by either an avidin-biotin complex (ABC method) or by streptavidin that is conjugated to either an enzyme or a fluorochrome.

For single labeling of different proteins and quantification with light microscope, we have used the ABC kit where a complex of avidin and the enzyme horseradish peroxidase (HRP) is created by incubation prior to addition to the tissue. The avidin-HRP complex is added following incubation with biotinylated secondary antibody. In the final stage, a solution with 3,3´duaminobenzidine (DAB) is added and the HRP enzyme coverts DAB to a colored product (brown). In the current thesis we have also added $NiCl_2$ that makes the color black instead of brown.

In paper III we used streptavidin amplification for visualization of tomato lectin. Neither lectin nor streptavidin are conventional antibodies. Lectins are proteins that bind more or less selectively to carbohydrate-moieties on glycoproteins. In the case of tomato lectin the target is glycophorin and Tamm-Horsfall glycoprotein. Furthermore, streptavidin bind to biotin with high affinity. The primary "antibody" (tomato lectin) was conjugated to biotin and added to the tissue where it bound its "antigen". This was followed by addition of a secondary "antibody" consisting of streptavidin conjugated to a fluorophore.

Fluorescent molecules

Fluorescence is the phenomenon arising through a three-step process that occurs in specific molecules called fluorophores. First, the fluorophore absorbs a photon that results in that an electron changing from the ground state to an excited vibrational state with higher energy. Secondly, the excited molecule collides with other molecules and thereby loses energy until it reaches the lowest excited state. This step is called the excited state life time. Thirdly, the electron decays back to the ground state and at the same time releases a photon. Since energy is lost when the electron is in its exited state, the emitted photon is always of lower energy (longer wavelength) than the excitation photon. The signal that you visualize in a microscope is the emitted photon.

All fluorophores have characteristic wavelengths for both excitation and emission. The traditional fluorescent dyes (e.g. FITC and PI) have broad fluorescence spectra which makes them difficult to use when doing multicolor labeling. These are also very sensitive to photo bleaching. Photo bleaching is an irreversible process meaning that the fluorescent probe permanently loses its ability to fluoresce. Newer fluorophores such as the Alexa Fluor® dyes instead have narrow spectra and are more photostable. In this thesis, we have therefore almost exclusively used the Alexa fluorophores.

Some antigens are more easily visualized than other. In addition, different fluorophores produce differently strong signals (Alexa 488 is for example generally stronger than Alexa 555 or Alexa 594). In order to optimize the staining and be able to visualize all antigens, it is therefore preferable to combine the weakest antigen with the strongest fluorophore, and vice versa. With indirect IHC, a relatively small number of secondary antibodies are needed in order for you to decide from analysis to analysis what fluorophore

that you want to use in order to detect a certain antigen. However, for the direct IHC you need to label each primary antibody with the desired fluorophore, hence significantly increasing the amount of antibodies needed.

Rapid photo bleaching of the fluorophores can be inhibited by using an antifade mounting media. In the current thesis we used ProLong Gold antifade reagent (with and without DAPI as nuclei stain) which cure within 24 hours, forming a semi-rigid gel. Following this, samples can be stored for months.

Antigen blocking

Non-specific binding of antibodies needs to be prevented by blocking. This is done by adding an excess of blocking agent to saturate any nonspecific binding sites that your antibody could otherwise bind to. The blocking is done prior to the addition of antibodies, and the blocking is present in all antibody steps during the staining procedure. Commonly used blocking agents are bovine serum albumin, gelatin or normal serum from the same animal species that the secondary antibody was raised in. In the current thesis we blocked unspecific binding by the use of donkey serum since all secondary antibodies used were raised in donkey.

LABELING OF PROLIFERATING CELLS

In all tissues in our body, new cells are continuously being produced to replace old, damaged or dying cells. This is accomplished by cell division that is intimately regulated during normal homeostasis. New cells are generated from preexisting cells by several defined stages in the cell cycle. The cell cycle consists of growth phases (G1 and G2), synthesis of DNA (S) and cell division (M). During the cell cycle, the DNA is duplicated and the two copies are then divided into two identical daughter cells. The cell cycle

also contains several crucial checkpoints that regulate the continuation or interruption of the cell cycle (Figure 6).
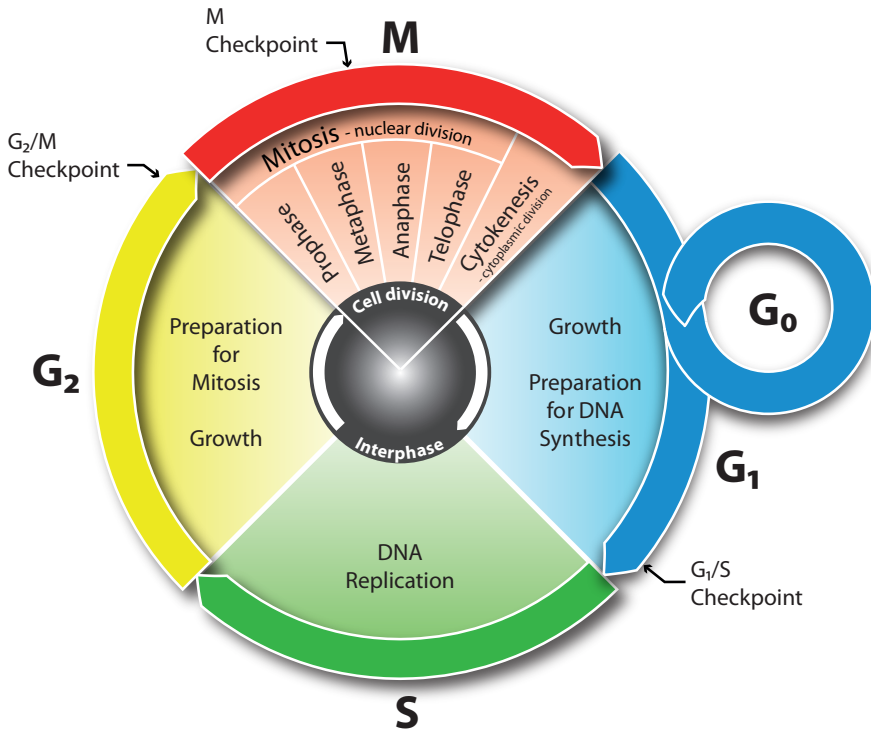
Figure 6. *The cell cycle with three important checkpoints outlined. Note that the true duration of the different phases is not correlated to its size within the figure. The M phase is for example very short while longest time is usually spent in the G1 phase (illustration made by Simon Lundholm @ 300Kelvin).*

BrdU labeling

Labeling of newborn cells can be accomplished by incorporation of thymidine analogs into the DNA during the S phase of the cell cycle (Kuhn and Peterson, 2008). Thymidine is unique in the sense that it exists in DNA but not in RNA (Kuhn and Cooper-Kuhn, 2007). The incorporated thymidine analogs can later be detected by IHC and thereby allow a "birth-dating" of

cells (Kuhn and Peterson, 2008). A cell will be permanently labeled with thymidine analogues as long as the signal is not diluted by subsequent divisions. Therefore, after a long period of time, detectable cells are those that have survived and at the time of thymidine analog injection were going through its final cell divisions (Kuhn and Cooper-Kuhn, 2007).

The most commonly used thymidine analog is 5-bromo-2-deoxy-uridine (BrdU) which can be given repeatedly for the purpose of labeling additional cells. BrdU is a synthetic nucleoside that competes with endogenous thymidine for incorporation into the DNA during the replication phase. BrdU is exogenously administered by i.p. injection. Since BrdU is incorporated into the cells during the S phase, it is important to be aware of the fact that BrdU can not only be incorporated into dividing cells but also into postmitotic cells undergoing DNA repair. It is therefore of great importance to use recommended low doses of BrdU (50 mg/kg body weight) to make sure that cells that undergo DNA repair do not incorporate enough BrdU to be detected during IHC (Cooper-Kuhn and Kuhn, 2002). An interval of about 4 weeks between labeling and detection is sufficient to recognize which cells that have survived and differentiated into neurons (Kempermann et al., 2003). In that sense BrdU incorporation can be used to measure survival of cells as well as differentiation of e.g. neurons. However, in the current thesis, BrdU incorporation was only used as a measurement of cell survival.

Cell cycle markers

Proliferating cells can be detected by different antibodies with the help of IHC. Antibodies that label either all active stages of the cell cycle or part of the cell cycle can be used. Therefore, the antibody of choice depends on what target protein you are interested in looking at. If you aim at identifying all proliferating cells or know that you have very few proliferating cells, PCNA

could be a good choice since it is expressed during all active phases of the cell cycle (G1/S/G2/M). However, if you instead want to narrow your analysis or reduce the number of labeled cells you could use Ki67 that is expressed during S/G2/M phases. Furthermore, phospho-histone H3 is only detected during G2 and M phases and therefore offers an even more specific resolution of the cell cycle. A combination of these markers could also be used in order to specifically target certain phases of the cell cycle.

STEREOLOGY

Stereology is a method that uses random and systematic sampling to generate unbiased and quantitative data. Stereological principles were used for cell counting, volume measurements and blood vessel analysis. All stereological analyses were performed with a Leica light microscope on DAB-stained sections, which has the advantage that they do not bleach by light. The Leica microscope was connected to a computer with the stereology system Stereo Investigator from MBF Bioscience.

Landmarks

Well defined landmarks are needed to ensure that all analyses are performed in an identical and objective manner possible. All histological analyses such as cell counting, volume measurements etc always started at the first section with a clearly separated dorsal and ventral hippocampus. Accordingly, the analysis ended at the last mid-sagittal section with a clearly curved GCL.

All analyses were performed by an investigator blinded to the treatment of the animals. In addition, the same analysis were always performed by one single person since individual variations otherwise could interfere with the outcome of the analysis.

Volume measurements

For volume measurements, the area of interest was traced (usually at 10x magnification) for all sections with separated dorsal and ventral hippocampus. Total volumes were then calculated according to the Cavalieri principle with the following formula: $V = SA \times P \times T$, where V is the total volume, SA is the sum of the area measurement, P is the inverse of the sampling fraction and T is the section thickness (25 µm in this thesis). Volume measurements of the different areas in the DG are shown in Figure 7.
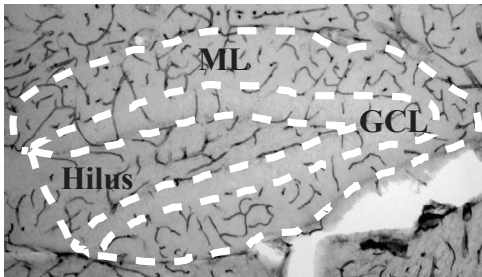
Figure 7. Representative picture showing the CD31 microvessel staining in the dentate gyrus and the schematic drawings of how the different regions were traced (granule cell layer; GCL and molecular cell layer; ML). Figure modified from Bostrom et al., 2013 (paper I).

Cell counting

When using a microscope to count cells, several different criteria can be used to define if a cell is considered as positive or negative. You start by looking at the intensity of the staining, secondly at the morphology of the cell and finally where the cell is located. Some antibodies used to define stem cells are for example present in multiple different cell types except NSPCs. In order to identify the true stem cells you therefore need to make sure that they are present in the neurogenic areas of the brain. In addition, some markers need colabeling with other marker to be considered specific for NSPCs. In that case confocal microscopy is needed (reviewed later). All cell counts in

the current thesis were performed on every 12th section throughout one hemisphere of the brain. The counted cells were then multiplied by 12 to acquire the total amount of cells in the brain.
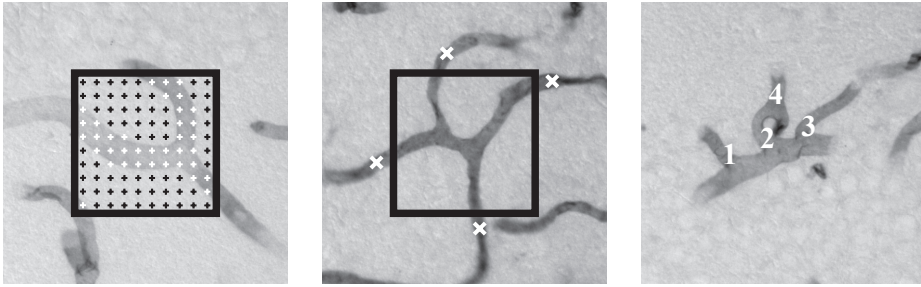
Vessel morphology

To our knowledge, assessment of vascular changes after moderate doses of irradiation to the developing brain has not been thoroughly studied previously. In the current thesis we therefore investigated the anatomy of blood vessels in two different regions of the brain; the hippocampus and the corpus callosum. Vessel morphology and density were assessed by two different methods.

Manual vessel analysis

In paper I, vessel analysis in the hippocampus was performed manually using the area fraction fractionator module in the Stereo Investigator program. The area of interest was traced and the computer overlaid a grid on top of the structure in which counting frames were distributed. In that way, counting frames were randomly distributed within the structure. The investigator then counted the cells/objects of interest within the counting frame. Four different vessel parameters were quantified; vessel area, numbers of vessels, vessel diameter and vessel branching points (Figure 8). The grids and counting frames were manually determined in order to acquire consistency throughout the analysis. In paper I this was performed by testing different settings on the same animal and monitoring how consistent the analysis was. The goal was to quantify enough vessels in each sample, without oversampling, so that your calculated total number of vessels comes very close to the actual amount of total vessels.

Figure 8. *Manual vessel morphology analysis was performed with the area fraction fractionator module. First, area was measured by marking all crosses that touched a vessel (left panel). Second, vessel number was quantified by counting all vessels that touched any of the borders of the box (middle panel). Furthermore, the diameter of all the counted vessels was measured using the quick measurement tool (not shown). Third, because there were few branching points and they were unevenly distributed, all branching points within each region were quantified without the area fraction fractionator module (right panel). Figure modified from Bostrom et al., 2013 (paper I).*

## Automated vessel analysis with Metamorph

During manual analysis, the investigator can adjust for minor discrepancies in staining intensities. However, in paper III, a semi-automated analysis was performed with the Metamorph Offline software to measure vessels in the corpus callosum. Images were acquired with the virtual tissue module in the Stereo Investigator software, which automatically acquired images within in the traced region of interest and stitched them together into one single image. Simultaneously, background subtraction within the software was used in order to subtract possible intensity differences within the microscope. These intensity differences could otherwise significantly affect the downstream analysis.

Within the Metamorph software, the threshold for what was considered positive staining was manually set by the investigator for each individual image. It is however possible to set a threshold and let the program import

all images according to the set threshold. Nevertheless, regardless of how good the staining is, there will almost always be slight differences between sections. With the automated analysis you therefore need to be even more critical in order to avoid false positive or false negative results. After setting the threshold, the software automatically measures different parameters chosen by the investigator such as area, number of objects, length, perimeter etc (Figure 9). Since the analysis are measured in pixels, the values have to be recalculated into µm. Information about your specific pixel versus µm ratio can be found within the Stereo Investigator program. Note that the ratio is different for different objectives on the same microscope.
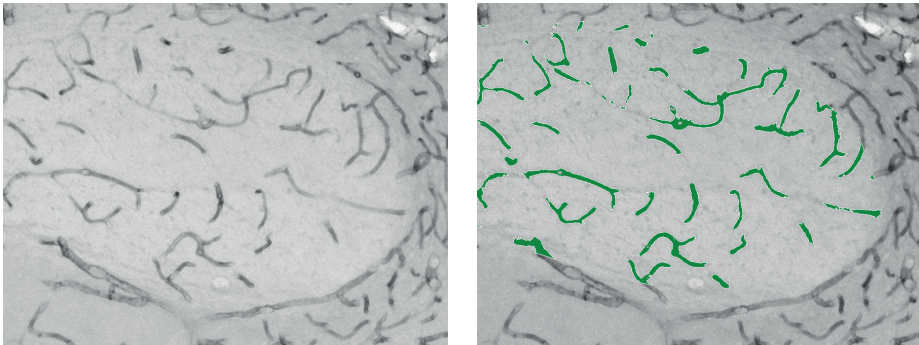
Figure 9. Microvessel parameters were measured with Metamorph on sections stained with CD31. The left picture illustrated the CD31 staining and the right picture represents microvessels that were analyzed with the Metamorph software in green.

CONFOCAL MICROSCOPY

One problem with regular fluorescence microscopes is that the complete sample is illuminated by light resulting in every plane of the sample fluorescing simultaneously. This is not a major problem if you are only interested in looking at one antigen but if you are interested in colocalization of several different antigens this creates a problem. Colocalization means that two or more molecules are present at the same physical location in a

specimen. For samples less than 5 μm thick, conventional fluorescence microscopy is appropriate for colocalization. However, colocalization in thicker samples should be analyzed using a confocal laser scanning microscope. A confocal microscope can collect light from a thin focal plane and thereby obtains thin optical sections without signals from remote locations in the specimen. By using a confocal microscope you can therefore determine if a colocalization actually exists or not. However, spectral bleed-through is a potential problem during multilabeling that arises as a consequence of emission spectral overlap between the fluorophores. Simultaneous excitation of the fluorophores should therefore not be used in order to avoid bleed-through and false colozalization. In the current thesis sequential scanning mode were therefore exclusively used, which excites the dyes separately, thereby minimizing bleed-through.

Neurovascular niche

For papers I and II, the distance between different cell types in the SGZ and its nearest microvessel was measured to reveal potential effects on the neurogenic niche after irradiation. Confocal images from one focal plane of the section were acquired with the cell/s and its nearest vessel in their best focus. Since the images were acquired from one focal plane only, extensive zooming up and down within the section had to be done in order to make sure that a vessel located closer to the cell/s was not excluded from the image. Following acquisition, the distance between cells and microvessels was measured with Adobe Illustrator CS6 in pixels and manually converted into μm. From all measurements, an average distance was calculated.

Pericyte coverage

Pericyte coverage was quantified by measuring the CD13$^+$ area (pericytes) within the CD31$^+$ area (microvessels). Confocal z-stacks were acquired and

maximum intensity projections were generated with the help of the ZEN 2012 software. The region/s of interest was manually determined and pericyte coverage was assessed with the Velocity software. First, the $CD31^+$ area was determined and the area was closed within the program to avoid gaps in the vessels due to possible weaker staining in some areas. Secondly, the $CD13^+$ area within the $CD31^+$ were determined. For both stainings, objects smaller than 5 µm were excluded to avoid single pixels. The outcome of the analysis was hence percentage pericyte coverage of vessels.

FLOW CYTOMETRY

Flow cytometry was used to evaluate the relative amount of ECs within the whole cell population at different time points after irradiation. Furthermore, nuclear staining was used to analyze the amount of cells within different stages of the cell cycle. Finally, flow cytometry was used to isolate ECs for further gene expression analysis.

Single cell suspension

In order to analyze and isolate ECs with flow cytometry, a single cell suspension is needed. The isolation of a single cell suspension from tissue is however not without complications. First, the regions of interest, in this case hippocampus and cerebellum, are dissected from the brain. This is followed by both mechanical and enzymatic digestion to acquire a single cell suspension. However, due to the numerous synaptic connections, extracellular matrix, myelin etc within your tissue, the cell suspension will also contain a lot of debris. The presence of debris complicates further downstream analysis and isolation.

Basic procedure of flow cytometry

Briefly, a flow cytometer could be explained as a fluorescence microscope that analyses cells in a suspension. However, in a microscope you receive an image of the cell while the flow cytometer gives you automated quantification of different parameters of the cell. Briefly explained, a single cell suspension flows through an illuminated flow cell where the cells scatter light in different directions dependent on size (Forward Scatter; FSC) and granularity (Side Scatter; SSC). Simultaneously, fluorescent antibodies attached to the cells become excited by the lasers and emit fluorescence signals that are collected by multiple detectors. Importantly, a flow cytometer is similar to the sequential scan mode of a confocal microscope, thereby limiting bleed-through between different fluorophores. Consequently, as long as you have fluorescent antibodies excited by different lasers there will be no bleed-through even if the emission spectrum is overlapping. However, during multicolor analysis you might need to use fluorophores with spectral overlap. This needs to be compensated, which is done by running single stained tubes one-by-one to detect the amount of spill-over.

Due to there being a large proportion of debris within each sample, a DNA stain (7-AAD) was included to identify cells. 7-AAD cannot pass through the intact cell membranes of living cells, but can be used to detect cells that have died, or cells which have been fixed and permeabilized, as the cells in this thesis. Firstly, 7-AAD was used to gate the cell population. Secondly, a sample with just secondary antibody was used to detect autofluorescence within the cell population. Gates were set above the autofluorescence so that a maximum of 0.2% of the cells were false positive (i.e. within the gate). Last, positively stained ECs within the defined gate were sorted into tubes and used for gene expression analysis.

Cell cycle analysis

As previously described, proliferation of cells can be analyzed with regular IHC. However, if you want to distinguish between cells in different stages of the cell cycle multiple antibodies need to be used. A quicker and a different way of doing this is with flow cytometry. Analysis of where in the replication phase a cell is can be achieved by fluorescent labeling of the cell nucleus. Cells in the G1/G0 phase of the cell cycle will have one copy of the DNA whereas cells in the G2 and M phase will have two copies of DNA. Cells in the S phase are continuously synthesizing DNA and will therefore have between one and two copies of the DNA. In the current thesis, cell cycle analysis was used in paper IV with the help of ModFit LT, a program that automatically determines the percentage of cells in each phase.

GENE EXPRESSION ANALYSIS

In paper IV, gene expression analysis was performed on ECs that had been isolated with flow cytometry.

RNA purification

The ECs were fixed prior to staining and isolation, and we therefore used the RNeasy FFPE kit (Qiagen) that is specifically optimized for RNA purification from formalin-fixed tissue. Formalin fixation usually leads to highly fragmented nucleic acids that are chemically modified by the formalin. The primary aim of the kit is therefore to reverse as much formaldehyde fixation as possible without further degrading the RNA. Formalin crosslinking is partially reversed by a short incubation at high temperature (80°C).

It should be noted that nucleic acids from fixed tissue should not be used for downstream applications that requires full length RNA. Instead, RNA isolated from fixed tissue can be used in applications that use small amplicons, such as reverse transcription quantitative PCR (RT-qPCR). In fact, many of the probes/primers used for RT-qPCR are short with amplicons of $\sim$ 50 bases to $\geq$ 200 bases that allows even significantly degraded RNA to be used for analysis without detectable negative effects.

RNA integrity is significantly affected by RNases that degrade RNA into smaller components. Intrinsic RNase activity can vary significantly between different tissues. For example, the pancreas has 181,000-fold higher RNase activity compared to the brain (Krosting and Latham). Furthermore, different tissues harbor different amounts of RNA. The effective RNA yield is therefore influenced by both the total RNA amount and the intrinsic RNase activity.

Furthermore, genomic DNA present in the sample could highly affect downstream gene expression analysis. Therefore, the kit has a DNase treatment included for maximal removal of genomic DNA.

cDNA synthesis

For synthesis of cDNA from the extracted RNA, the SuperScript VILO cDNA Synthesis Kit were used; a kit that is optimized to generate cDNA for use in RT-qPCR. It provides enhanced cDNA synthesis efficiency and was therefore optimal in this thesis since small amount of RNA was extracted from the ECs. To maximize the cDNA yield, the incubation time at 42°C was increased. It should be noted that an amplification step can be used to further amplify your cDNA. However, introducing this step will also introduce a possible variation between your samples. This step was therefore not used in the current thesis.

Reverse transcription quantitative PCR (RT-qPCR)

Quantitative polymerase chain reaction (qPCR) is a method that simultaneously amplifies and quantifies a specific DNA sequence of interest. For each cycle, the PCR exponentially amplifies the DNA, resulting in double amounts of cDNA after each cycle. In addition, with the help of fluorescent dyes the amount of DNA is monitored at the end of each cycle. After a number of cycles, the fluorescent signal reaches a threshold above background and the exponential phase of the reaction starts until a plateau phase is reached. To quantify the amount of mRNA present within your sample from the beginning, you determine the threshold (Ct) during the exponential phase and normalize it against stable genes within your sample, so called reference genes. The gene expression is therefore usually measured as relative amount of mRNA (ΔCt) between your gene of interest and reference genes.

It should be pointed out that changes in gene expression do not always result in an equivalent change in protein translation. Therefore, gene expression is often a first step that is later followed by protein quantifications.

Detecting genomic DNA

Finally, the possible detection of endogenous DNA should be addressed. If the DNase treatment during the RNA purification for some reason did not remove all endogenous DNA in the sample, the DNA could significantly affect the ΔCt value. To avoid this, negative controls can be used that include your RNA but not reverse transcriptase. Consequently, if DNA is detected within these samples, it is only due to endogenous DNA. If negative controls are included for each sample, the amount of detected DNA can be subtracted from the amount in the positive control to acquire the real ΔCt value, devoid of contaminated DNA. If there is a difference of 6 cycles between the

negative and the positive controls, it means that endogenous DNA accounts for roughly 1% of the detected gene expression. Furthermore, if there are 10 cycles between the positive and negative controls, endogenous DNA account for as little as 1 ‰ of the detected gene expression. In the current thesis, the amount of mRNA was too low to include both a positive and a negative control. However, one extra array was used to run negative controls for 8 random samples that had enough mRNA for both a positive and a negative control.

To avoid the complication of endogenous DNA to interfere with your qPCR you can also design primers and probes with care. The best probes to use are intron-spanning (_m1) that only detect spliced mRNA. For all cases possible, probes with the _m1 suffix were therefore used. If these were not present, probes with sequences located in different exons were used (_g1). This limits the detection of genomic DNA, but does not exclude it completely. The worst probes to use are those that a designed within a single exon (_s1) and amplification and detection can therefore occur from both mRNA and genomic DNA. These were only used when no other probes were available (information from Life Technologies web page, RNA isolation for qRT-PCR).

ELISA

Enzyme-linked immunosorbent assay (ELISA) is an antibody-based method used to determine the concentration of a protein based on color intensity. ELISA is a quick and easy method to use and is designed to rapidly handle large numbers of samples simultaneously. In order to measure the amount of a protein, ELISA could hence be used instead of IHC for quicker analysis. In paper III, ELISA was used to measure the concentration of myelin basic protein (MBP). An alternative way of measuring the amount of MPB protein

could have been accomplished by measuring the intensity stained by IHC. However, different staining intensities could possibly have affected the analysis greatly, and I therefore believe that the ELISA was the best analysis of choice for the MBP measurement.

STATISTICS AND SIGNIFICANCE

When performing statistical analysis you test the likelihood of two opposite hypothesis. The first hypothesis generally assume that no difference exist between the groups and is called the null hypothesis, H_0. Furthermore, the second hypothesis proposes that differences actually do exist (alternative hypothesis, H_1). You then analyze the data and decide whether to reject or accept the H_0 hypothesis. There is however a risk of error with rejection or acceptance of the H_0 hypothesis. A type I error occur if you reject H_0 although it is true. In addition, you might accept H_0 when in fact H_1 that is true, resulting in a type II error. The probability of making a type I error is called the significance level. A commonly used significance value is 0.05 and means that the probability of a type I error is 5 percent or that 1 out of 20 analysis will result in a significant result, although H_0 is true. The significance level is always set before the tests are calculated. Furthermore, the significance value does not describe the size of the difference and a low significance value is hence not equivalent with a great difference or effect but is instead a measurement of how high the demands for the test are. In the current thesis the following significance values were used: $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). The significance value is often confused with the p-value but these two are however not equivalent. In contrast to the significance value that is set before the test is calculated, the p-value it estimated afterwards. If the p-value if less that the pre-set significance values, you can reject the H_0 hypothesis which means that a difference has been detected.

RESULTS AND DISCUSSION

PERMANENT CHANGES IN NEUROGENESIS

In contrast to many other cells in the brain, stem and progenitor cells in the neurogenic areas have considerably elevated proliferative capacity. For that reason, they are highly sensitive to irradiation, and this has been shown in both rodents (Tada et al., 2000, Fukuda et al., 2004) and humans (Monje et al., 2007). The hippocampus is important for memory and learning by being responsible for the consolidation of short-term and long-term memories. It is however not an area for the storage of memories (Kempermann, 2011). The direct correlation, if any, between memory function and adult neurogenesis has been extensively debated, and still is (for review see Kohman and Rhodes, 2013). However, ample evidence exists, showing that neurons generated in adulthood form functional connections and that they might contribute to cognitive processes (Kempermann et al., 2004b, Grote and Hannan, 2007). It has been proposed that loss of these cells could contribute to the cognitive deficits observed after radiotherapy (Raber et al., 2004, Kuhn and Blomgren, 2011). Furthermore, limiting the radiation dose to the hippocampus has been associated with lower risk of long-term neurocognitive decline (Blomstrand et al., 2012, Gondi et al., 2013, Peiffer et al., 2013, Prokic et al., 2013).

It has been reported that irradiation-induced damage is more pronounced in the immature brain (Fukuda et al., 2005). By quantifying doublecortin-positive (DCX$^+$) cells in paper I, we have confirmed that a moderate irradiation dose of 8 Gy to the developing brain reduces the number of neural progenitor cells in the hippocampus. This finding has been reported previously in both young and adult rodents (Monje et al., 2002, Fukuda et al., 2005, Naylor et al., 2008, Hellstrom et al., 2009, Kalm et al., 2013). We were

also able to verify the natural age-related decline in hippocampal neurogenesis (Kuhn et al., 1996). The key finding of paper I was the relative loss of neural progenitor cells that progressed more quickly after irradiation, without any signs of recovery (Figure 10). This is in stark contrast to the SVZ, which is able to recover after irradiation, at least partly. The acute response to irradiation is however similar in both neurogenic areas (Hellstrom et al., 2009).
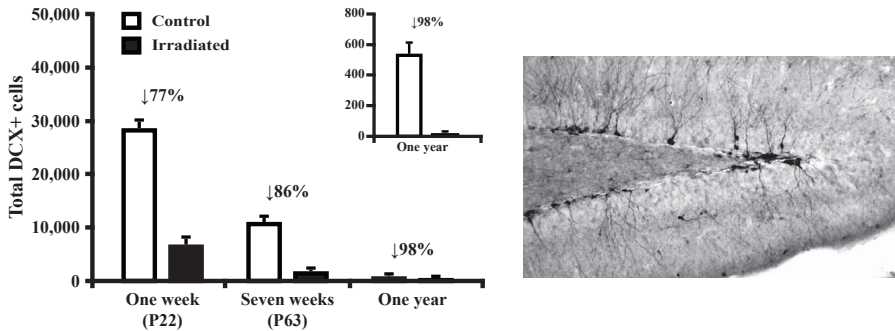
Figure 10. The number of DCX$^+$ cells in the DG were decreased in irradiated mice at all 3 time points after irradiation. Furthermore, the relative loss increased with time, resulting in almost complete ablation of neurogenesis 1 year after irradiation (left panel). The right panel is a representative image of the DCX staining. Figure modified from Bostrom et al., 2013 (paper I).

STRUCTURAL CHANGES IN THE VASCULATURE

In paper I we investigated irradiation-induced effects on microvessel structure and complexity in the hippocampal DG. We found that a single dose of irradiation reduced total number of microvessels and microvessel branching points at all three investigated time points after irradiation (1 week, 7 weeks and 1 year). In addition, we also observed significant growth retardation in the DG, an effect that persisted in both the GCL and the ML until 1 year post-irradiation. This is consistent with previous reports of

smaller hippocampal volumes in rodents (Fukuda et al., 2004, Naylor et al., 2008, Huo et al., 2012), as well as volume loss in children following radiotherapy (McDonald and Hayes, 1967). When differences in volume were taken into consideration by calculating vascular densities, we observed an increased vascular density 1 week after irradiation. This indicates that the surrounding neural and glial tissue initially was more affected than the vascular tissue. However, at the two later time points, the densities in irradiated mice gradually normalized compared to control mice, demonstrating that the vasculature was capable of adapting to the surrounding tissue. Our results contradict a previous study that reported a decreased capillary density in the DG after 8 x 4.5 Gy of cranial irradiation (Warrington et al., 2011). The radiation dose in that study was however considerably higher than the dose used here, which is considered a clinically relevant dose, and they used adult mice. However, in another study where adult rats were subjected to a single dose of 25 Gy they saw no difference in vessel density (Ljubimova et al., 1991). Difficulties exist in the comparison between studies, because of variation in the outcomes after different doses, different ages of the animals and different evaluation methods.

CHANGES IN THE NEUROVASCULAR NICHE

As previously described, irradiation-induced damage to hippocampal neurogenesis has been widely studied. However, many studies neglect the possible important role of the neurovascular niche. Previous studies performed in the adult rat brain have shown an alteration in the neurovascular niche where the distance between BrdU$^+$ cells and microvessels was increased in the irradiated rat brain (Monje et al., 2002). However, as the response to irradiation in the immature and adult brain varies, further studies are needed to confirm or reject the hypothesis that irradiation also affects the

neurovascular niche in the immature brain. The neurovascular niche was therefore investigated in papers I and II.

In paper I we measured the distance between microvessels and undifferentiated stem cells (BLBP$^+$) in the hippocampal SGZ 7 weeks after irradiation. However, in contrast to the study by Monje et al. we did not detect any alterations in the distance between BLBP$^+$ cells and microvessels 7 weeks post-irradiation (Bostrom et al., 2013). This challenges the idea of microvessel injury being an upstream event in irradiation-induced loss of neurogenesis. In paper II we extended the first study by analyzing different time points after irradiation, as well as different cell types. We then observed an increased distance between DCX$^+$ cells and microvessels 6 hours post-irradiation. In addition, an increased distance between proliferating Ki67$^+$ cells and microvessels was observed 1 week post-irradiation. The changes in the neurovascular niche however normalized with time, as shown by no difference between irradiated and control mice 7 weeks post-irradiation. The transiently increased distance for both proliferating cells and neural progenitor cells coincided with extensive cell death and inflammation within the SGZ, as well as edema in patients. Furthermore, earlier observations have shown that mainly proliferating cells in the neurogenic zones are sensitive to irradiation (Peissner et al., 1999, Tada et al., 2000, Huo et al., 2012, Roughton et al., 2012).

All microvessels have an inner lining of ECs surrounded by solitary perivascular mural cells termed pericytes. Pericytes extend processes that wrap around the walls of microvessels and they are considered important for microvessel maturation and stability (Lindahl et al., 1997). Due to the proposed important role of pericytes for the vasculature, and the lack of research performed within the area, we measured the pericyte coverage at different time points post-irradiation. Pericyte coverage was reduced acutely

(6 hours post-irradiation) in the SGZ of irradiated animals. This difference, however, normalized with time, as judged by the lack of difference both 1 and 7 weeks post-irradiation. Low pericyte coverage is linked to increased endothelial cell turnover (Allt and Lawrenson, 2001, Lee et al., 2009, Armulik et al., 2011) and reduced pericyte coverage could theoretically lead to increased endothelial cell proliferation. In fact, cell cycle analysis of ECs in paper IV showed an increased amount of ECs in the actively cycling phases (S/G2/M) compared to "resting phases" (G0/G1) in the cerebellum 7 days post irradiation. This is in line with the reported transient increase in proliferation observed 7-14 days after irradiation (Tada et al., 1999).

Furthermore, pericyte loss in Pdgfrβ$^{+/-}$ mice results in blood-brain barrier disruption and the accumulation of multiple neurotoxic and/or vasculotoxic molecules. Neuronal damage and memory deficits have also been observed in these mice, secondary to vascular damage (Bell et al., 2010). In the light of these results, our results regarding the transient reduction in pericyte coverage acutely after irradiation could perhaps coincide with increased BBB permeability (Soussain et al., 2009). This, in turn, could allow neurotoxic molecules into the brain, hence contributing to the neuronal damage seen after irradiation.

ENDOTHELIAL APOPTOSIS

It is well known that highly proliferating cells are extremely susceptible to irradiation (Fukuda et al., 2004). ECs in the adult brain are generally considered as stable cells with infrequent cell turnover (Hirschi et al., 2002) and would therefore be considered as a more radioresistant cell type. It has however been proposed that non-proliferating cells in the SGZ are also sensitive to irradiation and undergo acute apoptosis (Tada et al., 2000). Moreover, the current thesis shows that a larger proportion of ECs are in a

cycling phase of the cell cycle compared to the whole brain cell population at the age of P14. This was seen in both the hippocampus and the cerebellum and hence shows that ECs at the age of irradiation were still proliferating. From the age of P14 to the age of P21, ECs gradually adopted a more mature phenotype with infrequent cell turnover. This is consistent with a previous study showing that the maximal proliferation of ECs in rodents occurs between P5 and P9, followed by a gradual decrease. In their study, proliferation of ECs at the age of P14 was still significantly elevated compared to the adult (Robertson et al., 1985).

In paper I we investigated endothelial cell death by cleaved caspase-3 immunoreactivity. Activation of multiple caspases, including caspase-3, has been reported to be involved during endothelial cell apoptosis (Granville et al., 1999). We did not observe any significant cell death of ECs in irradiated animals acutely after irradiation. In fact, compared to the thousands of dying stem and progenitor cells in the SGZ (Huo et al., 2012), we detected only a handful of dying ECs in the entire hippocampus.

To further evaluate irradiation-induced apoptosis, we isolated ECs using flow cytometry in paper IV and investigated the gene expression of several genes involved in endothelial-specific apoptosis. While apoptosis in a number of cell types is dependent on and mediated by p53 (e.g. apoptosis of neural progenitor cells), the apoptosis of ECs is considered to be p53-independent. Instead, endothelial cell apoptosis is mediated by the acid sphingomyelinase (ASMase) pathway and knockout models for the gene that encodes ASMase shows protection against irradiation-induced endothelial cell apoptosis (Li et al., 2003, Fuks and Kolesnick, 2005, Li et al., 2010). In addition, ECs have 20-fold greater secretion of ASMase compared to other cell types in the body (Marathe et al., 1998). Endothelial apoptosis mediated by ASMase is considered to be independent and distinct from the p53-mediated pathway

(Santana et al., 1996). However, genes in the ASMase pathway were not induced in ECs of irradiated animals. Kolesnick and Fuks report that ASMase is rapidly activated within minutes to hours after irradiation (Kolesnick and Fuks, 2003), proposing that posttranslational modifications and subsequent activation might have occurred although no difference was detected on the gene level, or that the 6 hour time point were too late to detect any upregulation.

In summary, our data proposes that ECs are more radioresistant than other cell types. In general, cells are more vulnerable to irradiation when they are dividing (Fukuda et al., 2004). However, we showed increased amount of actively dividing ECs at the time of irradiation (P14) compared to the whole cell population in both the hippocampus and the cerebellum. Furthermore, no apparent EC death was detected acutely (6 hours) after irradiation. The question therefore remains how and if ECs are affected by *in vivo* irradiation if they do not undergo apoptosis. In addition to apoptosis or mitotic catastrophe, a consequence of irradiation is a state of irreversible growth arrest, so called senescence (Gudkov and Komarova, 2003). A recent article proposed that ECs enter a state of senescence after *in vitro* irradiation. Furthermore, the ECs had an increased extent of DNA damage that was repaired less efficiently than in other cell types, such as microglia, astrocytes and neurons. In addition, the authors showed that *in vitro* irradiation of rat cerebromicrovascular ECs led to the acquisition of a senescence-associated secretory phenotype, meaning that they secreted proinflammatory cytokines and chemokines (Ungvari et al., 2013).

GENE EXPRESSION IN ENDOTHELIAL CELLS AFTER IRRADIATION

In paper IV we utilized flow cytometry to specifically isolate ECs and investigate a selected panel of genes after irradiation to the developing brain. Furthermore, by comparing the results from the hippocampus and the cerebellum, we have identified both similarities and differences in the EC response to irradiation between these two regions. The hippocampal DG and the cerebellar cortex mainly consist of postnatally generated granule cells (Altman, 1969). In addition, immature cerebellar granule cells grafted into the developing DG shows features of residential dentate neurons. It was therefore proposed that factors present in the environment control the regional fate of neuronal precursor cells (Vicario-Abejon et al., 1995). However, completely different gene expression profiles during postnatal development have been observed in the hippocampus and the cerebellum (Saito et al., 2002).

Medulloblastoma is the most common malignant brain tumor in children and is located in the cerebellum. After surgery and chemotherapy to reduce the tumor burden, patients receive craniospinal irradiation together with a higher radiation boost delivered to the tumor bed. When it was revealed that long-term survivors of medulloblastoma often experience progressive neurocognitive decline, treatment protocols were adjusted to reduce radiation both in regards to volume and dose. Similar cognitive impairments, albeit not as pronounced, have been seen in non-irradiated patients hence proposing that radiotherapy alone does not account for all the observed negative effects. In addition to radiation and chemotherapy, surgery and the tumor itself can cause significant damage that affects cognitive function (Cantelmi et al., 2008).

In paper IV we show that ECs upregulated proinflammatory genes such as CCL-11, CCL-2 and IL-6 acutely after irradiation in both the hippocampus and the cerebellum. It has previously been shown that the inflammatory response after irradiation is transient in the immature brain (Kalm et al., 2009) but chronic in the adult brain (Monje et al., 2002, Monje et al., 2003). We observed the largest upregulation 6 hours after irradiation, after which we observed a gradual decline in gene expression until 7 days post irradiation when most genes were back to baseline levels. Our data hence confirm previous studies about transient inflammation in the immature rodent brain. However, it should be noted that gene expression does not always correlate with protein translation. Further studies are required in order to confirm the irradiation-induced inflammatory response of ECs.

As previously mentioned, following *in vitro* irradiation ECs adopt a senescence-associated secretory phenotype that is characterized by the upregulation of proinflammatory cytokines and chemokines such as IL-6 and eotaxin (CCL-11) (Ungvari et al., 2013), i.e. two of the genes that were upregulated in endothelial cells after *in vivo* irradiation in the current thesis. Furthermore, ionizing radiation of human ECs *in vitro* induced a similar proinflammatory phenotype with e.g. upregulation of IL-6 expression (Hneino et al., 2012). Since it has been proposed that reduced hippocampal neurogenesis is secondary effects to inflammation (Monje et al., 2002), it would be of great interest to explore whether ECs after a clinically relevant dose of *in vivo* irradiation would acquire a similar proinflammatory phenotype. In the study by Monje et al, non-irradiated neural precursor cells transplanted into an irradiated hippocampus failed to differentiate into neurons, at least not to the same extent as in non-irradiated tissue (Monje et al., 2002), indicating that the microenvironment are involved in the regulation of hippocampal neurogenesis. Interestingly, upregulation of CCL11 plasma

levels has been linked to age-related decline in hippocampal neurogenesis (Monje et al., 2002, Villeda et al., 2011). It is therefore tempting to speculate that inhibition of CCL11 or its receptor/s would decrease the inflammatory response after irradiation and reduce the negative effects on neurogenesis. To investigate this, transgenic mice and conditional knockout strains would be very useful tools.

WHITE MATTER DAMAGE

It has been proposed that white matter harbors an oligovascular niche, similar to the neurovascular niche in gray matter. Oligodendrocytes and ECs are closely associated in white matter and reports have suggested that a crosstalk between these two cell types are involved in the angiogenic response after white matter damage (Pham et al., 2012). In paper III we investigated vascular structure in the corpus callosum and found fewer vessels and smaller total vessel area after irradiation. However, this decrease was accompanied by decreased total volume of the corpus callosum, resulting in unchanged densities for all of the measured vascular parameters. As in the hippocampus (Bostrom et al., 2013), this indicates that the vasculature adjusts to the need of the growth-impaired corpus callosum, at least morphologically.

The time of irradiation in all papers in the current thesis coincides with increased growth of the corpus callosum, proliferation of oligodendrocytes and myelination in mice. The highest growth rates of the corpus callosum occur during the first years in life and the biggest difference in growth rates between males and females are observed during adolescence (Lenroot and Giedd, 2006, Lenroot et al., 2007). Moreover, myelination in the human brain continues during childhood and into the second and third decades of life (Semple et al., 2013). Oligodendrocytes are responsible for the generation of myelin sheaths and in rats oligodendrogenesis peaks during the second

postnatal week, just prior to the peak of myelination (Skoff and Knapp, 1991, Krinke, 2000, Fukuda et al., 2005). It has been reported that females have more unmyelinated fibers than males (Cerghet et al., 2009). In our study we did however observe that females had higher levels of myelin basic protein compared to males. The significance of this is however beyond the scope of this thesis.

Oxidative stress is considered a major damage-mediating effect of irradiation (Limoli et al., 2004). In addition, fully matured oligodendrocytes have a high metabolic rate and are therefore sensitive to oxidative stress (Bradl and Lassmann, 2010). In paper III we show that the numbers of oligodendrocytes decrease after irradiation, and that the effect was more pronounced in females. Interestingly, proliferation of glial cells is twice as high in female as in male rodents (Cerghet et al., 2009). This could perhaps partly explain the observed differences between females and males. Furthermore, the BrdU quantification followed a similar pattern as the Olig2 quantification, thereby indicating that pre-oligodendrocytes and proliferating cells might be equally targeted by irradiation in the developing brain. Supporting this, oligodendrocytes with myelination capacity can be generated from NSPCs in the SVZ. Following differentiation, the newly generated cells migrate to a great extent into the corpus callosum (Alvarez-Buylla et al., 2008). Since irradiation consistently causes damage in the two neurogenic areas of the brain (Fukuda et al., 2004), it would be tempting to speculate that ablation of NSPCs in the SVZ affect the ongoing myelination at the time of irradiation.

The role of the vasculature in secondary damage following radiotherapy has been extensively debated. By irradiating the vasculature specifically or the parenchyma uniformly with the boron neutron capture model, quite opposite effects have been reported. In spinal cord irradiation, similar white matter necrosis was observed both when the vasculature was targeted specifically

and when the parenchyma was irradiated uniformly (Morris et al., 1996). However, another study has shown that selective irradiation of the vasculature resulted in decreased loss of hippocampal neural progenitor cells compared to when irradiation was delivered uniformly to the parenchyma. They hence speculated that neural progenitor loss is an effect of direct irradiation to the precursor cells, rather than a secondary effect to vascular damage (Otsuka et al., 2006). However, the abrogation of acute apoptosis of ECs and/or NPCs did not prevent the decline in neurogenesis commonly observed after irradiation. It was therefore concluded that acute apoptosis of these cell types does not have a direct role in the decline in neurogenesis occurring after irradiation (Li et al., 2010).

It has previously been proposed that white matter is more susceptible to irradiation since white matter has a lower vascular density (McDonald and Hayes, 1967). In the current thesis we investigated vascular structure in both the hippocampus and the corpus callosum. However, vascular density was unchanged in both structures long-term after irradiation; hence arguing against that white matter is more susceptible to irradiation than gray matter. Interestingly, vascular density in this thesis was similar between the corpus callosum (white matter) and the DG (gray matter), at least for the hilus and the ML. The lowest density was found in the GCL, which could perhaps partly explain the fact that vascular structures were more affected in the GCL compared to the hilus and ML after irradiation (paper I).

VASCULAR VERSUS NEURONAL HYPOTHESIS

Vascular abnormalities are observed in many neurodegenerative diseases, but the question remains whether the vascular changes are the cause or a consequence of disease (Quaegebeur et al., 2010). In the case of radiotherapy, it was long hypothesized that damage observed in the brain was due to

irradiation-induced vascular damages that in turn caused ischemia and white matter necrosis (Myers et al., 1986). During the years, the cellular mechanism and primary source for irradiation-induced damage have however been extensively debated. Since the discovery that adult neurogenesis actually does exist in discrete areas of the brain, attention has to a great extent switched to the neuronal point of view. Proliferating NSPCs are extremely sensitive to irradiation, but it is not known if this is due to damage to the cells directly and/or through damage to and changes in the microenvironment.

More than a decade ago it was proposed that hippocampal NSPCs reside in a specialized vascular niche that regulates their proliferation and survival (Palmer et al., 2000). Furthermore, it has been proposed that radiation-induced disruption of the interaction between ECs and NSPCs results in loss of neurogenic potential (Monje et al., 2002). In addition, endothelial apoptosis has been observed to precede stem cell dysfunction after irradiation to the intestine, and stem cell damage could be rescued by preventing EC apoptosis (Paris et al., 2001). However, when irradiation targeted vessels specifically instead of being uniformly delivered to the parenchyma, reduced loss of NPCs was observed (Otsuka et al., 2006).

In the current thesis we report transient changes in the neurovascular niche that normalized with time. Neurogenesis, on the other hand, did not recover, instead the loss of NPCs in irradiated animals increased with time. This demonstrates dissociation between neurogenesis and the morphological patency of the hippocampal neurovascular niche. Furthermore, we also demonstrate that the vasculature seems to be able to adjust to the surrounding tissue. Together, these findings speak against the vasculature being an upstream event in reduced neurogenesis after irradiation. However, our gene expression analysis indicates that ECs may play an active role in the inflammatory response after irradiation. This reveals a potential novel role for

the vasculature, since it has been shown that inflammation negatively affects NPC differentiation.

Before the identification of irradiation-induced late effects, much higher irradiation doses were used both during radiotherapy and in animal models. However, during the last decades treatment protocols have been adjusted to reduce both radiation volume and dose in the hope of reducing negative side effects of radiotherapy. Consequently, many animal models have been redesigned, using lower irradiation doses in order to more closely mimic the clinical settings. In the current thesis we have therefore exclusively used relatively moderate doses of irradiation. This could perhaps partly explain the fact that we were unable to show such vascular damage that previous studies have reported following irradiation. Although ample evidence historically has supported the hypothesis that vascular damage is the primary cause of late radiation-induced brain injury, much evidence is now emerging, demonstrating late effects without apparent vascular damage or changes. The data presented in the current thesis suggest that the observed irradiation-induced damage cannot exclusively be due to vascular damage. However, the data do not rule out that the vasculature still has an important role in the toxic microenvironment after irradiation.

CONCLUDING REMARKS

As discussed in this thesis, all cell types in the brain have important and unique roles and a tightly regulated coordination between these cells is needed in order to maintain brain homeostasis and regulate the local environment for the cells to function properly. Many disorders within the CNS exhibit disruptions in one or both of the neural and vascular systems. A better understanding of the relationship between the two systems will be important in order to develop more effective and specific approaches to CNS disorders.

In many pathological situations, research focuses mainly on how neurons are affected. However, it is now generally agreed on that this is not enough and that it is important to consider all cell types within the neurovascular niche/unit. In order to protect one cell type, you may need to consider cell-cell interactions with other cell types to succeed.

SPECIFIC CONCLUSIONS TO GIVEN AIMS

I. When investigating microvessel structure and complexity we found that the vasculature adjusts to the needs of the surrounding neural and glial tissue.

II. We have observed that irradiation to the developing brain has long-lasting detrimental effects on neurogenesis and that neurogenesis deteriorates more rapidly after irradiation, without any signs of recovery.

III. We reported that the neurovascular niche was transiently affected by irradiation, while neurogenesis was permanently disrupted, thereby questioning the importance of the vascular niche in the regulation of neurogenesis.

IV. Finally, we showed that endothelial cells appeared to be more radioresistant than other cell types, at least within the hippocampus and cerebellum, but that they may be involved in the acute inflammatory response after irradiation through the upregulation of inflammatory genes.

CLINICAL PERSPECTIVE AND FUTURE DIRECTIONS

This thesis indicates that the ECs are involved in the inflammatory response acutely after irradiation. However, our study so far only includes gene expression data and therefore only partially unravels the importance of the vasculature in the inflammatory response after irradiation. In future studies, we wish to investigate the protein expression of the most strongly regulated inflammatory genes. We intend to do this by isolating ECs as previously described in paper IV and then quantitate protein expression of the most interesting genes using ELISA.

We are interested in the functional implications of this inflammatory response observed in ECs. This could be investigated by use of *in vitro* experiments, co-culturing ECs and other cell types. For example, co-cultures with irradiated ECs and pericytes, as well as irradiated ECs and NSPCs would help clarify if ECs are responsible for secondary effects on these other cell types. Alternatively, conditioned media from irradiated cultured ECs could be added to cell cultures of microglia and the microglial response be observed by change in morphology and gene and protein expression. We would thereby be able to confirm or reject the hypothesis that ECs are involved in the massive activation of microglia occurring after irradiation, which was shown to be detrimental for hippocampal neurogenesis.

The current thesis demonstrates reduced pericyte coverage 6 hours after irradiation. No solitary pericytes were observed that did not colocalize with microvessels, hence indicating that they did not undergo apoptosis, and/or that they were not recruited or stimulated to migrate to nearby sites of injury, at least not at the time point investigated. It is thereby unclear if the reduced

pericyte coverage was due to apoptosis, reduced numbers of pericytes or rearrangements of their structure. Unfortunately it is not trivial to determine the exact numbers of pericytes per vessel, since, to our knowledge, there is no available nuclear antibody specific for pericytes. Reporter mice, where pericytes are readily identified by the expression of an easily visualized marker or enzyme, could be used for this, together with staining for apoptotic markers after irradiation, either on sections or by flow cytometry. If using flow cytometry, pericytes could be isolated, whereby expression of selected genes or markers could be analyzed. The effect on pericytes after irradiation is so far unexplored and these experiments would therefore be a novel contribution to the radiation research field. Furthermore, our data do not exclude that there are ultrastructural changes in the pericyte – endothelial interaction, either at the time of reduced pericyte coverage or at later time points when coverage was normalized. This would be interesting to investigate by electron microscopy.

The work carried out in this thesis is oriented towards developing a better understanding of what causes the negative effects observed after cranial radiotherapy in children. It is important to investigate both acute and late effects since even very low doses of cranial radiation during infancy showed a negative correlation with intellectual capacity in adulthood (Hall et al., 2004). Furthermore, it has been proposed that cognitive decline following radiotherapy could, at least partly, be due to loss of hippocampal neurogenesis (Raber et al., 2004, Kuhn and Blomgren, 2011). In order to develop better strategies to ameliorate the negative effects, it is important to characterize how neurogenesis is regulated both under normal and pathological situations.

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