Does C3-deficiency alter the amyloid precursor protein expression and thalamic structure following cranial radiotherapy to the developing brain?

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Citation

"The pathologies that damage the human brain are rarely restricted to single anatomical structures. Stroke, trauma and particularly degenerative diseases do not respect functional anatomical boundaries. But there is much to be learned from observation of what occurs in humans whose brains are damaged by such insults" [1].

Populärvetenskaplig sammanfattning på svenska

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Kan immunbrist påverka uttrycket av APP och thalamusstrukturen efter strålbehandlingen mot den omogna hjärnan

Hjärntumörer är ovanliga hos barn men ändå utgör en tredjedel av all barncancer i västvärlden. Lyckligtvis kan mer än 70 % av dessa hjärntumörer botas med strålbehandling ofta i kombination med cellgiftbehandling och/eller operation. Strålbehandling är en effektiv metod vid behandling av barn med hjärntumörer, men ger förutom akuta även svåra kroniska biverkningar. Nedsatt kognitiv funktion är ett exempel på en sådan biverkan som kan försämra barnets livskvalité livet ut. De kroniska biverkningarna tros bero på den akuta inflammationen som stör hjärnans normala aktiviteter bl. a. minnets funktioner. Drabbade barn får då t.ex. inlärningssvårigheter.

En viktig del av immunförsvaret är det medfödda immunförsvaret som spelar roll i nervssystemets utveckling under både fosterutvecklingen och barndomen. Tidigare studier har visat att möss som saknar del av det medfödda immunförsvaret och som genomgår strålterapi haft bättre inlärningsförmåga jämfört med möss med bevarad komplett medfödd immunförsvar och som också genomgår samma strålterapi. Varför? Man har visat att strålterapin orsakar en inflammatorisk reaktion med massiv nervcelldöd. Vidare studier kunde man inte koppla förbättringen av inlärningsförmåga till limbiska systemet. Med detta projekt vill man undersöka om denna förbättring kan beror på någon del av mellanhjärnan istället.

Does C3-deficiency alter the amyloid precursor protein expression and thalamic structure following cranial radiotherapy to the developing brain?

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Abstract

Brain tumours in children preserve certain characteristics of the original stem cells that develop during foetal life. How do cancer cells acquire these characteristics is unknown. New research suggests that cell origin and its genetic alterations play a huge role in tumour development, tumour aggressiveness and prognosis. It is well known that more than 70 % of brain tumours in children can be cured with *cranial radio-therapy* (CRT) in combination with chemotherapy and/or surgery. Thereby, CRT is an effective cornerstone of the treatment of paediatric brain tumours but it also causes severe chronic adverse effects, such as neurocognitive decline. It is believed that CRT causes acute neuro-inflammation that making it difficult for the brain to heal and thereby disturbs the plasticity of the brain.

C3 is an influential component of the complement system. Several previous studies highlighted the importance of *C3* in both foetal and adult neurogenesis as it has an immune-dependent ability to protect/repair damaged cells and removal of damaged synapses in the brain. It has also been shown that mice lacking *C3* have a better cognitive outcome following *cranial radio-therapy* (CRT). The part of the brain called the ***thalamus*** is a collection of relay stations with extraordinary function between the senses in our body, and our reaction to these senses via the cortical and subcortical brain and it rich in ***APP***-cells. ***Amyloid precursor protein*** (APP) is an integral membrane protein expressed in many tissues, including the thalamus. APP has been shown to have an important role as a regulator of synapse formation and modulation of neural plasticity (memory and learning ability).

This project used wild type mice and mice lacking *C3* to highlight how CRT will affect the thalamus and its synaptic functions. Also, four groups were used in this study; control wild type, CR-treated wild type, control *C3-genmodified* and CR-treated *C3-genmodified* ($C3^{-/-}$) mice. Significant reduction in the volume of the thalamus was noted following CRT in both wild type and *C3-genmodified* mice. The expression of APP in the thalamus appears to be preserved in spite of CRT in both wild type and *C3-genmodified* mice. We conclude that modulating the immune system did not protect the thalamus from CRT-induced injury. In addition, *C3-defiicency* ($C3^{-/-}$) did not alter the APP expression in this region. The protection of memory function seen in *C3*-deficient ($C3^{-/-}$) mice after CRT must therefore depend on another region in the brain.

Introduction

Each year, nearly 70 children develop a serious brain tumour in Sweden. Most of these children survive at least five years after radiotherapy treatment [2,3]. However, many live with motor and intellectual disabilities [3]. Cranial radio-therapy (CRT) aims to cure children with brain tumours with as few side effects as possible and to get them an optimal quality of life afterwards. It has been shown that Cranial radio-therapy damages both tumour cells and normal cells in the brain (for example oligodendroprecursors, stem cells and microglia) [4-6]. This CTR-induced injury leads to different neurotoxic processes that are possible to measure in the cerebrospinal fluid (CSF) [7]. Beside the cell death that CTR generates, it has also been shown to negatively influence the chemical milieu of the brain. After CRT, inflammation is the unavoidable process to remove dead and injured cells. Furthermore, a chronic inflammatory response after CRT could affect the healing of the brain negatively. The challenge is to optimize radiotherapy, to make injury to normal cells as little as possible, and to provide an opportunity to restore as so much normal activity as possible in the brain, without compromising cancer treatment.

Paediatric brain tumours – PBT

Every year there are about 300 children in Sweden who get some type of paediatric cancer. It is often a severe and aggressive disease [2, 8, 9]. In general, children cancers have an embryonic origin such as neuroblastoma and glioblastoma. Therefore, it has been suggested that both environmental factors and hereditary factors tend to be involved in different degrees in the development of these rare tumours. However, the cause of the majority of paediatric CNS-tumours is still unknown. Leukaemia and lymphoma are the most common cancer forms in children representing around 42% of the cases, while brain, spinal tumours and solid tumours (blastomas and sarcoma) correspond to 25-31% each [2, 8, 10] **(**figure 1**)**. The incidence of childhood cancer and the disease panorama is age related. In the USA, many paediatric brain cancers are diagnosed in the first 4 years of life, the most common being astrocytoma [10, 11] **(**figure 2**)**. Different incidence rates have been reported from different parts of the world [8, 10, 12].

Figure 1

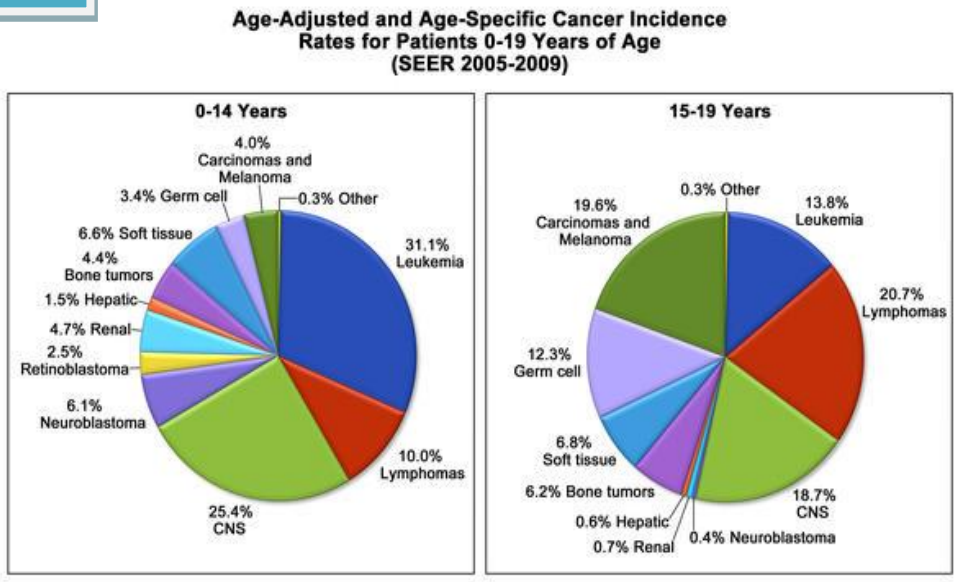

Figure 1 Incidence of cancer in childhood (up to the age of 19 years). CNS cancer represents 18.7-25.4 % and this is age-related [13].

Figure 2

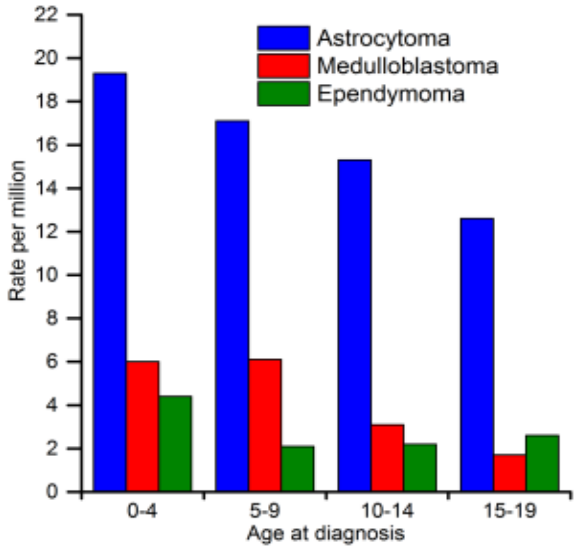

Figure 2 Age-related incidence rates for common CNS tumours, United States, 2006 to 2010. Astrocytoma is the most common brain cancer of childhood in USA [8].

Childhood brain cancer can anatomically and radiologically be subdivided in supra- and infratentorial. Supratentorial and infratentorial tumours occur overall in nearly equal frequency but supratentorial are more common in children < 2 years, whilst infratentorial are more common in the ages 4-10 [9, 12]. As for embryonic CNS tumours, they are distributed evenly between the different age groups. Most childhood cancers are primary tumours and their aetiology is mostly unclear, but they may be associated with certain diseases which have genetic aetiology; for example, neurofibromatosis and Li-Fraumeni Syndrome. As for the anatomy, in comparison to adult brain tumours, paediatric brain tumours are mostly localized in the posterior fossa and display diversity in their patho-histological features (cellular genesis and cellular biology). For this reason and to facilitate specific diagnosis, therapy and prognosis of brain tumours, the different types are subdivided into several grades – WHO 2007 classifications, where grade I is almost benign and grade 4 is the most aggressive types – anaplastic with poor prognosis [9, 12, 14]. Astrocytic tumours are the most common neuroepithelial tumours and mostly affect the cerebellum or hypothalamic/optic tracks [9, 12].

Brain cancer therapy

Recent studies have shown that the survival rates of children with paediatric brain tumours have arisen during the last few decades (figure 3) [2, 12]. The survival rate of CNS-tumours in children has increased dramatically since 1951. CRT, chemotherapy and surgery have played essential roles in the therapy of this group of tumours. For best reduction of long term morbidity and to achieve optimal survival, this multidisciplinary neuro-oncological approach plays an important role.

Figure 3

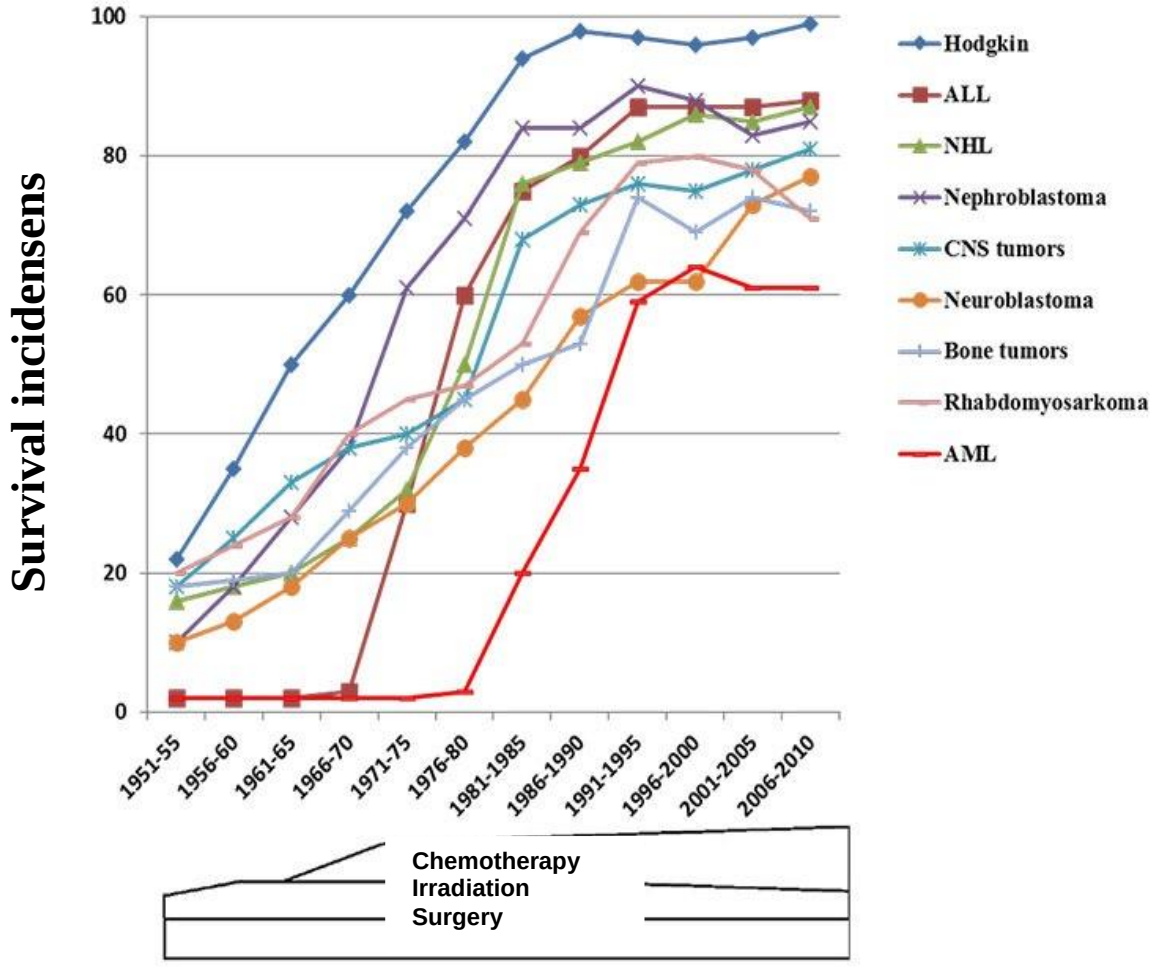

Figure 3 shows the different survival rates after paediatric cancer therapy between 1951 and 2010 [2]. CNS paediatric patients had a better chance of survival in the years 1951- 1965 when multiple modalities of therapy started being used. In 1965 chemotherapy took a more dramatically important place but CRT remained the first therapy modality.

Chemotherapy

Chemotherapy is a limited treatment method, thereby different types are given to cure CNS cancer or to limit and slow down cancer progression. Chemotherapy can also be used to relieve symptoms when a cure isn´t possible. In some cases, and under certain circumstances, it is recommended to use chemotherapy to reduce the risk of recurrence, known as adjuvant treatment. Such therapy is standard after some surgeries, such as breast- or colon cancer-surgeries. Many pharmacological studies first aim to adjust or make more effective choice of doses and types of chemotherapy so that side effects and damage to normal cells will be as minimal as possible, while the effect on the tumour becomes optimal [15-20]. Therefore, such chemotherapy treatment is different from the traditional therapy. Chemotherapy agents such as nitrosoureas, platin compounds and nitrogen solutions bind selectively to target tumour cells reacting with DNA, RNA and -molecular polypeptide's receptors thereby helping healthy cells to repair themselves and survive to a greater extent, and thereby the risk of side effects would be reduced. Side effects extend from renal to transient hepatotoxicity for nitrosoureas; nausea, vomiting and hypersensitivity reactions for platin compounds; and syndrome of inappropriate anti-diuretic hormone secretion (SIADH), infertility and myelosuppression for nitrogen solutions [15, 21].

Neurosurgery

Neurosurgery is an essential cornerstone in neuro-oncology but it is highly dependent on tumour localization in the brain and its accessibility for surgery. Another important factor to be considered is the risk of different forms of surgery related disabilities, such as paralysis, and this must be accepted before proceeding. The prognosis of medulloblastoma and glioma is correlated with the extent of resection and the response for adjuvant chemotherapy [15, 21, 22]. In some cases, surgery is not the best option and treatment with either CRT or chemotherapy or both is used instead, as the case for low grade tumours [22, 23]. The postoperative complications of brain tumour surgery are approximately 10 % while the mortality rate is thought to be in the range of 0-20 % [23-25]. Diverse factors influence the outcome of childhood brain tumour surgery such as the child's age, health condition, the type of tumour and its grade [24-26]. The major complication after neurosurgery include epilepsy, mutism, ataxia and speech abnormalities [25, 26] while endocrine complications include severe conditions as diabetes insipidus and hypocortisolism [25, 26].

Cranial Radio-therapy (CRT)

Cranial radio-therapy is an advanced cornerstone of the treatment of childhood brain tumours and it involves the use of ionizing radiation for cancer and some other conditions or diseases [4, 6]. Generally, it's known that radiation transforms water molecules to chemically reactive hydroxyl ions that damage DNA and cell nuclei repair systems of both normal and cancer cells. Radiation therapy is given to many kinds of cancer patients, some of which are cured by it [27-29]. Recently the therapeutic IR has been given through the use of particles; electrons (e^-) or photon energy, protons (p^+), and neutrons (n) [30, 31]. Electrons or electron beam is produced by a Linac; a linear accelerator, often used in the treatment of brain malignancies but used more frequently in craniospinal therapy [30, 32]. Photon energy or X-rays in range of 4-18 MV produced by a medical linear accelerator (Linac) is often used in radiotherapy of childhood brain tumours [31, 33]. Delivered CRT range of about 6 MV photons pass through the brain to induce the max effect on the tissue in question in order to achieve the best cure [7]. Radiation reactions in the body will certainly vary both in range and in intensity, and are often seen from 2-3 weeks after the first treatment session (in humans a total dose of 20-30 Gy delivered as 2 Gy per fraction) [4, 7, 34].

In recent years, attention has been drawn to the increased risk of secondary malignancies and the neurocognitive effects of CRT on the brain, especially in infants and small children. Lifelong late effects are neurocognitive decline such as decline in IQ, attention deficits, depression, anxiety, antisocialism, inattentiveness, slowness of executive function, reduced ability of judgment and insight and somnolence (after partial CRT) [35-37]. These can be correlated to the histopathologic changes that affect both pathological and normal tissue such as demyelination and necrosis of normal cells.

Another common adverse effect of cranial radio-therapy for brain tumours is the loss of one or several hormones. The brain of prepubertal children is more sensitive to CRT than adults, primarily because they are developing and growing. It can also lead to different degrees of hypopituitarism. Uncommon side effects induced by CRT are visual and hearing impairments that have a destructive influence on the quality of life of affected children [38, 39]. All the side effects are dose-dependent and the fractionation of CRT is an extremely important factor that seems have a reductive effect in almost all types of cranial radio-therapy [40].

The basic radiobiological effects of CRT-photons are DNA base damage, DNA-protein cross linking, and single and double stranded DNA breaks. CRT interacts with target cells in question directly or indirectly by free radicals and all these can be associated with a cascade reaction

leading to cancer cell death [5]. CRT kills the tumour effectively despite sub-lethal injures to normal tissues whilst using chemotherapy in combination takes care of micro-metastases [41-43]. The acute side effects of cranial radio-therapy that might be experienced by the children vary and some of them are treatable. Many precautions are taken to treat the acute side effects before they occur, for example medications such as anti-nausea and steroids (Table 1) [44, 45]. There is still a complexity in understanding late effects of radiation therapy. It can be affected by the type of tissue which is treated, fraction size, quantity and quality of the fractions, total dose or fractions dose, and total treatment course. Such limited CRT therapy leads to significant reduction of normal brain tissue injury. Others factors which affect and confound the complexity in the understanding of late effects is related to the patient's general condition such as the patients' age, comorbidities (especially genetic diseases such as neurofibromatosis type 2) and previous CRTs [46-49].

Table 1 Common acute and subacute side effect after CRT to paediatric brain tumours. Some can be totally cured.

Skin: Erythema, hyperpigmentation and hair loss.
CNS: Headache, vomiting and nausea.
Eye and Ear: Non-infectious conjunctivitis. Serous otitis media.
Parotid: Acute parotiditis.
Haematological: Leucopoenia and anaemia.

The complement system- C3 and C5 - role in the immune system

The complement system, which *C3* is part of, has been considered for a long time to be a humoral branch of the immune system, being responsible for recognizing, phagocytosis, cytolysis, and elimination of cellular debris and residues [50]. Additionally, it helps to sanitize the nerve system via microglia mediated mechanism that removes insulated and inaccurate synapses during brain development before adulthood [51-53]. There are many cell types in the brain which generate and express receptors responding to complement agents; microglia, astrocytes and others cells [54, 55]. All these cells are able to synthesise or express vast majority of the proteins (> 40 proteins) of the complement system [56, 57]. It is also well-known that the complement system is engaged in the innate immune system and has a central role to promote an inflammatory response both to reduce further damage to the tissue and to eliminate the pathogenicity of the injury mechanisms. When the C3 system is activated, it is converted to C3a by different enzymes that induces increased concentration of intracellular Ca^{+2} and even elevated expression of neutrophin in vivo. Thereafter, C3a manages the development of neural progenitor cells in the brain but other functions have also been shown. C3a is a small peptide with anaphylatoxic properties [58, 59].

Both C3a and C5a are generated via proteolytic cleavage of C3 and C5, respectively. Both C3a and C5a have potentially anaphylactic and chemoattractant profiles when they bind to specific receptors via a cascade of protein cleavage and activation [60-62]. Moreover, C3a is more selective and has anti-inflammatory effects while C5a is thought to have a wide range of pro-inflammatory effects **(**figure 4**)** [63-66]. This procedure ends with a collection of a membrane attack complex (MAC) that acts in variety of ways leading to either apoptosis of the cells or inducing an inflammation. This type of complement activation via C3a and C5a triggers the removal of damaged and unsuitable synapses, and encourages basal neurogenesis and ischemia induced neurogenesis [52, 67].

Figure 4

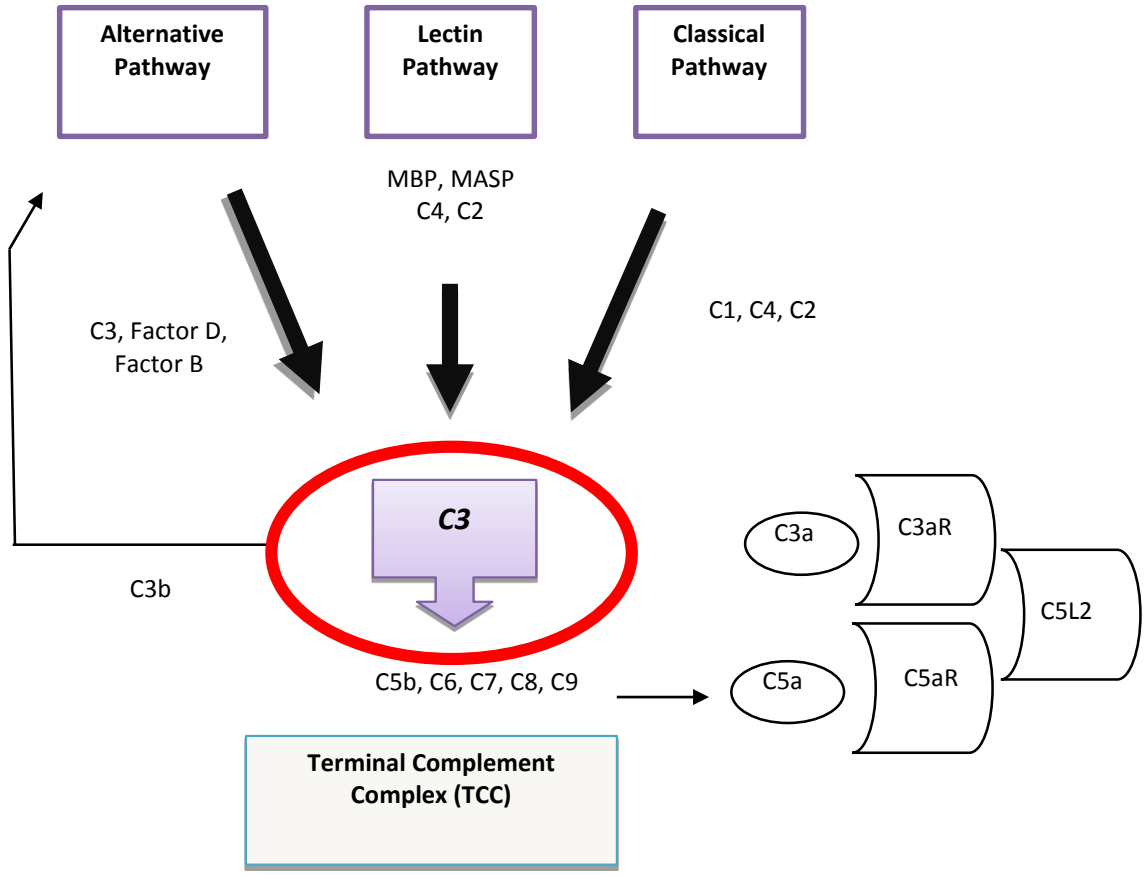

Figure 4 shows a representative outline of the three complement activation pathways depending on factor's stimulation. The alternative pathway activates C3 via activating surfaces, the lectin pathway activates C3 via sugar residues found on the pathogens and the classical pathway via antigen-antibody complex. ***C5L2*** is "A controversial receptor of the complement anaphylatoxin, *C5a*" [5, 7, 68, 69].

In recent years, it has been shown that the complement system is involved in tissue regeneration in, for example, limbs of amphibians and liver regeneration of mammalian but how, remains unclear [70, 71]. Adult mice lacking *C3* have been shown to be associated with retro-cognition ability after brain injury and even do not show signs as spontaneous epileptic activity in the hippocampus. The mechanism behind this is suggested to be related to the regulation of excitatory glutamatergic synapses and entails enhanced spatial learning [58, 72]. This reflects

that *C3* has a crucial role in synaptogenesis and neural plasticity. However, other studies have shown that in children who lack *C3* activity or when the expression of the complement is deranged or incompetent, this increases susceptibility to harmful conditions such as meningococcal meningitis [73, 74]. Therefore, much research has suggested that the complement system's polypeptides mainly provide protection against infections. Mice lacking *C3* system are incapable of generating *C3a* and *C5a*, and therefore are unable to have basal and ischemia induced neurogenesis [67]. Other studies have shown that *C3* affects the mount of synaptic loss during aging. Therefore $C3^{-/-}$ mice prove to be more protected and thereby have better hippocampal cognitive performance [72, 75].

When mice are treated with CRT, it can induce an inflammation which is believed to affect the decline and the differentiation of neural progenitor cells. Six hours after CRT, $C3^{-/-}$ mice had an increased inflammatory response such as increased levels of *IL-1β* and *IL-6* but this did not occur in control mice [76]. Furthermore, one study showed that C3a manages the development of neuronal progenitor cells in the brain [77].

Many CNS cells including microglia, astrocytes and nerve cells are able to produce the majority of the complement proteins. When brain tissue is injured, for example by CRT, it may activate the complement system, including *C3,* in the injured area [78]. Yet *C3* activation is associated with neuronal loss after cerebral injures and in the long term helps neural protection and tissue healing [79], neural differentiation and migration of neural progenitor cells [77], and migration of hematopoietic stem or progenitor cells [80]. One aim of this study is to investigate why *C3*-deficint mice have better learning and memory after treatment with CRT compared with control mice [76]. Marie Kalm et al. have shown that the hippocampus was not protected from CRT-induced injury long-term, measured as stem cell survival [4, 76]. Therefore another region, the thalamus, can be involved in the memory process and was therefore investigated in this study (figure 5).

Figure 5

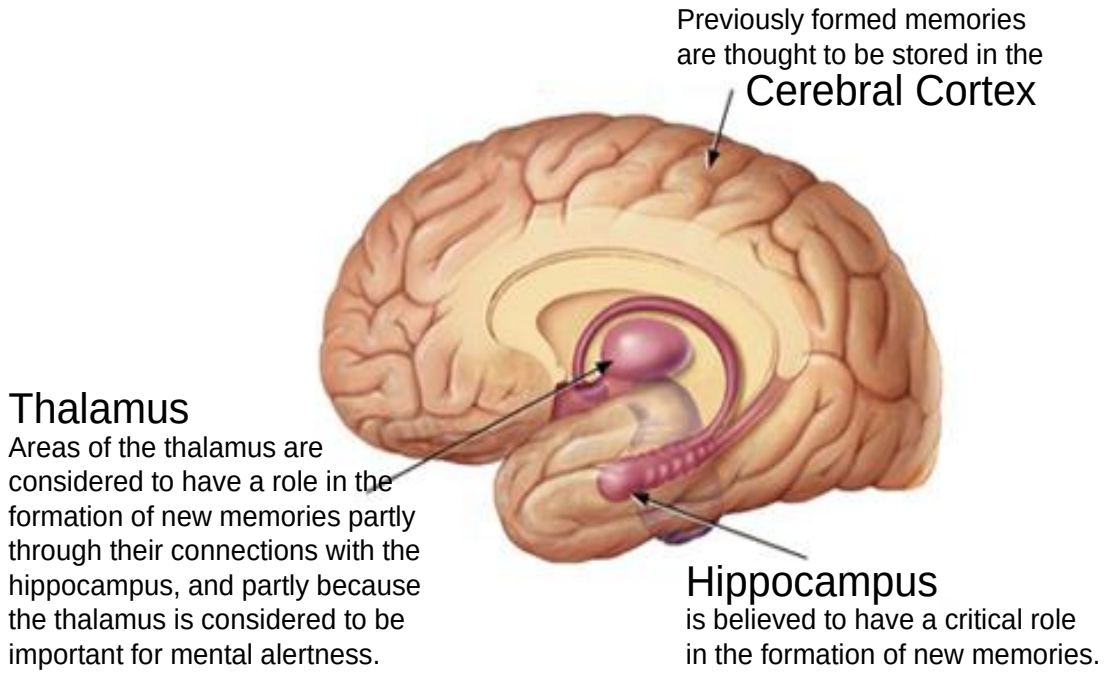

Figure 5 shows how the interaction between different parts of the brain involved in the memory process occurs. The fornix and mammillary bodies act as connections between the hippocampus and thalamus [81].

Thalamus

The brain is still an extremely fascinating part of the body despite all years of research using high technologic methods. It is still a mystery. The area of interest in this thesis is the diencephalon, and more exactly the thalamus.

The diencephalon includes the epithalamus, subthalamus, hypothalamus and thalamus. These are formed by the alar plates. The alar plates form three parts that have been described as neuromeres (prosomeres), which are similar to the rhombomeres of the hindbrain. Firstly, the rostral neuromere will form the prethalamus and hypothalamus. Secondly, the middle neuromere will form the thalamus and epithalamus. Lastly, the caudal neuromere will form the pretectum [82, 83].

The thalamus is connected to both the hippocampus and cerebral cortex and thereby affects cognitive function and memory **(**figure 5**)** and it consists of approximately 50-60 nuclei **(**figure 6**)** [84, 85]. It is considered to be the gateway to the cerebral cortex. This gateway has a critical role for spreading input to brain areas that influence cognitive functions, such as the neocortex and hippocampus. The human thalamus has two symmetrical parts affiliated with each other by connections intermedia and separated by the third ventricle. The nuclei in the ventral thalamus consist of the zona increta, the nucleus reticulare and nucleus pregeniculate. The nuclei in the dorsal thalamus consist of the allothalamic part even so called intra laminar nuclei and isothalamic part within subdivision parts. These nuclei have straight communications with the cerebral cortex except for nucleus reticularis and the allothalamic parts. The thalamus has many known but also unknown functions. Well-known functions are somatosensory, motor, consciousness, attention and neurocognitive (e. g. language and memory). Thalamic nuclei might have both contralateral and ipsilateral connections to the other parts of brain [86, 87].

Figure 6

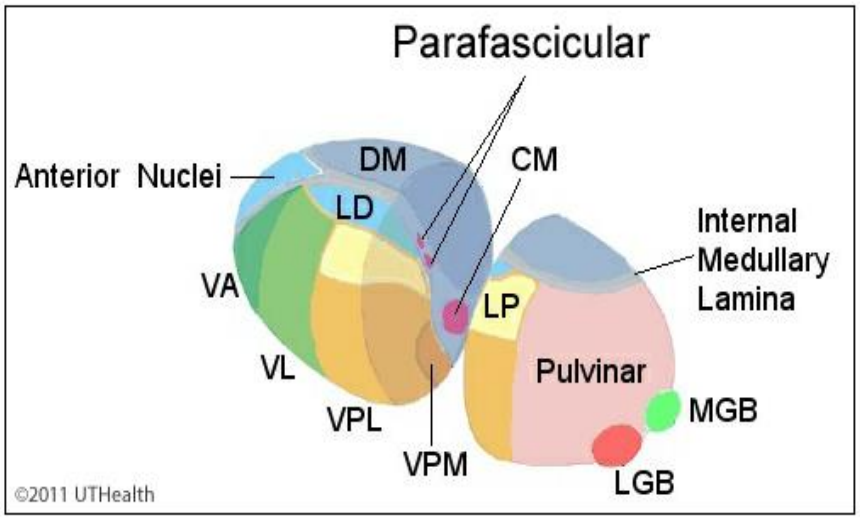

Figure 6 shows the most studied nuclei of the thalamus of human brain [88]. The nuclei have various connections to other parts of the brain. For example, the cortex, hippocampus and basal ganglia.

Amyloid Precursor Protein - APP

APP is substantially expressed normally in the brain and even in other parts of our body and it has different isoforms (APP_{695}, APP_{751} and APP_{770}) which are expressed in various proportions [89]. APP is suggested to have neurotrophic properties and acts as an neuroprotective actor where it enhance synaptogenesis, memory formation, survival, outgrowth of the neuronal-cells and neurite outgrowth in both the cortex and hippocampus. APP even has antagonistic effects as neurotoxic Aβ [90-92]. The study by Kalm et al. demonstrated that the levels of CSF-sAPPα/β were reduced, 3 and 12 months after prophylactic CRT in patients with SCLC (small cell lung cancer) [7]. Therefore, there is interest to explore the role of APP in the brain, more precisely in the thalamus. APP is an important transmembrane protein and is expressed in large amounts in the brain [93, 94]. APP may function as a receptor with a key role in many cellular mechanisms [95, 96]. Its intracellular part is small while its extracellular part is much larger in spite of the fact that this small domain acts as receptor [97]. APP is cleaved via sequential proteolytic procedures and various metabolites can be obtained. In general, the cleavage of APP peptides generates and releases an extra cellular form of soluble APPα/β peptides [98, 99]. Also, the rest of APP is a membrane bound C-terminal fragment which includes a transmembrane and a short cytoplasmic peptide part called C99/C83. In the Amyloidogenic pathway, the APP, cleaved by β- following by γ-secretase, results in the production of an extra cellular sAPPβ and Aβ and an intra cellular domain ACID. AICD has been suggested to operate as a transcriptional regulator. sAPPβ is less active but even suggested to play a role in normal neural development and neuroprotection of developed CNS. Aβ in small aggregates is believed to cause cell membrane disturbance [100, 101] and in large aggregates is associated with Alzheimer's disease [100, 102]. In other conditions such as Lewy body dementia, there is excess accumulation of neurotoxic substances in the CNS [100, 101, 103, 104].

In the Non-amyloidogenic pathway, APP can be cleaved by α-secretase following by γ-secretase to form sAPPα released in the extra cellular matrix and p3 and ACID released in the cytoplasmic space. sAPPα is essential for learning and memory, synaptic transmission, axonal transport, and cell proliferation. sAPPα has many fold potency in terms of neuroprotective properties such as spatial memory performance and neural survival than sAPPβ does [105]. Other types of APP isoforms cleaved by other caspases leads to the accumulation of various APP metabolites in the intracellular space [106, 107]. APP as a polypeptide is composed of nearly 700 amino acids and its gene transcription has been investigated in the human brain and it could also be found in PNS (peripheral nervous system) and skeletal muscles [108-110].

Other analyses of cDNA of APP disclosed that at least four kinds of mRNAs originated via alternate splicing of exons [97, 111] and the product is named after its number of amino acids. For example, APP 751 or APP 770 due the spicing of exons 7 and 8 but other splicing alternative was established [111-113]. Aβ is a short peptide that contains between 37 to 49 amino acids residues and its region is suggested to be localized in the EC- and transmembrane domains **(**figure **7**) [114].

Figure 7

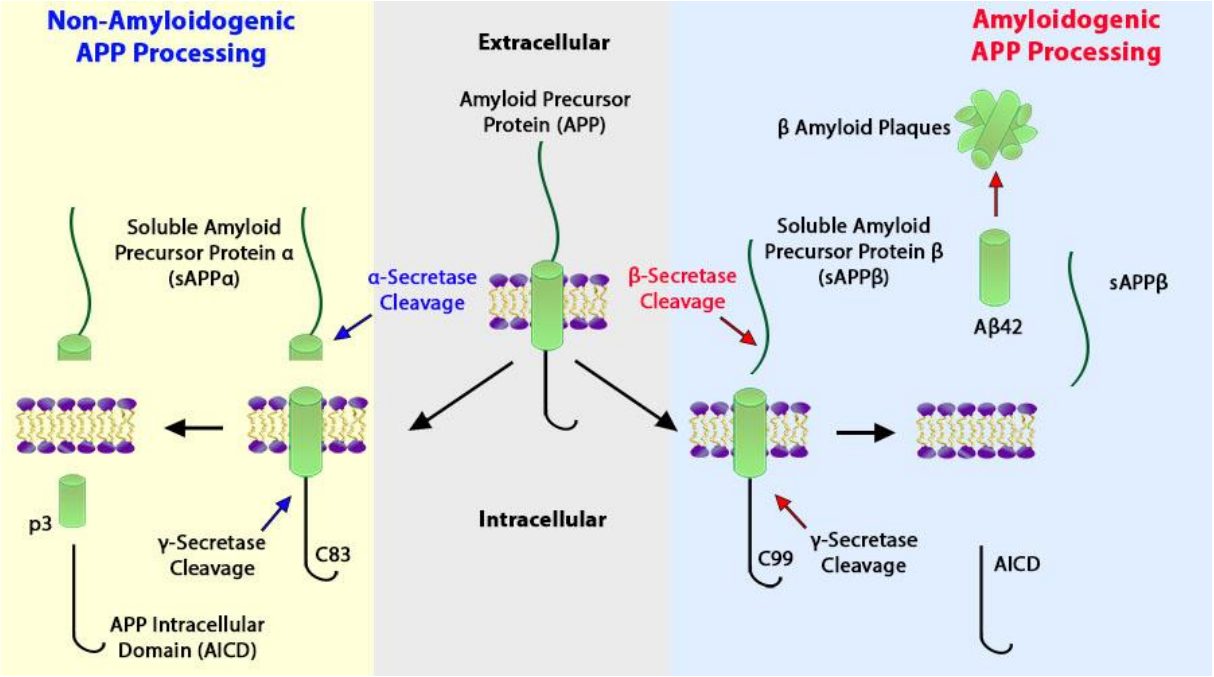

Figure 7 Shows the processing of the amyloid precursor protein (APP) by α-, β- and γ-secretases. Here we find two pathways (β/γ or α/γ) of APP proteolysis. APP can be cleaved by either α- or β-secretase, which is then followed by γ-secretase cleavage [103, 115].

Purpose

Our main goal is to reduce injury to normal tissue following cranial radiotherapy, to improve cognitive function, and to promote recovery in patients, especially children, who survived brain tumours.

Scientific issue:

To test the hypothesis that modulating the complement system during and after radiotherapy can prevent damage in the thalamus and potentially affect the expression of APP.

Material and Methods

Animals - Male and female mice

We have investigated CRT-induced injury in control (WT- C57BL/6) and *C3*-deficient mice (backcrossed on C57BL/6 - Charles River Laboratories, Sulzfeld, Germany). All mice were bred in-house. Mice were maintained on a 12-hour light cycle with food and water provided "ad lib". All mice were anesthetized with isoflurane and implanted with micro-transponders (DATAMARS, PetLink, and Youngstown, USA) to ensure identification in the IntelliCages. After weaning, the mice were kept in groups of up to 10 separated in according to gender and genotype. All the mice had daily injections of BrdU (Bromodeoxyuridine, 50mg/kg) for three consecutive days for 4 weeks before the mice were euthanized. The procedure had been approved by the Gothenburg committee of the Swedish Animal Welfare Agency (30/2008) [4, 76].

CRT-procedure

When the mice became 10 days old, both hemispheres were irradiated using a linear accelerator (Varian Clinac 600 CD; Radiation Oncology Systems LLC, San Diego, CA, USA) with 4 MV nominal photon energy using a dose rate of 2.3 Gy/min at Jubileums-kliniken, Sahlgrenska universitetssjukhus in Gothenburg. The total absorbed dose was 8 Gy administered in a single dose. The mice were anaesthetized with an intraperitoneal injection of 50 mg/kg tribromethanol (Sigma, Stockholm, Sweden) during CRT. The head was covered with one cm tissue equivalent material for a more precise CRT dose (dose variation was assessed to be +/- 5 %) passing through the brain. The mice were placed in the prone position on an expanded polystyrene bed during CRT. The CRT field was 2*2 cm. The whole procedure was completed in 10 minutes. Control mice were sedated but not subjected to CRT (figure 8) [4, 76].

Figure 8

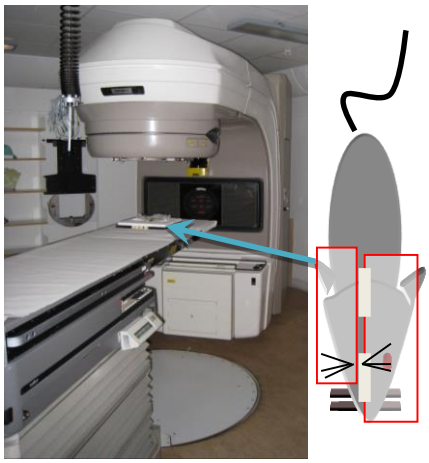

Figure 8 CRT procedures. The picture shows an apparatus which was used to investigate 2.3 Gy per minute. Mice were anesthetized before irradiation. Control mice were only anesthetized.

Procedure Pattern

See the overview of the procedure pattern below:

Figure 9

Wt- and *C3*$^{-/-}$ CRT-treated
Compared to C3$^{-/-}$ and Wt.

CRT P10

Four months later – thalamus and APP-positive cells analysis.

Figure 9 The timeline of the procedure. Mice were exposed to radiation on P10. After 4 months the mice were sacrificed and the tissue was used for immunohistochemistry.

Immunohistochemistry

The mice were deeply anesthetized with sodium pentobarbital 60mg/ml, 2 ml/ 100 g body weight i. p. (Pentothal, Electra-box Pharma, Tyresö, Sweden) before being transcardially perfused with Histofix (EMD Chemicals Inc., an Affiliate of Mereck KGaA, Darmstadt, Germany) in 0.1 M phosphate-buffered saline (PPS) 4 months after CRT. The brains were immersion-fixed in Histofix for 24 h at 4^{o}C and then separated into right and left brain parts. These were then kept in 0.1 M phosphate-buffer with 30% sucrose solution pH 7. Before further processing, the brain stem was cut at the base of the cerebellum. One hemisphere was cut into 25-µm sagittal sections in a series of 12, using a sliding microtome. To maintain the quality of

the tissue, the sections were stored in a cryo-protection (CPS) solution, containing 25% ethylene glycol, 25% glycerine and 50% 0.1 M phosphate-buffer at 4 °C, until staining [4, 76].

Under immunohistochemistry procedure, all sections were washed at least three times in TBS (Tris-buffered saline (TBS, 50 mMTris-HCl in 150 mM NaCl, pH 7.5)) and incubated in 0.6 % H_2O_2 in TBS for 30 minutes for blocking of endogenous peroxidases and for optimal specific staining. Next, all sections were incubated in a 10 mM sodium citrate solution, pH 9.0in a preheated water path (80^{o}C) under 30 minutes for releasing of cell membranes. The sections were then washed three times in TBS and incubated in a TBS block solution with 3 % donkey serum (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA.) and 0.1 % Triton X-100 (Merck KGaA, Darmstadt, Germany) for 30 minutes to facilitate antibody binding and would be incubated in the same block solution with APP (Amyloid precursor protein – anti body against APPs N-terminal), 1:1000 (Rabbit polyclonal anti-APP, Sigma- Aldrich, St. Louis, MO, USA.) at 4^{o}C for a whole night. The following day, the sections were washed three times in TBS and incubated within block solution containing biotinylated a secondary antibodies (donkey anti-rabbit, 1:1000, Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA) for one hour. Again sections were washed with TBS at least three times and then incubated in avidin-biotin-peroxidase (10 µl/ml TBS of A and B, Vectastain Elite ABC Kit Vector Laboratories, Bulingame, CA, USA.) for one hour to enable binding of avidin-biotin-peroxidase complex to the secondary antibodies. The sections were then washed up in TBS and this time the sections were developed for three minutes in 3, 3'-diaminobenzidine adulterated with TBS, H_2O_2 and $NiCl_2$ to intensify the reaction. Subsequently, the sections were rinsed for at least 4 times with lukewarm tap water and then mounted from 0.1 M phosphate buffer, pH 7.5, on glass slides (Menzel-Gläser, ThermoScientific, Microscope slides 25*75*1.0 mm, Mediate Gmbh, Burgdorf, Germany). The sections were left to dry overnight. The next day, the glass slides would be cover slipped (X-Tra-Kitt, and Menzel-Gläser cover slips 24*60 mm, Mediate Gmbh, Burgdorf, Germany) [4, 76].

Cell counting of APP-positive cells in the thalamus

The project was based on cell counting and was accomplished by quantifying APP-positive cells for the N-terminal throughout the thalamus in all sections containing a clear vision of

thalamus. This was accomplished using stereological principles (Stereoinvestigator, MicroBrightField, Colchester, VT, USA.). The thalamus area was identified at × 5 magnification (Using a Leica DM6000 B microscope – Leica microsystems, Wetzlar, Germany) with a DIC filter in the stereo Investigator program (MBF Bioscience Inc., Williston, VT, USA.). A micro-fire camera (Optronics, Goleta, CA, USA.) was used for visualization.

All APP-positive cells in the thalamus were counted in every section in the right hemisphere in × 20 magnifications, resulting in 5–7 sections per animal (n=7–8). Total volumes were calculated according to the Cavalieri principle, using the following formula: V=SA × P × T, where V is the total volume, SA is the sum of area measurements, P is the inverse of the sampling fraction and T is the section thickness. The total number of cells was obtained by multiplying the number of counted cells with the sampling fraction. The structure of the area of the thalamus and the APP-positive cells of the thalamus of every mouse was carefully analysed [4, 76].

The thalamus in sagittal cut sections was traced at 5x magnification in the Stereo Investigator program (MBF Bioscience Inc., Williston, VT, USA). APP-positive cells for the N-terminal within every tracing were counted at 20x magnification for each animal in all groups.

Statistical analysis

All data from the cell counting and volume measurements were analysed using a two-way ANOVA. The results were shown as mean ± standard error of the mean (SEM), with $P < 0.05$ considered statically significant. Treatment (control and cranial radio-therapeutic) and genotype (wild type and $C3^{-/-}$) were considered as main effects. Statistical analysis was performed using SPSS 21.0 (SPSS, Chicago, IL, USA).

Medical relevance and Ethical considerations

We hope with our study that if we can alleviate the late effects seen after radiotherapy, we would improve the quality of life for children who survive brain cancer. This research program used a preclinical setup with a rodent model. Cell cultures were used if possible, but unfortunately cell culture as a model can't replace the animal experiments fully considering the important cross-talk that happens in the body after a CRT-induced injury. All mice experiments were approved by the local committee of the Swedish Animals Welfare Agency (30/2008).

Results

There was marked difference in the expression of APP^+ cells between different regions in the brain of the mouse. APP-positive cells in the thalamus vary in density with maximum number in the superior – frontal plane and less in the inferior - posterior plane. It is also accumulated in some parts of the thalamus (LP, LD and RT superior portion) which have several functions, while in the other parts APP^+ dots are missing partially or completely.

The APP^+ N-terminal staining displayed clear, dark oval shaped dots **(**figure 10**)**. It has been shown that the distribution of APP throughout the thalamus is altered by CRT in Wt mice [116]. In this study, wild type and *C3*$^{-/-}$ mice were treated with CRT (8 Gy) at postnatal day 10. At the age of four months the mice were sacrificed and the thalamus volume and APP expression was further analysed. APP (N-terminal) expression was analysed using stereological procedures following CRT in the thalamus.

Figure 10

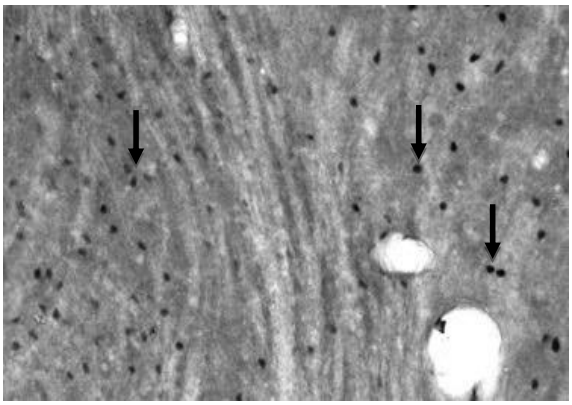

Figure 10 a representative microphotograph of the thalamus taken at 20x magnification. The N-terminal of APP is seen as black oval shaped dots (↓). APP cells from Wt Female mouse after CRT-treatment. All APP-positive cells were quantified throughout the entire thalamus.

Figure 11

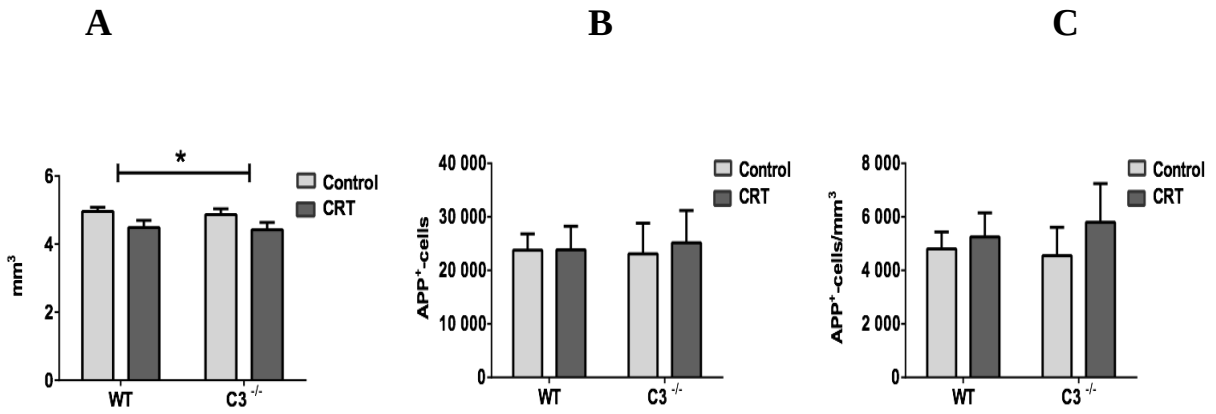

Figure 11 shows different result of thalamus volume, APP-positive cells and APP density 4 months after CRT between the genotypes. A: show the volume of the entire thalamus. B: show the total expression of APP. C: shown the density of APP-positive cells/mm^3. All data shown as mean ± S.E.M. n=8 per group.

Change of the thalamus before and after CRT: The volume of the thalamus was estimated at 4 months after CRT for these different groups; Wt, CRT-Wt, *C3*$^{-/-}$ and CRT-*C3*$^{-/-}$. There was a significant lack of growth in the thalamic structure following CRT. There was no significant difference between the genotypes. Four months after CRT the volume of the thalamus was significantly reduced (*p=0.016*). The volume was reduced by 10.6 % in the Wt-mice and 17.3 % in the *C3*-deficient mice. Neither the total number of APP-positive cells nor the density of APP in the thalamus was affected by genotype or CRT. The APP-positive cells were expressed to the same amount in irradiated and controls for wild type and *C3*$^{-/-}$ mice. There was however a slight increase of APP expression following CRT. The increase in density was 9.4% for the Wt mice and 29.8 % for the *C3*-deficient mice. A higher number of animals per group would be necessary to distinguish if there is a difference or not.

Discussion

This study aimed to investigate how inflammatory modulation before and after CRT influence cognitive functions and learning in young mice. It has been shown that *C3*$^{-/-}$ mice have improved learning and memory following cranial radiotherapy compared to Wt mice. This difference was however not due to a protection in the hippocampus. Hence, we assessed the effects of *C3*$^{-/-}$ on the volumes of the thalamus and also the APP expression in this area. Here we demonstrated that Wt and *C3*$^{-/-}$ mice have a reduced thalamic volume 4 months after a single dose of CRT at the age of 10 days. This lack of growth could presumably have a high impact on the cognitive decline seen after cranial radio-therapy in childhood. It has been demonstrated that anti-inflammatory treatment can improve some of the adverse side effects seen after CRT, such as memory and learning [5, 7, 76, 117]. The complement system is an important mediator of the CRT-induced inflammation but it doesn't affect the change in the volume of thalamus after CRT. In this study, the volume of the thalamus was affected 4 months after CRT in the developing brain. These results indicate disruptions in the growth of the thalamus due to cell death, decreased cellular proliferation etc. caused by CRT. It has been shown that other brain regions are highly affected and have less growth after CRT [118]. It has also been shown that CRT limits the CNS capability to regenerate, for example in hippocampus [119]. In this study, cells of the thalamus expressing APP tend to be unaffected or slightly modulated. Since the entire volume of thalamus is affected it would be interesting to quantify the proportion of the different cell types present in this region. This is out of the scope in this study but it has been shown that neurogenesis, oligogenesis and angiogenesis can be affected by several pathologic disturbances such as CRT [4, 120, 121].

This study suggests that the APP-positive cells of thalamus in mice can resist the effects of CRT at this moderate dose (8 Gy). It is still unclear what role of the APP positive cells have in the thalamus and if there is a link between the decreases of sAPPα/β in CSF seen in patients. There are however preliminary results that the APP expression investigated in this study is located to astrocytes. Further investigations are needed to find out why the N-terminal of APP is located in the nucleus of astrocytes and the potential influence it has on the late effects after cranial radiotherapy. A clinically adapted dose of 8 Gy to the brains of juvenile mice has been shown to induce late side effects and can profoundly diminished neurogenesis and disturbed growth of the granule cells layer in the hippocampus [118, 122]. Paediatric brain tumours are planned with a higher dose of CRT, even up to 55 Gy to the malignant tissue. A CRT dose of 8 Gy, which correspond to 18 Gy when allocate 2 Gy fractions, corresponds to what the healthy tissue

surrounding the brain tumour receives [123, 124]. Therefore, this model is a good method to study the late effects on the healthy brain tissue.

Mice that lack *C3* used in former study have been shown to have better circumstances for hippocampal growth during the subacute phase (7 days after CRT) and improved learning months after CRT [76]. This improvement in learning was not due to a long term protection in the hippocampus. Moreover, the results from this study could not demonstrate a protection in the thalamic region. Nevertheless, *C3* deficient mice are protected from CRT-induced learning problems indicating the important role of the microenvironment in this kind of injury. Many anti-inflammatory drugs could be using for this purpose. Cortisone, estradiol and androgen therapy could have neuro-protective features and can influence the functions of hippocampus [125-128].

This thesis reviews recent insight into the diverse types and function of APP and the cleaving mechanism in both normal physiology and pathology, especially the relation to higher cognitive functions and thereby synaptic plasticity. It would be intriguing to further develop this research, and reveal the secret of APP mechanisms. In this study, the effect of *C3* deficiency on the volume of the thalamus was affected but the amount of APP-positive cells in the thalamus 4 months after CRT was unaffected. The CRT-induced lack of growth in thalamus is however a new finding and increase the understanding of the cognitive decline in a better manner due to its important role in connecting the different brain regions but also its role in memory and learning. Furthermore, another study has shown that the APP protein levels was increased in the thalamus after CRT and could be linked to the decrease of sAPPα/β seen in cerebrospinal fluid in patients following prophylactic radiotherapy [7]. N-terminal APP is normally greatly expressed in the anterior part of the thalamus and most part of the medial regions and it has the special relation to the hippocampus and cortex. These connections are associated with memory and therefore to both learning and cognition [129]. The thalamic nuclei and their connections to the cortex and hippocampus are associated with most of inputs and outputs, so called drivers and modulators responsible for attention, awareness, memory and language [130-132].

In summary, this study has shown that there is a trend toward an increase of APP expression and smaller thalamic volume following CRT. The increase was 9.4 % for the Wt mice and 27.3 % for the *C3* deficient mice; however the density of APP-positive cells/mm^3 was not significant. A higher number of animals per group are needed to elucidate if that is the case. It is however a possibility that the growing thalamus devoid of physiologic active complement

component (*C3*) has a better opportunity to recover. A lot of research focused on how to avoid the late effects of CRT. This is important with the increasing number of long term survivors seen in today's society. With a better understanding of the pathophysiology of brain tumours, consequences of therapy, influence of age and gender, it might be possible to find better therapeutic strategies to improve the quality of life later on. We hope that further research will elucidate how the thalamus and APP could influence the long term side effects after CRT. We can so far only speculate whether APP expression in the thalamus has a protective role in the brain in general or if it has a mechanism of compensation for cell loss and affected neurogenesis after cranial CRT. Collectively, this study and a part of Kalm et al. studies cover a broad field of research on CRT effects on normal and tumours tissues. These studies review novel insight into the diverse function of CRT and its long term side effects. Significantly, the decline in volume of the thalamus after CRT in juvenile mice cannot alone be associated with cognitive impairment, but can be correlated with adverse long term side effect together with injuries in other brain regions caused by CRT.

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