IRRADIATION-INDUCED INJURY TO THE JUVENILE BRAIN

-ROLES OF SEX AND INFLAMMATION

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ABSTRACT

Radiotherapy is commonly used in the treatment of pediatric malignancies, but unfortunately it is associated with negative side effects, both long-term and short-term. Negative side effects following cranial irradiation involve intellectual impairments, where gender and age at treatment are important factors for the outcome. Younger age at diagnosis and female gender are associated with more severe late effects. We investigated sex-dependent differences in the response to cranial irradiation both acutely and long-term, in the presence or absence of lipopolysaccharide-induced inflammation.

Differences in the response to irradiation (IR) between the sexes were detected in the acute phase, for proliferation and cell death, in the hippocampus and in the corpus callosum. Looking at the long-term effects of IR we show that females display a more pronounced lack of growth in both the granule cell layer of the hippocampus as well as in the corpus callosum. We also show that females are more susceptible to IR, as judged by reduced proliferation and neurogenesis in the hippocampus, and reduced oligodendrogenesis in the corpus callosum, compared to males. At the behavior level, we show using the IntelliCage setup that learning and memory are impaired after one single dose of 8 Gy, more so in females. Female mice also display a more anxious behavior in the open field and elevated plus maze compared to their male counterparts.

Pre-treatment with LPS prior to IR reveals a sex-dependent difference, where females display a higher general inflammatory response and caspase-3-dependent cell death compared to males in the acute phase. We further show that LPS prior to IR sensitizes the brain in both male and female mice long-term. LPS-treated animals display a more pronounced lack of growth of the GCL and reduced hippocampal proliferation and neurogenesis. We also show that microglia density is highly up-regulated in the DG four months post-IR in both vehicle- and LPS-treated female mice.

In conclusion, female mice are more susceptible to IR which is consistent with clinical observations. This demonstrates that gender is an important factor to take into consideration in both the rodent and human brain. We also show that an ongoing inflammation at the time of IR aggravates the injury, which may enhance cognitive deficits in pediatric patients undergoing radiotherapy.

Key words: Cranial radiotherapy, neurogenesis, dentate gyrus, corpus callosum, sex, inflammation, microglia, LPS, IntelliCage, open field, elevated plus maze
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POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Tack vare förbättrade behandlingsstrategier, som bland annat strålbehandling, har antalet barn som överlever sin cancer ökat kraftigt under de senaste decennierna. Joniserande strålning används för att slå ut de maligna tumörcellerna genom att förändra det genetiska materialet i kärnan. Strålningen påverkar även de normala cellerna men dessa celler har en bättre förmåga att reparera de skador som strålningen orsakar. Strålning mot hjärnan ger bestående skador, bland annat i form av tillväxt- och hormonrubbningar, inlärningssvårigheter och en nedsatt kognitiv förmåga. Ju yngre barnet är vid strålningstillfället desto allvarligare komplikationer kan uppstå senare i livet. Unga flickor har visat sig drabbas av svårare komplikationer till följd av strålningen jämfört med unga pojkar. Det är idag känt att stamceller är känsliga för strålning och att celldöd sker i de områden där dessa celler finns. Även omogna oligodendrocyter, som är viktiga för en välfungerande signalöverföring mellan nervceller, är mycket känsliga för joniserande strålning. När barn drabbas av en nedsatt kognitiv förmåga efter strålbehandling kan detta vara associerat med en minskad nybildning av nervceller (neurogenes) i hippocampus samt skador i den vita substansen, som bildas av oligodendrocyter. Strålning orsakar även en akut inflammation som kan vara bestående och därmed påverka hjärnan under lång tid.

Vi har med hjälp av en musmodell visat att strålningen orsakar en akut inflammation samt celldöd i den omogna hjärnan. Vi har sett att celldelningen minskar kraftigt i den akuta fasen efter stråltillfället. Månader efter stråltillfället är både gyrus dentatus i hippocampus och corpus callosum tillväxthämmade av strålningen, framför allt hos honmössen. Neurogenesen i hippocampus samt oligodendrogenesen i corpus callosum påverkas också av strålning hos framför allt honor. Honorna har en högre basnivå under normala förhållanden men efter strålning minskar dessa till samma nivå som hos strålade hanar. Detta skulle kunna vara bidragande faktorer till den ökade strålkänsligheten som man ser hos unga flickor. Även hanmössen påverkas negativt av strålningen men inte i lika hög grad som honorna. Detta reflekteras även i olika beteendetester vi genomfört. Vi har då sett att de strålade honorna har svårare med inlärning och är mer ängsliga än hanarna. De underliggande orsakerna till könsskillnaderna efter strålning är ännu oklara och behöver belysas av ytterligare forskning

I denna avhandling har vi även undersökt hur en pågående inflammation vid strålningstillfället påverkar skadorna både på kort och lång sikt. Detta gjorde vi genom att behandla mössen med lipopolysackarid, en glykolipid från Gram-negativa bakterier som sätter igång en inflammation. Vi såg ingen ökad känslighet i den akuta fasen, men på lång sikt såg vi hos båda könen att de djur som haft en inflammation vid stråltillfället hade en större skada i hippocampus, med lägre neurogenes, jämfört med de djur som bara hade behandlats med strålning. Detta belyser vikten av att undersöka hälsotillståndet hos patienter innan strålbehandlingen påbörjas och i den mån det går försöka vänta med strålbehandling tills en infektion eller inflammation av annan orsak har gått över. Man skulle även kunna göra en cytokin-profil, d.v.s. mäta halten av inflammatoriska ämnen i blodet för att undvika att stråla en patient med en pågående infektion eller inflammation.

Sammanfattningsvis är det viktigt att undersöka effekterna av olika behandlingar hos båda könen, då de kan påverkas olika. Dessutom kan vi genom att undersöka skillnader mellan könen underlätta möjligheten till en effektivare behandling som är mer individualiserad för både flickor och pojkar.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers:

I. Karolina Roughton, Marie Kalm, Klas Blomgren
Sex-dependent differences in behavior and hippocampal neurogenesis after irradiation to the young mouse brain
European Journal of Neuroscience (2012) 36, 2763–2772

II. Karolina Roughton, Martina Boström, Marie Kalm, Klas Blomgren
Irradiation to the young mouse brain impaired white matter growth more in females than in males
Cell Death and Disease (2013), in press

III. Karolina Roughton, Ulf Andreasson, Klas Blomgren, Marie Kalm
Lipopolysaccharide-induced inflammation aggravates irradiation-induced injury to the young mouse brain
Developmental Neuroscience (2013), Epub Aug 20

IV. Marie Kalm*, Karolina Roughton*, Klas Blomgren
Lipopolysaccharide sensitized male and female juvenile brains to ionizing radiation
Submitted manuscript
*these authors contributed equally to the study

Additional papers not included in the thesis:

Qian Li, Hongfu Li, Karolina Roughton, Xiaoyang Wang, Guido Kroemer, Klas Blomgren, Changlian Zhu
Lithium reduces apoptosis and autophagy after neonatal hypoxia-ischemia
Cell Death and Disease (2010) 15 (1): e56, Epub July 15

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ABBREVIATIONS

ANOVA - analysis of variance

BBB - blood-brain barrier

BrdU - bromodeoxyuridine

CC - corpus callosum

CD31 - cluster of differentiation 31

CNS - central nervous system

CRT - cranial radiotherapy

DAB - 3-3'-diaminobenzidine tetrahydrochloride

DCX - doublecortin

DG - dentate gyrus

ELISA - enzyme-linked immunosorbent assay

EPM - elevated plus maze

GCL - granule cell layer

GEE - generalized estimating equations

Gy - Gray

HR - homologous recombination

Iba-1- ionized calcium-binding adapter molecule 1

IHC - immunohistochemistry

IL - interleukin

IR - irradiation

LPS - lipopolysaccharide

MBP - myelin basic protein

ML - molecular layer

NHEJ - non-homologous end joining

Olig2 - oligodendrocyte lineage transcription factor 2

OF - open field

P - postnatal

PBS – phosphate-buffered saline

PHH3 – phospho-histone H3

ROS - reactive oxygen species

S100 - S100 calcium binding protein

SGZ - subgranular zone

SVZ - subventricular zone

TBS - Tris-buffered salin

BACKGROUND

The male and female brain

Male and female human brains are similar, but differences do exist between the two, influencing many brain areas as well as behavior. Differences observed include memory, emotion, hearing, vision, the processing of faces, pain perception, neurotransmitter levels and navigation, to mention a few (Cahill, 2006). Some neurological disorders are much more common in boys than in girls, for example dyslexia, attention deficit hyperactivity disorder (ADHD) as well as autism spectrum disorder. Women are more prone to suffer from major depressive disorder, anxiety, panic disorders, anorexia nervosa and anorexia bulimia (Budziszewska et al., 2010, McCarthy et al., 2012). The differences between the male and female brain are thought to partly depend on sex hormones and experience during development, but sex hormones cannot fully explain all differences between males and females in the adult brain since many of these differences persists even in the absence of these hormones (Purves et al., 2008). However, there is evidence indicating that menstrual cycle hormones influence learning and memory processes through interaction with stress hormones, and in humans the menstrual cycle influences performance in verbal and spatial tasks (Cahill, 2006).

In adolescence, males have been reported to have 9-12% larger brain volumes compared to females. When different brain areas are accounted for overall differences in total brain size, the amygdala and the hippocampus have been shown to differ between genders. Imaging studies have shown a larger hippocampus in females and a larger amygdala in males. It has further been shown that the amygdala have a greater density of androgen receptors and the hippocampus has a greater density of estrogen receptors. The hippocampus and the amygdala are structures involved in depression and anxiety disorders,

disorders where gender has been shown to be of importance (Lenroot and Giedd, 2010).

Sex differences in the brain are complex but important to bear in mind. There are underlying biological differences between males and females under normal conditions which are important to consider when investigating different treatment strategies, since the outcome might differ depending on gender. It is of great importance to investigate both males and females, since the two genders might respond differently to different treatments.

Fig 1. The male and female brain

Children and cancer

Cancer is rare before the age of 20, but out of all pediatric tumors, brain tumors and leukemia are the most common malignances in children under the age of 15 (Vizcaino et al., 1998, Steliarova-Foucher et al., 2004). During the last decades, due to improved treatment protocols, the survival rates have increased and today 75% of all children that suffered from a brain tumor are still alive 5 years after diagnosis (Gustafsson et al., 2013). The treatment strategy for brain tumors varies depending on the tumor location and histology, but a combination of

surgery, local or craniospinal radiation with or without chemotherapy is a common strategy (Butler and Haser, 2006).

Cranial radiotherapy (CRT) is an effective tool in the treatment of brain malignancies, but is also associated with late-occurring negative effects, so called late effects. Common late effects seen after CRT are hormonal imbalances, perturbed growth, and reduced information processing, but also persistently impaired learning and memory function (Lannering et al., 1990). When looking into long-term consequences of CRT, men and women who have survived their childhood brain tumors are less likely to get married or get an employment compared to their peers, an effect that is linked with the CRT dose that the patient received (Armstrong et al., 2009). These findings were confirmed in a Swedish population-based study (Boman et al., 2010). Young age at the time of diagnosis is an important factor when looking into the negative late effects seen after CRT, where younger children are more liable to show a decline in cognition and processing speed compared to older children, at least partly because the growth of the young brain is retarded (Fouladi et al., 2005). Another risk factor for negative late effects is gender, where girls are more sensitive to the effects of CRT compared to boys (Ris et al., 2001, Lahteenmaki et al., 2007).

Radiotherapy

Even though irradiation is associated with negative late effects, it is still a very useful and common tool in the treatment of malignancies. There are two types of irradiation (IR), ionizing and non-ionizing, but as a treatment tool the ionizing type is used. This means that the IR contains enough energy to produce ions in the tissue being irradiated. The goal with radiotherapy is to accomplish cell death in all tumor cells while at the same time keeping the non-tumor cells

healthy. To keep all healthy cells alive is practically impossible during radiotherapy, but there is a difference in sensitivity between healthy cells and tumor cells which is exploited in radiotherapy. Firstly, tumor cells do not have the same ability to repair the damages caused by IR, and secondly, the tumor cells are more sensitive to IR due to the high proliferation that is characteristic of these cells. To exploit this difference in properties between healthy and tumor cells, the dose of IR plays an important role. If the dose is too low, no cells will be affected by the IR, but if the dose is too high, all healthy and tumor cells will die. It is therefore important to find a dose where as many tumor cells die and healthy cells survive as possible. Tissues differ in the sensitivity to IR and the most radiosensitive tissues are those with high proliferation rates. Neurons in the brain are post-mitotic and thereby rather radioresistant. However, the neurogenic areas, the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ), where new neurons are born throughout life, are highly sensitive to ionizing IR, especially in the young brain (Fukuda et al., 2005b).

The cell cycle and it´s checkpoints

In the post-mitotic stage, cells are not as sensitive to IR. But during proliferation the cells expose their DNA and are thereby in a more vulnerable state, where the ionizing IR can more easily cause DNA damage.

The cell cycle is a process which all cells have to go through during their lifespan. At the end of each turn of the cycle, the cell is divided into two cells. The cell cycle consists of five different phases, G0, G1, S, G2 and M (Fig 2.). During the S phase (the synthesis phase), DNA duplication occurs, which normally takes about 10-12 hours in the human cell. After the S phase the cell moves into the M phase (the mitotic phase), where chromosome segregation and cell division occurs, which requires much less time compared to the S phase.

During the G1 and G2 gap phases, the cell grows and doubles its mass of organelles and proteins, a process that usually takes more time than DNA replication and division. G0 is a quiescent state of the cell cycle where the cell is not dividing (Gage et al., 2008).

Before a healthy cell is allowed to divide, it has to go through a set of checkpoints. These are points where progression of the cycle can be stopped until the conditions are favorable for the cell to enter the next step. There are three checkpoints in the cycle. The first checkpoint takes place between the G1and S phase, the second in the mid- S phase and the third between G2 and M phase (Alberts et al., 2002). The first two checkpoints protect against errors occurring in DNA replication, while the third checkpoint prevents the cell from dividing with chromosomal aberrations (Hoeijmakers, 2001).

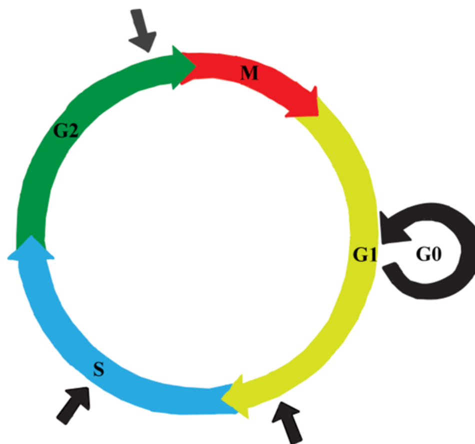

Fig 2. The cell cycle and its checkpoints. The cell cycle starts in the G1 phase, where the cell increases in size. The checkpoint between G1/S ensures that everything is ready for DNA synthesis. DNA is replicated during the S phase. The checkpoint in the mid S phase ensures that no errors in DNA replication occur. The cell continues to grow during the G2 phase and the checkpoint between G2/M ensures that everything is ready for entering the M phase, which is the stage where the cell can divide and become two identical daughter cells. G0 is a resting phase where the cell has left the cycle.

DNA damage and DNA repair

The DNA molecule is vulnerable to different environmental agents, such as UV light, ionizing radiation and numerous chemicals that can cause alterations in the DNA structure. These are challenges that our cells meet every day at low doses, and they are prepared for them. However, if these damages are left unrepaired they may lead to mutations, which in turn enhances the risk of cancer formation (Hoeijmakers, 2001). Ionizing radiation can harm the cell through the energy that it transfers to the tissue being irradiated, thereby causing damage to the DNA structure either directly or indirectly. Absorption of radiation energy to the DNA (direct effect) can cause structural DNA alterations while interactions of radiation and water molecules (indirect effect) produce water-derived free radicals that in turn can cause DNA damage (Fig 3.). IR-induced damages to the DNA molecule include base damage, single-stranded breaks, double-stranded breaks and cross-links, where double-stranded breaks are the most severe for the cell and enhance the risk of cell death (Suzuki and Yamashita, 2012).

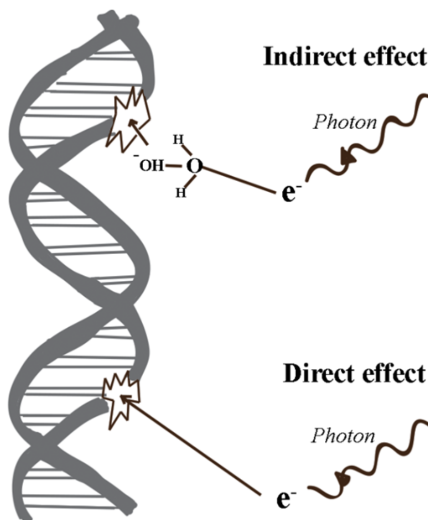

Fig 3. The indirect effect of IR is the production of free radicals. The IR affects water molecules, which are abundant in biological systems and creates excited water molecules, ionized water and free electrons. These free electrons are taken up by other water molecules and form unstable water ions, which in turn create hydrogen ions and free radicals, which can attack the DNA. In the direct effect of IR, the electrons directly interact with the atoms in the DNA chain.

A cell that becomes injured immediately starts to repair the resulting damages in the DNA. With non-severe damages, the repair machinery can use the other DNA strand as a template, but if a double-strand break occurs, the cell has no possibility to do so, since both strands are injured (Degerfält et al., 2008). In the latter case, the cell has to figure out which ends of the damaged strands that belong together, a very difficult task in a mammalian cell (Hoeijmakers, 2001). There are at least two major repair mechanisms when a double strand break occurs, homologous recombination (HR) and non-homologous end joining (NHEJ). In the NHEJ pathway the double-strand breaks are rejoined at an appropriate chromosomal end. The HR pathway uses homologous DNA as a template and relies on a sister chromatid and is thereby most efficient in the S and G2 phase when a sister chromatid is present. The NHEJ, on the other hand, does not require a sister chromatid and can be efficient during the whole G1 phase (Sakata et al., 2007).

Cells have different capacity to repair IR-induced damages to the DNA. One important factor is the efficiency of different repair enzymes which permit the cell cycle to halt and give time for DNA repair if damage has occurred. The ability to repair is dependent on where in the cell cycle the cell resides when the irradiation takes place. The S phase is the most sensitive phase in the cell cycle. Tumor cells have a poor repair system compared to healthy cells and this difference is used in radiotherapy and makes it to an advantageous therapy in cancer treatment (Degerfält et al., 2008).

Comment: In this thesis a linear accelerator was used as the source of IR. The linear accelerator is the most common device for radiotherapy used in Sweden. This device has the capacity to produce IR energies between 2 and 25 MeV and can deliver both electron and photon IR, and is therefore suitable for the most common types of treatments. In the linear accelerator electrons are accelerated in a linear path. Photon IR is created when the accelerated electrons slow down

in a metal sheath. The dose rate in a linear accelerator is high, usually between 2-4 Gy per min when the source-to-distance is 100 cm. This means that the treatment session can be kept short (Degerfält et al., 2008).

Effects of irradiation

IR can be harmful to many different types of tissues and cells, but this thesis will focus on the damages caused by IR particularly to the corpus callosum (CC) and the DG of the hippocampus.

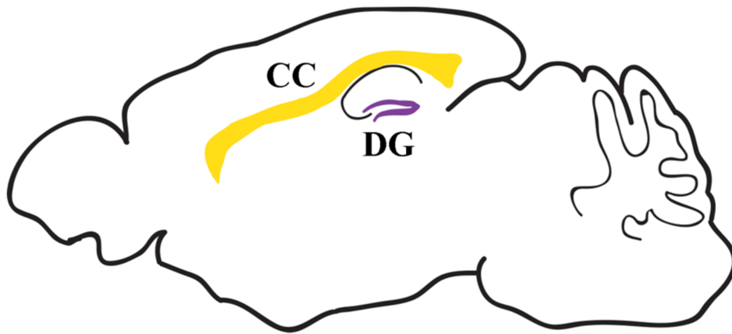

Fig 4. A schematic picture showing the corpus callosum (CC, in yellow) and the hippocampal dentate gyrus (DG, in purple) in a sagitally sectioned rodent brain.

Corpus callosum

The CC is located in the center of the brain and is the largest white matter structure (Fig 4.). In the human brain, the development of the CC is completed within the first four years in a child's life (Fitsiori et al., 2011). The growth rate of the CC continues throughout childhood and adolescence, but the highest

growth rate occurs during the first years (Lenroot and Giedd, 2006). The myelination of the human brain continues into adulthood in specific brain regions and is not complete until the fourth decade of a person's life (Semple et al., 2013). There is a difference between males and females in the brain (including the CC) growth rates, where females reach peak values of brain volumes earlier than males. During adolescence, males therefore show a steeper rate of white matter increase compared to females (Lenroot and Giedd, 2006, Lenroot et al., 2007). Gender differences in the CC have also been reported in the CC shape, where women showed to have a more bulbous and men a tubular-like shaped splenium (the posterior part of the CC). Women also were shown to have a greater width of the splenium compared to men in adulthood, but this gender difference was not observed in children 2-16 years of age (Allen et al., 1991). Another study investigating differences between genders showed that the CC midsagittal area was larger in females compared to males in the human brain when correcting for the effect of brain size. The larger area seen in females was more pronounced in younger adults. The CC area decreased with age in both genders when brain size increased (Ardekani et al., 2012). White matter integrity in the CC is important for cognitive performance, since it is involved in information processing (Palmer et al., 2012) and the development of white matter structures has been associated with increased reading ability and cognitive function (Paus et al., 1990, Casey et al., 2000).

Oligodendrocytes

Oligodendrocytes are a type of brain cells abundant in the CC and have as their main function to support neurons by producing myelin sheaths and insulate axons to enhance neuronal signaling in the CNS (Bear. MF et al., 2007) . Oligodendrocyte preogenitors are among the most vulnerable cells of the CNS. Oligodendrocytes interestingly only have a short time for myelination early

during differentiation and are thereafter relatively incapable of myelinating once they mature. Oligodendrocytes have a high metabolic rate, generating toxic byproducts and a low concentration of the antioxidative glutathione, and are therefore particularly sensitive to oxidative damage. Oligodendrocyte loss might occur by the exposure to inflammatory cytokines released during an infection (Bradl and Lassmann, 2010) and inflammatory cytokines released in IR-induced inflammation in the brain has been shown to cause oligodendrocyte degeneration in mice (Nakazawa et al., 2006).

IR to the brain leads to decreased white matter volume and demyelination (Oi et al., 1990, Reddick et al., 2000). The younger the child is when going through radiotherapy, the more negative effects on the white matter volume. It has been shown in the rat brain that lack of normal myelination occurs to a greater extent in younger animals when the brain is not fully developed (Fukuda et al., 2005a, Reddick et al., 2005). The CC is affected in children treated with radiotherapy and especially the most posterior parts of this structure have been shown to be radiosensitive (Palmer et al., 2002). It has been reported that IR affects both the rodent and the human brain through the loss of oligodendrocyte precursor cells and late demyelination. The same study also showed a permanent decrease in the number of proliferating cells in the neurogenic SVZ, the CC and the cortex in IR rats, indicating that IR causes a loss of oligodendrocytes which cannot fully be compensated for due to the depleted stem cell compartment (Panagiotakos et al., 2007).

Hippocampus

The hippocampus is located in the medial temporal lobe in the human brain and belongs to the limbic system. Mammals (including humans) have two hippocampi in the brain, one on each side (Purves et al., 2008). The components of the hippocampus include the CA3 and CA1 regions and the DG (Fig 3.),

which receives input from the entorhinal cortex (Monje and Palmer, 2003). The hippocampus is one of the areas in the brain where neurogenesis occurs throughout life. Neurogenesis (the generation of new neurons) takes place in the SVZ along the lateral walls of the lateral ventricles and in the subgranular zone (SGZ) in the hippocampal DG (Kempermann et al., 2004a). Neural stem cells in the hippocampus continuously generate neurons, astrocytes and oligodendrocytes, while at the same time undergoing self-renewal (Gage et al., 1998).

The hippocampal formation (Fig 5.) is part of a neuronal network that plays an important role in learning and memory (Eichenbaum, 2000, 2001). The function of the hippocampus is not to store memories long-time, but rather process the information before sending it further for long-time storage in the neocortex. A functional hippocampus is essential for the ability to remember facts and the order of events, a phenomenon called episodic memory (Eichenbaum, 2013).

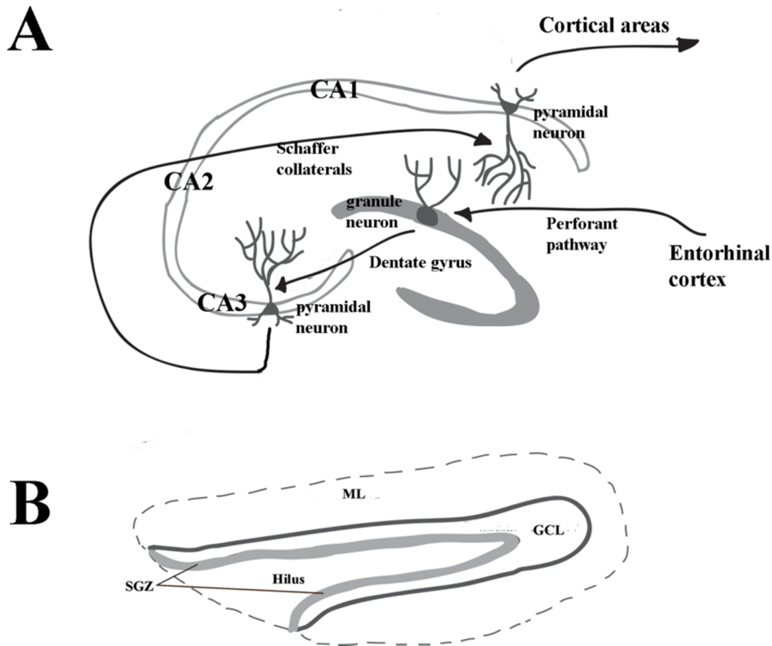

Fig 5. A shows the circuitry of the hippocampal DG. The granule cells in the DG receive information via the perforant path from the entorhinal cortex and project in turn to pyramidal neurons of the hippocampal CA3 region. The CA3 neurons project axons via the Schaffer collaterals to pyramidal neurons of the CA1 region which in turn send the information further for long-time storage in cortical areas. **B** shows an overview of the different subregions of the DG (GCL= the granule cell layer, ML= molecular layer). The SGZ resides between the GCL and the hilus.

Hippocampal neurogenesis

The SVZ and the hippocampal DG, i.e. the neurogenic areas in the brain are sensitive to irradiation, but differences in recovery between these two regions have been shown, where stem and progenitor cells in the DG are severely affected long-term, while progenitor cells in the SVZ appear to recover, at least partly (Hellstrom et al., 2009). This thesis will solely focus on neurogenesis and the effect of irradiation on the hippocampal DG. In the adult brain new neurons are born in the SGZ in the DG, a region between the GCL and the hilus. The

stem cells in the SGZ are believed to be radial glia-like cells expressing nestin, GFAP and the stem cell marker SOX2 (Brown et al., 2003). The newborn cell migrates into the GCL and integrates into the neuronal network where it extends dendrites and axons and form synapses (Fig 6.) (Kempermann et al., 2004b). Many of the newborn cells in the SGZ die shortly after birth but the surviving cells mature and receive synaptic GABAergic input (Gage et al., 2008, Zhao et al., 2008, Kuhn and Blomgren, 2011). Hippocampal neurogenesis has been associated with learning, stress, enriched environment and different brain diseases such as depression (Curtis et al., 2012). Animal models support the findings indicating hippocampal neurogenesis to be important for normal cognitive function, and factors which increase neurogenesis in the DG, such as running and enriched environment, also improve hippocampal dependent memory. On the other hand, factors which decrease neurogenesis, such as chemotherapy or IR impair animals' performance in hippocampal-dependent tasks (Monje and Palmer, 2003, Rola et al., 2004, Will et al., 2004).

Irradiation impairs neurogenesis by blocking cell proliferation and eliminating the dividing precursor cells (Kempermann et al., 2004b). Stem and progenitor cells have a high proliferation capacity and this is one explanation to why hippocampal neurogenesis is highly affected by IR. Studies in rodents have shown that especially IR to the developing brain has negative effects on neurogenesis (Fukuda et al., 2004). A developing brain contains many cells undergoing proliferation, especially in the neurogenic regions, which make them vulnerable to external stimuli like IR. Studies have demonstrated that girls are more vulnerable to IR compared to boys, for example as judged by the capacity to learn a foreign language, a task where a functional hippocampus is of great importance (Lahteenmaki et al., 2007).

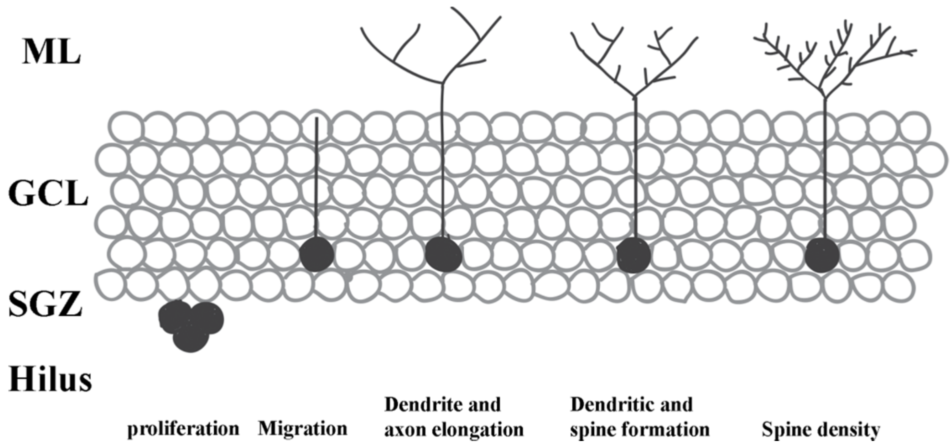

Fig 6. An overview of the proliferation and maturation of new neurons. Newly born neurons migrate into the GCL and integrate into the network.

Neuroinflammation

The immune system can be divided into two parts, the innate and the adaptive immune system, where the innate or the non-specific immune system is very rapid and serves as the first line of defense against any foreign threat. The adaptive immune system on the other hand is mainly driven by lymphocytes that create an antigen-specific immune response which takes time to develop but gives rise to a long-lasting immunity.

Microglia

Immune responses in the brain are mediated by microglia, which are the resident immune cells in the CNS (Fig 7.) (Abbas and Lichtman, 2005). Microglia migrate into the brain during early development where they sustain a resident population through proliferation (Kohman and Rhodes, 2013). Microglia are located throughout the whole brain, but the density varies between different regions and they are more abundant in gray matter than in white matter in the

adult brain (Lawson et al., 1990). In the resting state, microglia are constantly surveying the environment using many fine processes in the search of possible threat, but during pathological conditions such as damage or the presence of an infection, they initiate an inflammatory response through the secretion of pro-inflammatory molecules such as CCL2 and IL-6, and phagocytose dying cells (Ekdahl et al., 2003, Monje et al., 2003). Microglia are essential in hippocampal neurogenesis under normal conditions, where they are involved in phagocytosing apoptotic newborn cells (Sierra et al., 2010).

Ionizing radiation leads to a cascade of events that activates microglia, which start to release pro-inflammatory cytokines and chemokines which may lead to oxidative stress in the brain (Ballesteros-Zebadua et al., 2012). This response is thought to result in chronic oxidative stress where IR activates microglia and the infiltration of immune cells to the brain. These immune cells in turn generate reactive oxygen species (ROS) which activate more microglia and more immune cells that can increase the level of oxidative stress (Greene-Schloesser et al., 2012).

Not long ago, the brain was considered to be immunologically isolated, with no immune activation due to the presence of the blood brain barrier (BBB) (Lucas et al., 2006). It later became clear though that the CNS is specialized immunologically and contains immune cells both under normal and pathological conditions (Lucin and Wyss-Coray, 2009). The BBB consists of tight junctions around the capillaries in the CNS and serves as a selectively permeable barrier that separates circulating blood from the extracellular fluid in the CNS (Hamilton and Sibson, 2013). Irradiation to the brain may cause microvasculature damage. This damage leads to increased permeability of the BBB, infiltration of lymphocytes and edema and many patients who suffer from IR-induced edema are treated with glucocorticoids (Ballesteros-Zebadua et al., 2012). The pro-inflammatory cytokine CCL2, released by activated microglia, is

increased by irradiation to the young brain (Kalm et al., 2009a, Roughton et al., 2013). This cytokine seems to play an important role in the breakdown of the BBB and animal studies have shown that CCL2-deficient mice had smaller infarct volumes and decreased BBB breakdown in stroked animals (Strecker et al., 2013). The chemokine receptor CCR2 binds the ligand CCL2 and is expressed by microglia. CCR2 mediates the recruitment of microglia to sites of inflammation in the CNS. Recent studies have shown that mice lacking this receptor are protected from the cognitive impairment seen after IR to the brain in hippocampal dependent behavior tasks (Allen et al., 2013, Belarbi et al., 2013, Raber et al., 2013).

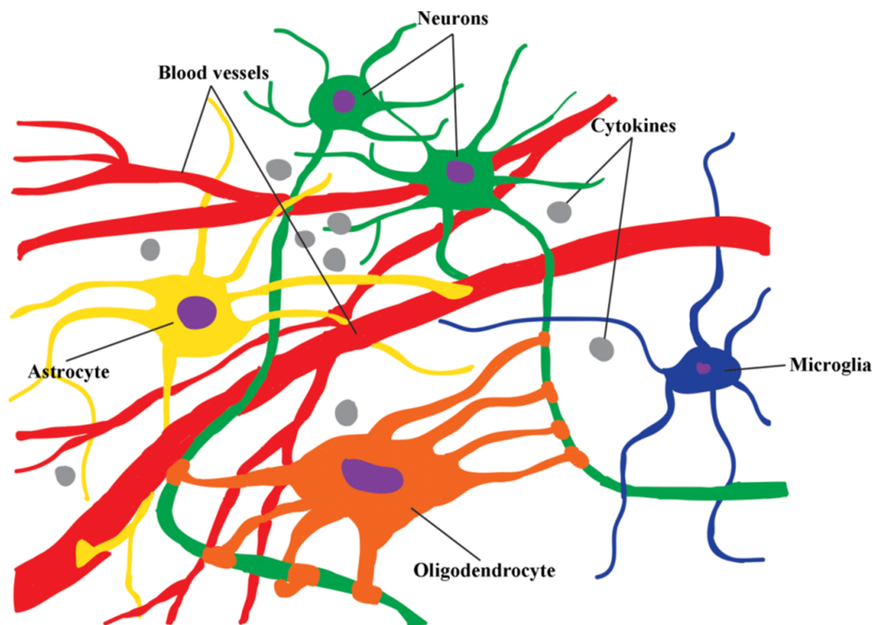

Fig 7. An overview of the different cell types in the CNS.

Lipopolysaccharide

Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria. LPS passes the BBB and triggers the innate immune system through the activation of microglia. After passing the BBB, LPS binds to the toll-like receptor 4, which is expressed by microglia and thereby directly activates the innate immunity. Studies where LPS has been used to stimulate microglia, indicate that microglial activation has negative effects on hippocampal neurogenesis (Kohman and Rhodes, 2013). Neuroinflammation induced by LPS leads to reactive microgliosis which partly alters neurogenesis by the release of neurotoxic mediators such as IL-6, TNF-α, nitric oxide and ROS. These pro-inflammatory mediators might lead to a disturbed microenvironment in the so called neural stem cell niche which in turn can lead to disrupted neurogenesis (Russo et al., 2011). Neuroinflammation caused by LPS was shown to negatively affect cognitive function and treatment with the non-steroidal anti-inflammatory drug indomethacin restored hippocampal neurogenesis in an animal study (Monje et al., 2003).

Despite the negative effects seen after LPS-induced neuroinflammation, it has also been demonstrated that low doses of LPS can be neuroprotective (preconditioning) if given before traumatic brain injury or stroke (Lin et al., 2009). This is thought to depend on that a low dose of LPS triggers defense mechanisms in the tissue and thereby induces tolerance if the new insult happens within a certain time window (Mallard and Hagberg, 2007, Rosenzweig et al., 2007). This means that under some conditions a systemic infection, or another pro-inflammatory stimulus, might be harmful while it under other conditions might be neuroprotective.

Both LPS and IR can separately cause neuroinflammation and in papers III and IV we wanted to investigate the effects of these two components combined.

Learning and memory

Learning is the acquisition of new information or knowledge while memory on the other hand is the retention of learned information. The hippocampus seems to play an important role in the temporal organization of memories (Eichenbaum, 2013). Memories can be either short- or long-term. Long-term memory holds a large amount of information for much longer periods than the short-time memory and comprises different kinds of memories. *Declarative memory* (also called explicit memory) is memory available to consciousness and can be divided into e*pisodic memory* which consist of memories of events and *semantic memory* which is the knowledge about language and facts. *Non-declarative memory* (also called implicit memory) is memory expressed through performance and is thought to operate unconsciously. *Procedural memory* is a non-declarative memory important for supporting cognitive and motor skills, such as tying shoes and riding a bike (Purves et al., 2008).

Important brain structures for declarative memory are located in the medial temporal lobe and consist of the hippocampus, entorhinal cortex, parahippocampus and perirhinal cortices (Delgado and Dickerson, 2012). The hippocampus has been reported to be crucial for cognitive processing and is involved both in the consolidation and acquisition as well as the retrieval of declarative memory. The hippocampus is also a key brain structure in *spatial memory* which is required to navigate through the environment (Castilla-Ortega et al., 2011). There are a number of studies supporting that hippocampus-dependent cognition involves neurogenesis in the DG and different strains of mice showed that greater neurogenic capacity correlated with better performance in spatial memory tasks (Kempermann and Gage, 2002). Gould *et al.* (1999) showed an association between adult-generated neurons and hippocampal-dependent learning, where trace eyeblink conditioning and spatial water-maze training doubled the number of newly generated neurons in the DG.

The same study also showed that non-hippocampal-dependent learning did not increase the number of newly generated neurons in the DG (Gould et al., 1999). It has been shown that part of these newly generated surviving cells functionally integrate in the DG in response to hippocampal learning (Castilla-Ortega et al., 2011).

The importance of the hippocampus in memory function was first understood in the 1950´s when a patient called Henry Molaison (HM) became amnesic after a bilateral medial temporal lobectomy which was performed to decrease the epileptic seizures he suffered from. After the surgery HM immediately showed a severe persisting amnesia characterized by a poor-functioning declarative memory. Despite the severe amnesia, HM´s intelligence, working memory and motor skill learning were not affected (Corkin, 2002). Clinical studies have reported that injury caused by IR to the hippocampus is an important factor in the decreased cognitive function seen in patients, especially deficits in learning, memory and spatial processing (Gondi et al., 2010, Blomstrand et al., 2012). This IR-induced deficit in cognitive function is particularly evident in children undergoing radiotherapy (Lannering et al., 1990). It is therefore important to try sparing the hippocampus from injury, especially in children receiving radiotherapy to the head and neck.

AIM OF THE THESIS

The aim of the thesis was to investigate sex-dependent differences in the response to irradiation of the juvenile brain, and how these responses are affected by an existing inflammation caused by lipopolysaccharide.

Specific aims of the thesis:

I. To investigate possible differences in behavior and hippocampal neurogenesis between the sexes after irradiation to the young mouse brain.

II. To investigate possible differences in white matter injury between the sexes after irradiation to the young mouse brain.

III. To investigate possible effects of combined lipopolysaccharide-induced inflammation and irradiation on hippocampal neurogenesis in the young mouse brain.

IV. To investigate possible effects of combined lipopolysaccharide-induced inflammation and irradiation on behavior and hippocampal neurogenesis in the young mouse brain in both sexes.

MATERIALS AND METHODS

Animals

Male and female C57BL/6 mice were purchased from Charles River Laboratories, Sulzfeld, Germany. In paper III, only male mice were used. The animals were ordered in litters containing 5-6 pups per litter. All animals were kept on a 12-hour light cycle with food and water provided *ad libitum.* Animals used for long-term experiments were weaned at P21. In paper I, animals used for IntelliCage were put together in groups of 10 animals per cage after weaning and RFID (Radio Frequency Identification) microtransponders (Datamars, Petlink, Youngstown, OH, USA) were implanted. These transponders were used to be able to identify the animals during the IntelliCage experiment.

Comment: In this thesis an animal model was used to be able to investigate IR-induced injury to the juvenile brain. Cell cultures can be very useful to some extent, but not when studying behavior and investigating the microenvironment in specific brain regions. The response to brain injury, including the immune response, is the combined result of multiple physiological mechanisms in the entire organism.

Ethical permissions

All animal experiments were approved by the Gothenburg committee of the Swedish Animal Welfare Agency. The application numbers used in this thesis were: 46-2007, 30-2008, 423-2008, 326-2009, 47-2011.

Irradiation procedure

A linear accelerator (Varian Clinac 600 D, Radiation Oncology Systems, LLC, San Diego, CA, USA) with 4 MV nominal photon energy was used for IR. All animals were anesthetized on postnatal day (P) 14 with an intraperitoneal injection of 50 mg/kg of tribromoethanol (Sigma, Stockholm, Sweden) to immobilize the animals during the procedure. The animals were placed in a prone position on a polystyrene bed and the head was covered with a tissue equivalent material to obtain an even IR dose throughout the underlying tissue. The whole brain was irradiated with 8 Gy, using a radiation field of 2 x 2 cm and a source to skin distance of 99.5 cm. The dose rate was 2.3 Gy/min and the variation of the absorbed dose was estimated to ±5%. An absorbed dose of 8 Gy is equivalent with 18 Gy delivered in 2-Gy fractions according to the linear quadratic formula and an alpha/beta ratio of 3 for late effects in the normal brain tissue (Fowler, 1989). A dose of 12-18 Gy is commonly delivered to children with CNS involvement in acute lymphatic leukemia, and is thereby a clinically relevant dose in our animal model. The dose used for treatment of brain tumors in children, such as medulloblastoma, is even higher, up to 55 Gy to the tumor bed. After the IR procedure, the pups were placed on a warm bed (~36°C) to be able to maintain the body temperature of the animals. When the pups recovered from anesthesia, they were returned to their dams in their original cages until sacrificed. Control animals used in the experiments were anesthetized but not subjected to IR.

Injections

Bromodeoxyuridine

To investigate proliferating and surviving cells, the animals were injected with an intraperitoneal injection of bromodeoxyuridine (BrdU, 50 mg/kg) (Roche

Diagnostics GmbH, Mannheim, Germany) dissolved in 0.9% sodium chloride for three (papers I and II) or four (papers III and IV) consecutive days, to be able to detect cells that were dividing at the time of the injection. Four weeks later the animals were sacrificed at an age of 4.5- (papers I and II), 2.5- and 4 months (papers III and IV), respectively). All BrdU injections were given in the beginning of the active period.

Comment: BrdU is a thymidine analogue which is incorporated into the DNA during replication and can thereafter be detected immunohistochemically in surviving cells. BrdU was administered after IR to be able to investigate the number of proliferating and surviving cells.

Administration of lipopolysaccharide

In papers III and IV, animals were administered an intraperitoneal injection of lipopolysaccharide (LPS) from *Escherichia coli* 126:B6 (sigma-Aldrich, St. Louis, MO., USA) dissolved in 0.9% sodium chloride, to a dose of 0.1, 0.3 or 0.9 mg/kg at P13, 24 hours prior to IR.

Comment: LPS is a component of Gram-negative bacteria which triggers the innate immune response, hence mimicking a systemic infection. LPS can pass the BBB and directly activate the innate immunity in the brain (Russo et al., 2011).

Immuno- and caspase activity assays

Different kinds of assays were used to investigate IR-induced effects on hormones (paper I), myelin basic protein (MBP, paper III), chemokines/cytokines (paper IV) and caspase activity (paper IV). The brains were quickly removed after sacrifice, cut in two halves (paper IV) and put on

dry ice to stop RNAse activity. In paper III the cortex (including the corpus callosum) was dissected out before quickly being put in liquid nitrogen and then stored at -80°C. The right hemisphere (paper IV) or cortex from both hemispheres (paper III) was homogenized by sonication in phosphate-buffered saline (PBS) containing Triton X-100, EDTA and protease inhibitor cocktail. After sonication the samples were centrifuged and the cytosolic fractions were stored at -80°C until evaluation. In paper I, animals used for blood collection were anesthetized with a mixture of isoflurane and oxygen. The chest was opened and blood was drawn from the heart with a syringe. The blood samples were put on ice, centrifuged and then serum was obtained, transferred to a new tube and stored at -80°C. The total protein concentration was measured according to Whitaker & Granum (Whitaker and Granum, 1980), adapted for microplates, and the samples were centrifuged to remove possible debris before running the immunoassays.

Enzyme-linked immunosorbent assay

In paper I, enzyme-linked immunosorbent assay (ELISA) was used to investigate hormones possibly affected by IR to the hypothalamic-pituitary axis. Five hormones were investigated using different ELISA kits: *Insulin-like growth factor-1* (IGF-1-R20; Mediagnost, Reutlingeen, Germany), *estrogen* (TKE21; Siemens Healthcare Diagnostics Ltd, Camberley, UK), *testosterone* (cat. No. 189102; MP Biomedicals, Costa Mesa, CA, USA), *free triiodothyronine* (T3; ELISA TF E-3100; LDN am Eichenhain, nordhorn, Germany) and *free thyroxine* (T4; Amerlex-MAB, Trinity, Biotech Co., Wicklow, Ireland). In paper II, the ELISA was used to measure the content of MBP in cortical tissue after IR (E90539Mu, Uscn, Life Science Inc, Wuhan, China). The analyses were performed according to the instructions of the manufacturers.

Luminex

In paper IV, a multiplex mouse assay (Bio-Plex Pro TM Mouse Cytokine Standard 23-plex, Group 1, Lot #5022433-1, Bio-Rad Laboratories AB, Sundbyberg, Sweden) was used to measure mouse cytokines/chemokines. The inflammatory mediators assayed were: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1 (CCL2), MIP-1α, MIP-1β, RANTES and TNF-α. The samples were analyzed using a Bio-Plex Protein Array System and the related Bio-Plex Manager (Bio-Rad, Sundbyberg, Sweden).

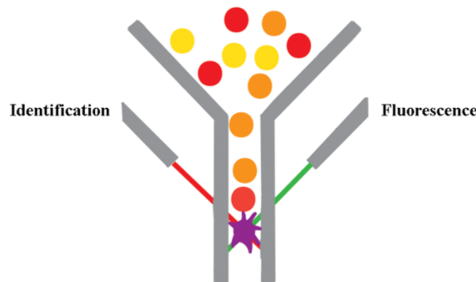

Fig 8. The luminex system. Fluorescently died magnetic beads with a covalently bound antibody binds to the biomarker of interest in the suspension. It has two lasers, one to detect the identification signal and the other to detect the fluorescence signal.

Comment: Luminex is a magnetic bead-based assay designed to measure multiple biomarkers of interest in small volumes (Fig 8.). The Luminex assay used in this thesis enabled us to quantify multiple cytokines/chemokines in the same run, in a 96-well plate. The principle behind the assay is based on fluorescently dyed beads with distinct colors which enable discrimination within the suspension. The Luminex assay is similar to a sandwich ELISA, where the captured antibodies directed to the biomarkers of interest are covalently coupled

to the beads. These beads then react with the sample solution (containing the biomarkers of investigation). The mixture is washed, removing unbound protein, and a biotinylated antibody for detection is then added to the assay. Finally, streptavidin-phycoerythrin (PE, reporter molecule) is added. Just like in flow cytometry, the magnetic beads in the Luminex assay pass through a detection chamber one at a time. There are two lasers in the detection chamber, one detects the identification signal of the bead and the other generates a reporter signal by exciting phycoerythrin. The concentration of the anlayte that is bound to each bead is proportional to the fluorescence intensity of the reporter signal.

Caspase activity assay

To measure IR-induced, caspase-dependent cell death in paper IV, we used a caspase activity assay. 20 µl of sample was mixed with 80 µl extraction buffer (containing 50 mM Tris pH 7.3, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 0.2% CHAPS, 1mM PMSF and 1% protease inhibitor) and incubated in a microtiter plate (Microfuor Dynatech, VA, USA) at room temperature for 15 min. After incubation, 100 µl assay buffer (50 mM Tris pH 7.3, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 4 mM DTT and 1 mM PMSF) containing the peptide substrate Ac-DEVD-AMC (50 µM, Peptide Institute, Japan) was added to each well. Cleavage of Ac-DEVD-AMC was measured at 37°C with an excitation wavelength of 380 nm and an emission wavelength of 460 nm, using a Spectramax Gemini microplate fluorometer (Molecular Devices, Sunnyvale, CA). The degradation was followed for 2 hours, at 2 min intervals and Vmax was calculated from the entire linear part of the curve. The activity was calculated as pmol AMC released per mg protein and minute.

Comment: Detection of DEVDase activity (caspase-3-like activity) is a method to measure the apoptotic activity in an experimental setup. Caspase-3 and -7 are

members of the *cysteine aspartic acid-specific protease* (caspase) family and play an important role during mammalian apoptosis. Apoptosis can be induced by a number of stimuli, for example exposure to radiation or chemotherapeutic agents. The caspases being activated participate in a cascade of cleavage events that stops important key repair enzymes and leads to cell degradation.

Immunohistochemistry

Tissue preparation

The animals were deeply anesthetized with sodium pentobarbital (Pentothal®; Electra-box Pharma, Tyresö, Sweden) before being perfused with phosphate-buffered saline (PBS) to clear the vasculature from blood cells. This was performed to avoid unspecific binding of antibody later when staining the tissue, since blood cells have a high capacity for antibody-binding and exhibit auto-fluorescence. When the vasculature was cleared, the animals were transcardially perfused with 6% formaldehyde buffered with sodium phosphate and stabilized with methanol (Histofix™; Histolab Products AB, Sweden). The brains were removed and immersion-fixed in Histofix for 24 h and then transferred to a 30% sucrose solution containing 100 mM phosphate buffer and stored at 4°C before further processing. The right hemisphere was cut in 25 µm sagittal sections in a series of 12 (papers I, II, and IV) or in a series of 10 (paper III) using a sliding microtome. The sections were stored at 4°C in a cryoprotection solution containing 25% ethylene glycol and 25% glycerol until staining.

Staining

All staining was performed using free-floating staining protocols. A list of all antibodies used in this thesis is found in table 1. Sections to be stained for

phospho-histone H3 were treated with 10 mM sodium citrate at 80°C for 30 min for antigen retrieval (papers I and II). To block endogenous peroxidases, sections used for immunostaining with diaminobenzidine tetrahydrochloride (DAB) visualization were treated with 0.6% hydrogen peroxide for 30 min and then washed several times using Tris-buffered saline (TBS). Sections to be stained for BrdU were treated with 2 M HCl at 37°C for 10 min followed by 10 min in borate buffer (100 mM, pH 8.0). Nonspecific binding was blocked by 3% donkey serum dissolved in TBS containing 0.1% Triton X-100 for 30 min. Sections were further incubated at 4°C overnight (sections stained for doublecortin (DCX) were incubated over three nights) with primary antibody diluted in 3% donkey serum in TBS containing 0.1% Triton X-100. After incubation with primary antibodies, the sections were washed in TBS and a biotinylated secondary antibody was added and incubated at room temperature for 60-90 min. The staining was then developed using DAB diluted in TBS containing hydrogen peroxide and nickel chloride for enhancement of the reaction. To stop the reaction, the sections were washed several times in tap water and then placed in TBS before mounting. In paper III, sections double-stained for Iba-1 and BrdU were incubated with fluorophore-conjugated secondary antibody for 2 hours before mounted in ProLong mounting medium.

Antibodies

Antibody	Dilution	Company
rabbit anti-**active caspase-3**	1:250	BD Bioscience Pharmingen
rat anti- **BrdU**	1:500	AbD Serotec
rat anti- **CD31**	1:2000	BD Bioscience Pharmingen
goat anti- **DCX**	1:125	Santa Cruz Biotechnology
rabbit anti- **DCX**	1:1000	Abcam
rabbit anti- **Iba-1**	1:1000	Wako Pure Chemical Industries
goat anti- **Olig2**	1:1000	R&D Systems
rabbit anti- **phospho- histone H3**	1:1000	Millipore/Chemicon
rabbit anti- **S100**	1:5000	Dako Cytomation

Table 1. List of primary antibodies

Cell counting and volume assessment

Cell counting was performed on free-floating sagittal sections. All immunopositive cells were counted in every 12th (papers I, II and IV) or 10th (paper III) section. In paper I, BrdU-positive cells were counted throughout the granule cell layer (GCL). In papers III and IV, BrdU-positive cells were counted throughout the whole DG, divided into the different subregions the GCL, the hilus and the molecular layer (ML). DCX-positive (papers I, III and IV) and phospho-histone H3-positive cells (paper I) were counted through the SGZ of the GCL, defined as ranging from three cell layers into the GCL to two cell layers into the hilus. In papers III and IV, Iba-1-positive cells were counted in the whole DG. In paper III, all immunopositive cells (active caspase-3, PHH3, BrdU, Olig2, S100 and Iba-1) were counted in the corpus callosum (CC) in all sagittal sections that contained a defined divided dorsal and ventral hippocampus. All counting was performed using stereological principles (Stereoinvestigator, MicroBrightField, Colchester, VT, USA). The GCL, hilus, ML and CC was traced in 10 x magnification using a DIC filter. All cells were

counted in every 12th (papers I, II and IV) or 10th (paper III) section at 40 x magnification throughout the whole DG. The total volume was assessed by multiplying the counted sections with the sample fraction (i.e. multiplied by 12 in papers I, II and IV and by 10 in paper III).

Behavioral studies

IntelliCage

In paper I, the IntelliCage platform was used. IntelliCage is an automated method for different behavioral studies, including the assessment of learning and memory. One advantage with the IntelliCage setup is that it provides the animals with a home-cage environment with a minimum of handling of the animals during the experiment (Fig 9.). The mice are able to live with their littermates during the whole experiment. Each cage consists of four conditioning corners where two water bottles per corner can be connected. To each corner a door is located which the animals have to open by performing a nosepoke. Visits are monitored using RFID transponders subcutaneously injected in each animal and antennas are located in each corner for detection. Nosepokes are monitored by sensors and the drinking in the corners is detected by a lickometer. Several IntelliCages can be connected making it possible to study a large set of animals at the same time. The cages are connected to a computer which stores the data of all visits, nosepokes and licks performed by each mouse individually.

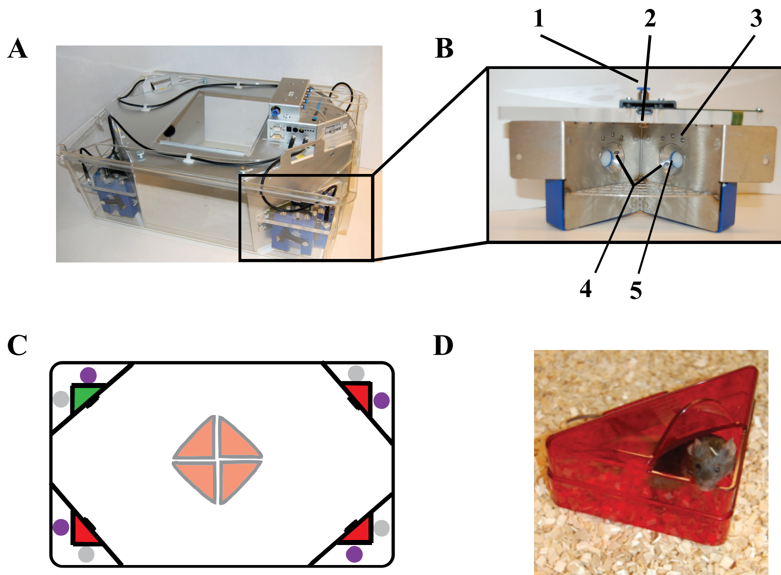

Fig 9. The IntelliCage platform used in paper I. **A** shows a representative picture of the cage. **B** shows a blown-up photo of the corner where 1 shows the air puff valve (not used in current study), 2 shows the presence detector, 3 shows the LED lamps (not used in current study), 4 shows the nose poke detectors and 5 shows a door that prevents access to one of the water bottles. **C** shows the corners, where the green color represents the allocated corner where the animal can drink and the red color represents the non-allocated corners where the animal cannot drink. **D** shows a red house which is placed in the middle of the cage (four red houses in each cage) to give the mice shelter.
Picture from (Karlsson et al., 2011).

The experimental design used in paper I was performed on three months old mice and lasted for three weeks. To avoid stress, animals of 8-10 per group that were put together from weaning, were also put together in the same cage during the experiment. Since the cages are not ventilated, males and females were tested separately on two different occasions, to avoid sex-specific odors that could interfere with the test. Animals which did not learn to drink or had a nonfunctioning chip implanted were excluded from the test. The experiment started with an introduction period (6 days) where all animals had access to all corners (i.e. could drink from the water bottles in all four corners in the cage).

The introduction period was used to let the animals explore the new environment and to learn how to open the doors in order to reach the water bottles (i.e. to perform a nosepoke). After the introduction period each animal was randomly assigned to one corner where that specific mouse was able to drink. All the other corners were then closed for that animal (corner period 1). This was performed to observe how long it took for each individual animal to learn this task (place learning). After five days, the animals were randomly assigned to a new corner, excluding the corner previously assigned (corner period 2) i.e. reversal learning was measured. Five days later this was repeated and the animals were assigned to the third and last corner during the experiment (corner period 3), again excluding the previously assigned corners.

Data from the IntelliCage were analyzed using the IntelliCage software (IntelliCage Plus, version 2.4; New Behavior AG, Zurich, Switzerland), Microsoft excel and SPSS 17.0 (SPSS inc, Chicago, IL, USA). Only the dark, active period was included in the analysis. Visits lasting longer than 180 s were excluded to minimize the risk of including visits during which the animals slept in the corners (Karlsson et al., 2011).

Open Field

The open field test (OF) was used in papers I and IV. Just like in the IntelliCage experiment, males and females were tested separately to avoid smells that could interfere with the results. Animals were tested at the age of three months. The test was used to measure general locomotor activity and anxiety. The size of the OF varies between laboratories, but the one used in in papers I and IV consisted of an automated OF arena of 50 x 50 cm. By measuring parameters like how much time the animals spend in the center of the arena, anxiety can be assessed (Crawley. JN, 2007).

In the OF test the animals are introduced to an unfamiliar arena, individually, and immediately recorded. The possibility to record the session has the advantage of the observer not being present during the test. In paper I the animals were tested for 30 min and in paper IV for 10 min. The central zone of the arena was defined as a 30 x 30 cm area in the center. Total distance moved and the percentage of distance moved in the central vs. the entire zone (paper I) or the distance to the walls (paper IV) was measured using VIEWER 3.0 Software (Biobserve, GmbH, Bonn, Germany). The middle of the body was defined as a point for tracking the entries of the zones. The more time the animal spends by the walls (i.e. less time in the center zone) the more anxiety it is thought to display. Four arenas were recorded simultaneously using a CCD camera. The arenas used in this test were made of gray Plexiglass covered with sawdust (paper I) or a light plastic material (paper IV) to be able to see the animals against the background. The C57BL/6 mice used in this thesis are black/gray and therefore a light background was needed to be able to track the animals. In paper I some of the sawdust was replaced and moved around and the walls cleaned with a mixture of water and ethanol between the trials. In paper IV the whole arena was cleaned with a mixture of water and ethanol between each trial to avoid odors from animals already exposed to the arena.

Elevated plus maze

In paper IV, an elevated plus maze (EPM) was used. The EPM has been described earlier (Komada et al., 2008). EPM is commonly used to introduce animals to a novel environment with an open area (the open arm) and a closed area (the closed arm). The maze is made like a cross with four arms (Fig 10.). The animals are placed in the intersection, in the middle of the EPM, enabling the animals to visit all four arms. Two of the arms are open, making the animal able to see the distance to the floor, and two are enclosed by walls. More entries

out onto the open arms of the EPM indicate a less anxious behavior (Crawley. JN, 2007).

The EPM used in this thesis was made from black Plexiglass and placed on an aluminum stand 60 cm above the floor. The test was performed in the mornings to avoid the daily variation of hormones that could interfere with the test and males and females were tested separately. The room was only dimly illuminated. Like described above, the animals were placed in the intersection of the EPM and then recorded individually for 5 min. The number of entries into the open arms and the time spent there was analyzed manually.

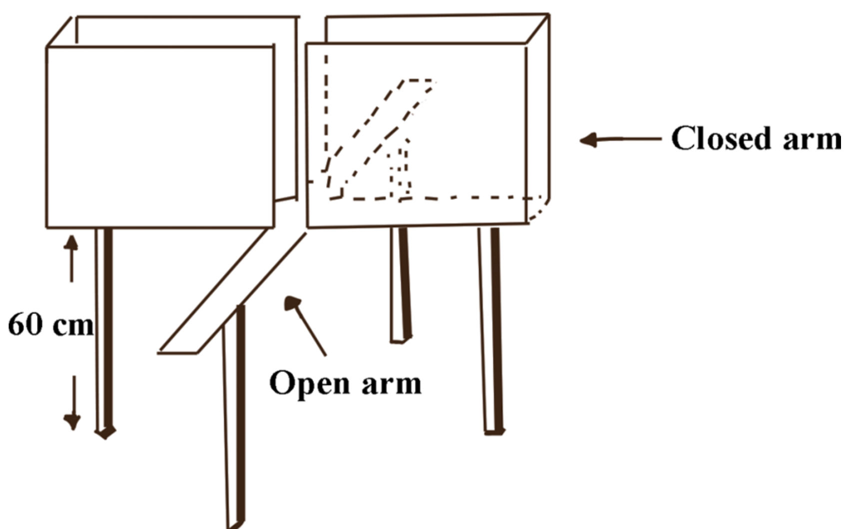

Fig 10. The Elevated plus maze (EPM) setup

Place recognition

In paper IV, place recognition was performed. Place recognition is a test where animals are able to explore a novel environment which contains objects, until

habituation. After a period of time, one of the objects can be replaced (object recognition) or placed in a new spatial location (novel place recognition). If the animal explores the moved object more than the original one, it indicates that the animal has a memory of the original configuration (Roberts and Hedlund, 2012).

The first day of testing, the animals were introduced to an unfamiliar arena individually for 10 min for habituation. Males and females were tested separately to avoid sex-specific odors, as mentioned above. 24 hours later (day one in the place recognition experiment) the animal was introduced to two identical objects in the arena for 5 min. The second day (24 hours later), one of the objects was moved to the opposite side of the arena and the animals were able to explore the new setting for 5 min. Four arenas were used simultaneously and recorded using a CCD camera and the time spent at each object was recorded and analyzed. The arena consisted of Plexiglass covered with a light plastic material that was cleaned with a mixture of ethanol and water between the trials (as described in the OF section). Visits to the objects were defined as exploration of the animal with a nose distance of two cm or less from the object.

Statistical analysis

For statistical analysis, three different tests were used, depending on the experimental setup and if the population was considered to be normally distributed. All analyses were performed using SPSS 17.0 and SPSS 19.0 (SPSS, Chicago, IL, USA) and statistical significance was considered if $p<0.05$. In papers I, II and IV, a two-way ANOVA was used. In the two-way ANOVA there are two independent variables (i.e. sex and treatment in papers I, II and IV). When using ANOVA, normal distribution is assumed in the population. The main effects involve the independent variables (sex and treatment). The

two-way ANOVA calculates if there is an interaction between the main factors, i.e. assessing the effect that one factor has on the other factor.

Student´s t-test was used in paper III, where normal distribution was assumed in the population.

Generalized Estimating Equations (GEE), a non-parametric test, was used for analysis of the IntelliCage and OF-data in paper I. These experiments generated al large set of data over an extended period of time. Since normal distribution could not be assumed in the populations, repeated measurement ANOVA was not applicable. The GEE is a statistical test that estimates average responses of the population for the different parameters that are investigated in the IntelliCage paradigm. A poisson model was used for integer values (i.e. nosepokes and visits), a binominal model for calculations of ratios and a normal log model was used for the residual values. Interaction between the main effects was analyzed (i.e. if treatment groups developed differently over time). If the interaction did not reach significance, the main factors (time and treatment) were analyzed separately.

RESULTS AND DISCUSSION

Irradiation acutely depresses proliferation in the dentate gyrus and corpus callosum and increases inflammation and cell death in the young brain

In this thesis we have investigated the acute IR-induced effects in both the DG and the CC after a single dose of 8 Gy to the young mouse brain. We have seen a decrease in proliferating cells in both the DG and the CC 6 h after IR as judged by the quantification of phospho-histone H3 positive cells (PHH3). Histone H3 is one of the core proteins that DNA can wrap around to form nucleosomes in the cell nucleus. In the late G2 and M phases in the cell cycle, phosphorylation of the serine 10 residue of histone 3 takes place, which can then be recognized by an antibody, hence only proliferating cells undergoing mitosis are recognized (Hendzel et al., 1997, Mandyam et al., 2007). We observed a decrease of almost 50% of the proliferating cells in the SGZ of the DG in irradiated males and females at this early time point. A decrease in proliferative cells was also seen in the CC of young animals, but this decrease was not as pronounced as the reduced proliferation in the hippocampus.

Besides the discovery of reduced proliferation, we observed increased cell death, as judged by the active caspase-3 activity 6 h after IR. Caspases are a family of proteases that are essential for cells undergoing apoptosis (programmed cell death). Caspases exist as inactive pro-caspases, but during activation they are cleaved in a cascade of events leading to cell death. In this thesis we studied IR-induced cell death in both whole brain tissue as well as in the CC separately, and there was an increase in cell death at both levels. Apoptosis in the CC has previously been reported to peak 6 h after IR, at least in adult female rats

(Bellinzona et al., 1996). It is also known that cell death occurs in the young hippocampus at this time point after IR (Fukuda et al., 2004, Fukuda et al., 2005b, Kalm et al., 2009b).

The cells most vulnerable to irradiation in the adult brain have been reported to be oligodendrocytes, endothelial cells and neural precursors (Siu et al., 2012), which partly explains the altered proliferation and cell death observed in the DG and CC seen in our study. We did not observe any differences in proliferation and IR-induced cell death between males and females at this point though, neither in the SGZ of the DG, nor in the CC. The IR procedure in our study took place at an age of P14, i.e. the animals had not yet reached sexual maturity, which could be one explanation.

There was no difference in the volumes of the DG or the CC in young animals exposed 6 h after IR. This was expected since the time of evaluation after IR was too short to affect the volumes. IR is known to retard growth of the developing brain (Fukuda et al., 2004, Fukuda et al., 2005b, Roughton et al., 2012).

Stem and progenitor cells are sensitive to IR due to their high proliferative capacity, and neurogenic areas such as the SGZ of the hippocampus are therefore particularly vulnerable areas in the brain. Since the hippocampus is involved in learning and memory, loss of cells in this area is thought to be of importance in the cognitive decline seen after IR. Since the developing brain harbors more stem and progenitor cells than the adult brain it is more radiosensitive, and pediatric patients who receive cranial and/or craniospinal IR usually suffer from cognitive impairments as they grow older (Lannering et al., 1990, Gondi et al., 2010).

Both irradiation and pretreatment with LPS trigger inflammation in the young brain

IR induces inflammation in both the human and rodent brain (Monje et al., 2002, Monje et al., 2007, Kalm et al., 2009a, Kalm et al., 2009b), but inflammation can also be triggered by the cell wall component LPS from Gram-negative bacteria, so administration of LPS can mimic some of the features of having a systemic infection. In papers III and IV we wanted to combine these treatments to see if the presence of a systemic inflammation at the time of IR would affect the outcome, both acutely and long-term. Both IR and LPS can activate neuroinflammation. IR to the developing brain causes a transient up-regulation of cytokines and chemokines, such as CCL2 and IL-1β, activates microglia and induces oxidative stress and cell death (Fukuda et al., 2005b, Kalm et al., 2009a, Kalm et al., 2009b). Administration of LPS, on the other hand, induces neuroinflammation by causing reactive microgliosis which contributes to neuronal dysfunction through the release of inflammatory factors such as IL-6, TNF-α IL-1β, nitric oxide and ROS (Turrin et al., 2001, Russo et al., 2011).

Interestingly, we observed differences between males and females in the acute phase after IR if the animals were pretreated with LPS 24 h before IR, where young females displayed a higher cell death (caspase) activity in the whole brain compared to young male mice. Moreover, we also observed that females pretreated with LPS generally had a higher levels of pro-inflammatory cytokines and chemokines compared to males. However, animals treated with IR alone also displayed an inflammatory response and increased levels of cell death, but no differences between the sexes was observed in the absence of LPS. Our results from papers III and IV show that an acute inflammation is caused by either IR or LPS but that the combined treatments do not enhance the negative effects in the acute phase. It is important to keep in mind that we only investigated one time point in the acute phase after IR which just gives

information about the cytokine/chemokine/cell death levels at that particular moment, possible changes in the temporal profile in the acute phase were not investigated. To better understand the inflammatory response induced by the combination of LPS and IR, this should be studied over a broader time period and include more time points, but papers III and IV give a snapshot of what is going on in the DG when exposed to inflammation.

Proliferation and neurogenesis in the dentate gyrus are hampered by irradiation, more so in female mice

Cognitive impairments, such as decreased verbal memory, spatial memory, attention and the capacity of novel problem-solving, are seen after cranial irradiation (Greene-Schloesser and Robbins, 2012). In paper I we investigated the long-term IR-induced effects on proliferation, survival and neurogenesis in the hippocampal DG. Proliferating cells are vulnerable to IR, particularly when the DG is still growing. IR to the young brain induces loss of proliferative neural stem and progenitor cells, which in turn hampers growth and the end result is a smaller DG volume after early exposure to IR. The DG does not shrink by the treatment of IR, but rather the growth is retarded. When we irradiate a brain which is still growing, we "hit a moving target" which enhances the negative effects seen in the young brain, and the younger the patient at the time of treatment, the greater the cognitive deficits (Packer et al., 1987, Fukuda et al., 2005b, Sarkissian, 2005, Hoffman and Yock, 2009).

In our study we observed that the proliferation rates in the SGZ of the DG were decreased four months after IR in both sexes. In the animals not exposed to IR, we noticed that females had a higher level of proliferation compared to males, but the relative loss of proliferative cells induced by IR did not differ between males and females. When we analyzed the GCL volume at this late time point,

we observed that non-irradiated female mice had a larger GCL volume compared to males. Both sexes showed a growth retardation induced by IR, but the relative growth retardation was more pronounced in female compared to male mice exposed to IR at a young age. In paper I we further investigated hippocampal neurogenesis and cell survival in the adult mouse brain. The survival of cells in the DG was analyzed by bromodeoxyuridine (BrdU) incorporation. BrdU was administered 3 months after IR and quantified 4 weeks later. As mentioned previously, BrdU is a synthetic thymidine analogue which incorporates into the DNA during replication and is commonly used to detect proliferating and surviving cells. BrdU differs from thymidine in that it holds a bromide group instead of the methyl group held by thymidine. During replication, BrdU can be passed on to daughter cells and be detected by antibodies and thereby the proliferative state and survival of cells from the time of injection can be investigated. The administration of BrdU at high doses may cause cytotoxic effects (Cavanagh et al., 2011). This is due to the larger size of BrdU compared to the methyl group in thymidine which increase the risk of mutations. One single injection of BrdU labels all cells in the S phase at the time of the injection. Repeated exposures of BrdU label the additional cells entering the S phase until a plateau is reached (Gage et al., 2008). For that reason we repeated the injections with BrdU. Further, BrdU can be detected immunologically for a long time after exposure depending on the proliferation rate of the cell. Every time the cell proliferates, the signal in the nucleus is diluted (Ward et al., 1991, Cooper-Kuhn and Kuhn, 2002). In our study we observed that non-irradiated females had a higher proportion of BrdU labeled cells compared to males. We further observed that IR induced a reduction of BrdU incorporation in both males and females, but this reduction was more pronounced in irradiated females.

Neurogenesis was measured as the number of doublecortin- (DCX-) positive cells in the SGZ of the DG. DCX is a microtubule-associated protein which is transiently expressed in neural progenitor cells and immature neurons and commonly used to measure neurogenesis (Gage et al., 2008). In paper I we found that hippocampal neurogenesis was highly affected by IR in both males and females. Just like the observation mentioned above, where non-irradiated females showed a higher proportion of BrdU-labeled cells and thereby a greater cytogenic capacity, we discovered the same pattern in neurogenesis, where female mice not exposed to IR had a higher number of immature neurons in the SGZ of the DG compared to males. Higher neurogenesis in females has been reported previously by Clark *et al*. (2008) who also demonstrated that running improved neurogenesis and females were more active compared to males and ran longer distances (Clark et al., 2008). Further, we observed that both sexes displayed IR-induced impairment in neurogenesis, but the reduction in neurogenic capacity was more pronounced in the female mouse brains exposed to IR.

It is known that young girls suffer more severe late effects compared to young boys when treated with CRT (Mulhern et al., 2004, Butler and Mulhern, 2005, Sarkissian, 2005, Lahteenmaki et al., 2007), but the underlying reason for these differences is not yet fully understood. Estradiol has been shown to have neuroprotective properties and affect hippocampal neurogenesis (Hurn and Macrae, 2000, Ormerod et al., 2003), but in this study we irradiated the animals at P14, i.e. before they reached sexual maturity, to prevent the estrous cycle from being a confounding factor. We only observed higher proliferation and neurogenesis in the hippocampus in adult animals, which possibly demonstrates that the normal decline in hippocampal neurogenesis occurs at a slower rate in females compared to males, but if this is dependent on the levels of sex hormones, such as estrogen, needs further investigation. We measured the blood

levels of estrogen in adult animals but did not find any IR-induced changes, so since the reduction in neurogenesis was greater in females after IR, at least for the dose used in our study, estrogen did not provide any protection against this type of cell death. As mentioned previously, we did not find any differences between the sexes in normal, baseline proliferation in young animals, where the levels were four times higher than in the adult animals. This sex-dependent difference was only observed at the later stage, where IR reduced proliferation, BrdU incorporation and the number of immature neurons to virtually the same numbers in both sexes, so the relative decrease was higher in female mice subjected to IR at a young age.

White matter integrity in the corpus callosum is affected by irradiation, more so in female mice

Irradiation to the brain is associated with subsequent cognitive impairments, and the hippocampus was shown to be of great importance in this process (Roman and Sperduto, 1995). The decline in IQ in patients is mostly a result of the inability to learn at a rate that is appropriate for a child at the age, rather than from a loss of previously learned knowledge. One mechanism that is believed to contribute to the decline in IQ observed in pediatric survivors is the loss of white matter or the inability to develop white matter at an appropriate rate (Mulhern et al., 2004). In this thesis we irradiated the whole brain and hence not only the hippocampus is affected by the treatment. The functional outcome observed after IR is not only dependent on injury to the hippocampus but also involves other cell types and areas, for example impaired myelination, which has been reported after IR to the young rodent brain (Fukuda et al., 2005b). IR has been shown to inhibit hippocampal neurogenesis, which might at least partly explain the cognitive impairments seen after radiotherapy, but today it is hypothesized

that the cognitive decline seen after CRT involves dynamic interactions between many different cell types in the brain including, astrocytes, microglia, neurons, endothelial cells and oligodendrocytes (Greene-Schloesser and Robbins, 2012). In paper II we investigated the effect on the corpus callosum (CC) after IR to the young mouse brain. As mentioned before, CC is the largest white matter structure in the brain and continues to grow until adulthood (Lenroot and Giedd, 2006). In our study we observed that the volume of the CC was affected by IR to the young mouse brain in both sexes, and moreover we observed that this structure was affected to a greater extent in females. The growth retardation observed in the CC was reflected in the total brain weight, where female mice were shown to be more radiosensitive, as judged by a greater lack of growth after a single dose of 8 Gy to the young brain. We did not observe any IR-induced difference in myelin basic protein (MBP) levels at this time point in either sex. Demyelination has been shown to occur after IR, but takes time to develop and another study reported that no loss of MBP was seen up to 9 months after IR to the female rat brain, but a drastic demyelination occurred at 15 months after a dose of 25 Gy (Panagiotakos et al., 2007). In our study we only used a shorter, four months, follow-up after IR, and a lower dose, which might explain the lack of demyelination. Even though we did not observe demyelination, as judged by the amount of MBP, we did see IR-induced loss of cytogenic capacity (BrdU incorporation) and oligodendrocytes (Olig2) in the CC at the later time point. Both the BrdU and Olig2 counts displayed a greater IR-induced reduction in the female CC. The loss of BrdU incorporation and pre-oligodendrocytes seem to be the main victims of the IR-induced injury to the mouse CC, resulting in fewer cells developing into mature oligodendrocytes. As mentioned before, oligodendrocytes are sensitive to oxidative stress (Bradl and Lassmann, 2010) and IR causes oxidative stress (Limoli et al., 2004, Spitz et al., 2004, Manda et al., 2008), so this might, at least partly, be one of the explanations to the loss of Olig2-positive cells observed in this study. In

addition to the observation that females were more susceptible to IR, as judged by the Olig2 counts and BrdU incorporation, non-irradiated females displayed a higher level of BrdU incorporation compared to male mice. This is consistent of what we observed in paper I, in the hippocampal dentate gyrus. In the DG, non-IR females displayed a higher level of cytogenic capacity but the IR-induced loss of BrdU incorporation virtually reached the same levels as in IR males. Since both hippocampal neurogenesis and white matter integrity are believed to be important for cognitive function (Palmer et al., 2012, Eichenbaum, 2013), the combined results from papers I and II, where females seem to be more radiosensitive, as judged by the greater reduction in neurogenesis and impaired cytogenic capacity in the white matter of the CC, is consistent with clinical studies where girls have been shown to be more susceptible to CRT (Butler and Mulhern, 2005, Sarkissian, 2005, Lahteenmaki et al., 2007, Raber, 2010).

It has previously been proposed that white matter in the brain is more radiosensitive because it harbors fewer capillaries compared to gray matter (McDonald and Hayes, 1967) and in paper II we wanted to study the vascular structure in the CC. We observed that irradiated animals had fewer vessels and smaller total vessel surface area compared to control animals, which was also the case for the mouse hippocampus (Bostrom et al., 2013). We did not see any differences in the vessel density after IR, though. This might be explained by an adjustment of the vasculature to the growth retardation observed in the CC, which speaks against the theory that the white matter would be more radiosensitive than gray matter. However, in paper II we show that IR to the young brain is more affected in the white matter of the female CC compared to males, which is consistent with the observations in paper I and from clinical studies where girls are more prone to suffer from more severe side effects caused by CRT.

Irradiation and hypothalamic-pituitary-related hormones

CRT has been shown to cause endocrine deficiencies in children (Sklar and Constine, 1995, Duffner, 2004) and since we irradiated the whole brain we wanted to investigate possible loss of function in the hypothalamic-pituitary axis. In paper I we therefore investigated hypothalamic-pituitary-related axis hormones in serum, expected to be affected by IR. The analyzed hormones included Insulin-like growth factor 1 (IGF-1), which plays an important role in childhood growth (Veldhuis et al., 2006); IGF-1 is in turn regulated by growth hormone (GH), and GH production is reduced in most children treated with high doses of CRT, the thyroid hormones free T3 (FT3) and free T4 (FT4), which are important for the regulation of metabolism. We did not observe any differences in these hormones after IR to the young brain, at least not four months after the treatment. Further, since we wanted to investigate sex-dependent differences in the sensitivity to IR, we analyzed the sex hormones estrogen and testosterone. As expected, we did find a sex difference in these hormones, where males showed higher levels of testosterone in both control and IR animals than females and females showed higher levels of estrogen in both control and IR animals than males, but no IR-induced differences were observed. As described earlier the animals were irradiated at P14, hence before sexual maturity was reached, which might be one explanation to the observations in paper I.

Inflammation at the time of irradiation aggravates the injury in the dentate gyrus

In papers III and IV we wanted to investigate how an ongoing systemic inflammation, caused by LPS administration, would affect the outcome after IR to the young brain. In the study performed in paper III we only used male animals and evaluated the IR-induced injuries in the DG after 2 months. In paper

IV we extended the study to include both sexes, and we also performed behavior assessment and waited 4 months after IR to evaluate morphological long-term changes.

It has been shown that inflammation caused by IR has major effects on hippocampal neurogenesis (Monje et al., 2002), but our aim with the studies was to explore how a pre-existing inflammation would affect the IR-induced injury in the young brain. In paper III, we evaluated three different doses of LPS in irradiated and non-irradiated animals to obtain the optimal dose for the induction of an immune response (0.1, 0.3 and 0.9 mg LPS/kg) (Roughton et al., 2013) and tissue cytokines were analyzed 6 h after IR. The 0.9 mg/kg dose was excluded since the general condition of the animals was negatively affected and we ended up with the dose of 0.3 mg LPS/kg which generated a robust cytokine response and was therefore further investigated in papers III and IV.

Iba-1 was used as a marker for microglia in both papers III and IV and evaluated 2 months and 4 months after IR, respectively. In paper III we evaluated if an LPS boost would affect microglia proliferation as judged by the quantification of BrdU and Iba-1 double-labeled cells, but we did not observe any effect on newly born microglia after LPS pretreatment. In paper III, where only male mice were used, there was no difference in the density of microglia in animals pretreated with LPS or vehicle, neither in IR nor in control animals. In paper IV, on the other hand, where both sexes were studied, we did find a difference in microglia density in the DG, such that irradiated females displayed a greater density in both vehicle- and LPS-treated animals.

LPS has previously been shown to be neuroprotective leading to a reduced injury and also a reduced neuroinflammation if administered at low doses as preconditioning before ischemic or traumatic brain injury (Mallard and Hagberg, 2007, Stenzel-Poore et al., 2007, Lin et al., 2009, Longhi et al., 2011). However, in our study LPS did not have any protective effect, but rather

aggravated the IR-induced injury in the DG. As expected, we observed growth retardation in the DG (including the subregions GCL, hilus and ML) in the IR animals, but the retardation was greater if the animals had been pretreated with LPS compared to vehicle-treated animals (paper III). In the control (non-IR) mice there was no difference in the DG volumes in vehicle- or LPS-treated animals. When the volume of the DG was investigated at the later time point (paper IV), we did not observe any IR-induced difference between animals exposed to LPS or vehicle, indicating a transient effect of LPS in the DG area. BrdU labeling and quantification four weeks later was investigated in papers III and IV and revealed a reduction of surviving cells after IR in the DG in animals pretreated with LPS. Loss of proliferation and survival (BrdU incorporation) in the DG was observed in both sexes, but in paper IV we observed that this loss was more pronounced in vehicle-treated females compared to males. In the LPS-pretreated animals we did not see any difference between sexes. As in paper I, BrdU quantification was used to measure the total cytogenic capacity and DCX was used to measure ongoing neurogenesis, revealing a greater reduction of immature neurons in the GCL in LPS-pretreated animals at both time points (2 months after IR in paper III, Fig 11. and 4 months after IR in paper IV).

The reduced hippocampal neurogenesis caused by IR was aggravated by LPS in both sexes and one explanation to this could be that the inflammation induced by LPS caused a neuroinflammation that sensitized the brain (Kalm et al., 2009a, Kalm et al., 2009b, Russo et al., 2011). It still remains to be shown if lower doses or the administration of LPS at other time points would have different effects after IR, maybe even protective effects, but in our studies we did not see any protection when LPS was administered 24 hours before IR and then evaluated at 2 and 4 months after IR in either sex.

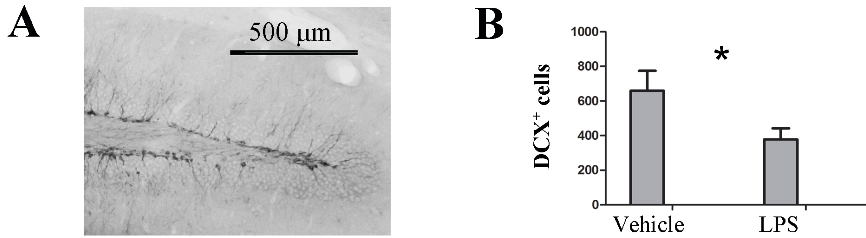

Fig 11. A shows a representative microphotograph of DCX-positive cells in the DG. **B** shows the quantification of DCX-positive cells in vehicle- versus LPS-treated animals exposed to IR. *Figure modified from paper III* (Roughton et al., 2013).

Females are more affected by irradiation-induced injury as judged by behavioral assessment

It is important to investigate any functional (behavioral) effects after IR and in papers I and IV we investigated both cognitive and motor behavior after IR.

Learning and memory are more affected in female mice subjected to IR (IntelliCage)

The IntelliCage platform has previously been shown to be a suitable method to investigate IR-induced impairments in learning and memory (Karlsson et al., 2011, Kalm et al., 2013). IntelliCage has been shown to be useful to measure place and reversal learning, which at least partly can be claimed to be hippocampal-dependent (Colgin et al., 2008). The parameters used in the test to measure learning was the *incorrect visit ratio* (the ratio of visits to the incorrect corners where the mouse could not drink divided by the total number of corner visits), the *incorrect nose poke ratio* (the ratio of nose-pokes performed in the incorrect corners divided by the total number of nose pokes in all the corners) and *nose pokes per incorrect visit* (the attempts to open the door in an incorrect corner where the mouse cannot drink) (Roughton et al., 2012). The *incorrect*

visit ratio showed that there was no difference between IR or control males, but both groups improved over time, which was interpreted as learning. However, IR females did not improve over time in the first corner period compared to control females. The same was seen for the parameter *nose poke ratio*, where no differences between treatment groups were observed in males but IR females displayed an impaired learning in the first corner period. For the parameter *nose poke per incorrect visit*, we observed an IR-induced effect in the first and second corner periods in irradiated males only. Both IR and control males improved over time, but IR males made more mistakes (more attempts to open the door in an incorrect corner) compared to control males. This was the only parameter where males performed worse after IR than females and persisted in performing nose pokes in the incorrect corners. Overall, the ability to learn where to find water when this was new to the animals (corner period 1) was only impaired in IR females, but not in males, while reversal learning (the ability to learn where the new corner is and not persisting trying to drink from previously correct corner, i.e. corner period 2 and 3) was not affected after IR in either sex.

Exploratory behavior was also investigated in IntelliCage by measuring how long it took for the animals to visit any corner during the introduction period. This revealed that males took almost four times longer to visit any corner compared to females, but no difference after IR was observed. This suggests that females have a stronger exploratory behavior compared to male mice.

Females subjected to IR display a more anxious behavior compared to irradiated males (open field and elevated plus maze)

The open field (OF) paradigm was used in both papers I and IV to measure anxiety, locomotor and exploratory behavior. The total distance moved in the OF arena showed that IR animals were more active and moved longer distances compared to controls. To measure anxiety we investigated the distance moved in

the center of the arena divided with the total distance moved in the whole arena. This revealed that females exposed to IR spent less time in the center of the arena compared to controls, an effect that was not observed in males, indicating that IR females were more prone to anxious behavior compared to irradiated males.

In paper IV we measured the distance to the walls in the OF paradigm after the administration of LPS. The parameter distance to walls is also a measurement of anxious behavior. If the animals are too anxious to get into the center they will stay close to the walls. The animals exposed to LPS did not show any IR-induced alterations in behavior, but in the vehicle-treated animals we observed an anxious behavior after IR in both male and female mice but the increase in anxiety was higher in female mice. In paper IV we also used the elevated plus maze (EPM) to investigate IR-induced anxiety in both sexes after administration of LPS. The parameter used in the EPM was time per visit on the open arm, which showed the same pattern as the OF. In the LPS-treated animals no IR-induced anxiety was observed, but in the vehicle-treated groups we detected an increased anxiety (hence shorter time per visit on the open arm), with a greater increase in the IR female mice.

Using the IntelliCage platform, the EPM and the OF paradigms, we have shown that IR has negative behavioral effects on the developing brain. Moreover, the negative outcomes seen after IR in our mouse model were shown to be more pronounced in females, both as judged by the impaired learning abilities measured in the IntelliCage and increased anxious behavior as judged by the OF and EPM (Roughton et al., 2012). Negative outcomes after IR in females have also been shown in adult animals where contextual fear conditioning was used (Villasana et al., 2010). Our results are consistent with clinical studies showing that young girls are more sensitive to CRT compared to young boys (Mulhern et al., 2004, Lahteenmaki et al., 2007) The reason why girls are more susceptible to

CRT is not known and needs further investigation, and our mouse model was demonstrated to be useful in the search for the underlying mechanisms.

It is important to consider that in our study we irradiated the whole brain with one single dose of 8 Gy and in the clinical situation the dose is usually fractionated to reduce the negative side effects on the normal tissue (Sarkissian, 2005), giving the healthy cells a chance to recover and repair the IR-induced DNA damage. Due to experimental setup with the IR source being located at the hospital, it would have been complicated to perform fractionated IR in our studies, but it is an important difference to bear in mind. Efforts were made to estimate what a single dose of 8 Gy is equivalent to, compared with fractionated IR. As mentioned above, an absorbed dose of 8 Gy is equivalent with 18 Gy delivered in 2-Gy fractions according to the linear quadratic formula and an alpha/beta ratio of 3 for late effects in the normal brain tissue (Fowler, 1989). A CRT dose of 12-18 Gy is commonly delivered to children with CNS involvement in acute lymphatic leukemia. The dose used for treatment of medulloblastoma, for example, is much higher, up to 55 Gy or more to the tumor bed. So 8 Gy as a single fraction is equivalent to a moderate 18 Gy dose in the clinical situation.

CONCLUDING REMARKS

This thesis concludes that IR to the juvenile brain has negative effects on hippocampal neurogenesis, white matter integrity and behavior, more so in female mice. It further concludes that LPS-induced inflammation prior to IR aggravates the injury to hippocampal neurogenesis

Responses to given aims:

I. We have shown that IR to the young brain causes a persistent decrease in hippocampal neurogenesis, negatively affects learning and memory, as judged by the IntelliCage experiments, and induces an anxious behavior, more so in female mice.

II. We have demonstrated that IR to the young brain affects white matter development in of the corpus callosum more in females than in males, as judged by increased cell death, hampered growth, as well as decreased proliferation and oligodendrogenesis.

III. We have shown that if a systemic inflammation induced by LPS 24 h prior to IR of the young brain, the long-term IR-induced injury in the DG is aggravated, as judged by hampered growth and decreased hippocampal neurogenesis in the male mouse brain.

IV. We have demonstrated that LPS administered 24 h prior to IR of the juvenile brain aggravates hippocampal neurogenesis in both males and females. We further demonstrated that females are more susceptible to IR-induced injury, as judged by behavior assessment, regardless of whether inflammation is present or not.

CLINICAL PERSPECIVE AND FUTURE DIRECTIONS

Radiotherapy is an effective tool in cancer treatment and since the treatment strategies have improved over the last decades, the number of long-term survivors after childhood cancer has increased. With the increasing number of survivors, more attention is paid to the negative side effects seen after radiotherapy. Since pediatric patients undergoing cancer treatment are more susceptible to the negative side effects of radiotherapy, it is important to try to limit the damage to areas most affected by irradiation. Neural stem and progenitor cells, with high proliferation rates, are radiosensitive and the hippocampal DG and the SVZ, where neurogenesis occurs throughout life, are therefore areas to be protected, if possible, during the treatment. Since girls treated with radiotherapy are more susceptible to IR-induced injury to the normal brain tissue and suffer from more severe late-occurring side effects compared to boys, it may be important to take this into consideration during the planning of cancer treatment.

Animal models are useful when investigating the effects of IR to the brain and in this thesis we have shown that IR impairs learning and memory, as judged by IntelliCage experiments. The impaired learning and memory observed in the behavioral tests correlated with reduced hippocampal neurogenesis and impaired white matter growth of the CC, more so in female mice, which is in accordance with clinical observations where girls have been shown to be more radiosensitive. Future studies are needed to investigate the underlying sex-dependent mechanisms. Our mouse model was demonstrated to be useful in this search. We did not observe any IR-induced differences in the sex hormones estrogen or testosterone in serum.

Since our studies have shown that a preexisting inflammation at the time of IR aggravates the injury to normal brain tissue, it may be of great importance to perform a cytokine profile before the treatment to make sure that a systemic inflammation, for example caused by a sub-clinical infection, is not present at the time of IR. The cytokine profile could easily be analyzed in a blood sample, by evaluating the levels of circulating cytokines/chemokines.

Since females had a higher density of Iba-1 positive cells and thereby a greater inflammatory potential in the DG after IR, it should to be further evaluated if the IR-induced inflammation is one of the reasons for the greater loss in neurogenesis seen in females subjected to IR. There are at least three different phenotypes of microglia, the “resting” or surveillance phenotype, the toxic, pro-inflammatory (M1) phenotype and the protective, anti-inflammatory (M2) phenotype, which have different functions in the brain. It would be of great interest to phenotype microglia at different ages and after IR. If the loss of M2 cells occurs at the same time as an increase of the M1 type occurs needs to be elucidated. If the response in microglia is the underlying reason why females show a more pronounced injury following IR, it is possible to explore prophylactic gender-specific treatments affecting microglia.

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