Neonatal Exposure to Low-Dose Ionizing Radiation in Mice

Developmental Neurotoxic Effects of Single and Fractionated Doses and Interaction with Nicotine

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Abstract

Buratovic, S. 2015. Neonatal Exposure to Low-Dose Ionizing Radiation in Mice: Developmental neurotoxic effects of single and fractionated doses and interaction with nicotine. 38 pp. Uppsala

This thesis aims to investigate the developmental neurotoxic effects of low-dose exposure to ionizing radiation, alone or together with nicotine, during a defined critical period of neonatal brain development in mice.

Investigation of neurotoxic effects following fractionated or acute low-dose radiation, resembling the clinical situation during repeated CT scans or radiation delivered to non-target tissue during radiotherapy, and possible interaction effects with other agents, is of great importance for risk and safety evaluation.

During mammalian brain development there are defined critical periods for induction of developmental neurotoxic effects. One of these critical periods is called the brain growth spurt (BGS) and involves extensive growth and maturation of the brain. It is known that neonatal exposure during the BGS to low doses of radiation, as well as nicotine, can have a negative impact on neonatal brain development, resulting in impaired cognitive function in the adult mouse.

The present studies have shown that developmental neurotoxicity following low-dose irradiation can be induced during the same critical period of brain development as previously has been shown for chemicals. The observed neurotoxicity was manifested as altered spontaneous behaviour and habituation capacity, independent of sex, as well as elevated levels of an Alzheimer-related neuroprotein in the adult mouse. Furthermore, fractionated dose regimes seem to be as potent for induction of neurotoxicity and behavioural disturbances as an equivalent single acute dose. The cholinergic system can be a target system for developmental neurotoxicity of ionizing radiation, either alone or in combination with the cholinergic agent nicotine. Co-exposure to ionizing radiation and nicotine exacerbated the behavioural disturbances and cholinergic system dysfunction observed in these studies.

Further studies on developmental neurotoxic effects of low-dose neonatal irradiation and interaction with medical drugs and environmental pollutants are important for the field of radioprotection.

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This thesis is dedicated to my mother and father,
for their never ending love and support

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Buratovic, S., Stenerlöw, B., Fredriksson, A., Sundell-Bergman, S., Viberg, H., Eriksson, P. (2014) Neonatal exposure to a moderate dose of ionizing radiation causes behavioural defects and altered level of tau protein in mice. *NeuroToxicology* **45:**48-55.

II Buratovic, S., Stenerlöw, B., Fredriksson, A., Sundell-Bergman, S., Eriksson, P. (2014) Effects of neonatal fractionated low-dose exposure to ionizing radiation and the interaction with nicotine on behaviour in mice. *Submitted*.

Contents

Abbreviations

ACh	Acetylcholine
ANOVA	Analysis of variance
BGS	Brain growth spurt
b.w.	Body weight
CaMKII	Calcium/calmodulin-dependent kinase II
ChAT	Choline acetyltransferase
CNS	Central nervous system
CT	Computerized tomography
GAP-43	Growth associated protein 43
Gy	Grey
IR	Ionizing radiation
mAChR	Muscarinic acetylcholine receptor
MeHg	Methyl mercury
MRT	Multiple range test
nAChR	Nicotinic acetylcholine receptor
NGF	Nerve growth factor
NMRI	Naval medical research institute
s.c.	Subcutaneous
SD	Standard deviation

Objectives

The objective of this thesis was to investigate the developmental neurotoxic effects of low-dose exposure to ionizing radiation during a defined critical period of neonatal brain development in rodents. This thesis specifically aims to explore:

- Whether a single low-dose exposure to ionizing radiation during neonatal brain development can alter adult mouse spontaneous behaviour in a novel home environment.

- Whether there is a sex difference between male and female mice in susceptibility to neurotoxic effects following neonatal low-dose irradiation.

- Whether neonatal low-dose irradiation has an impact on levels of essential neuroproteins in the neonatal and adult mouse.

- Whether neonatal fractionated low-dose irradiation can induce similar behavioural alterations to the equivalent single dose irradiation.

- Whether neonatal co-exposure to nicotine and irradiation alters the susceptibility of the adult mouse cholinergic system.

Introduction

This thesis focuses on the neurotoxic effects of neonatal exposure to low-dose ionizing radiation (IR) during a defined critical period of brain development and possible interaction effects with nicotine in mice.

Ionizing radiation

Medical imaging and tumour therapy are major sources of exposure to IR. Although IR has many benefits, exposure to radiation may also have negative consequences. Risk assessment of exposure to IR has predominantly been focused on high dose exposure and late risk for cancer. Less is known about low-dose exposure, non-cancer effects and possible interaction effects with different classes of toxicants. An epidemiological study investigating adult long-term survivors of low-grade glioma showed that patients treated with radiotherapy experienced a progressive decline in cognitive abilities (Douw et al., 2009). This observation was also valid for patients who had been treated with fractionated dose schemes to doses regarded as safe i.e. ≤ 2 Gy/fraction. This worsening of cognitive impairment was not observed in patients who did not receive radiotherapy. It is well known that children who undergo radiotherapy for tumours in the central nervous system (CNS) have an elevated risk of developing late cognitive dysfunction (Pollack et al., 1995, Mulhern et al., 2004) which may arise from radiation delivered to non-target tissue. Furthermore, it has been shown that young children, below the age of 18 months, irradiated to moderate doses for treatment of cutaneous haemangioma experience cognitive impairments in adulthood (Hall et al., 2004). The use of diagnostic and imaging techniques such as computerized tomography (CT) scans has increased during the past decade and exposure to IR for medical purposes has now become the major source of exposure (Mettler et al., 2000, Mettler et al., 2008, Bernier et al., 2012). The average dose delivered during a conventional CT scan has been estimated at 21-153 mGy/scan (Leitz and Almén, 2010, Pearce et al., 2012b). The relatively high radiation dose delivered during a CT scan, compared to conventional x-ray, has resulted in CT scans being accountable for 40-67% of the received medical dose in the population, albeit only making up a fraction of all radiological examinations performed annually (Bernier et al., 2012). Children below the age of 15 have been subjected to approximately 11% of all CT scans,

nearly half of which were directed towards the cranial area. In diagnostic radiology, CT scans of the head region contributed to almost 14% of the total collective effective dose in the general population (Mettler et al., 2000, Mettler et al., 2008).

Gestational exposure to 1-2 Gy of x-ray irradiation during the embryonic or early foetal period has been shown to affect nerve growth factors (NGF) and apoptosis in the foetal rat (Benekou et al., 2001, Bolaris et al., 2001).

Nicotine

Tobacco with its active substance nicotine is one of the most widely used dependence-producing substances (Henningfield and Woodson, 1989). Exposure to nicotine will lead to vasoconstriction and increased heart rate. If exposure occurs during pregnancy a reduced blood flow to the uterus will limit and decrease the oxygen and nutrient accessibility for the foetus. As a result, the most pronounced adverse effect of smoking during pregnancy is low birth weight of the foetus in relation to gestational age (Ellard et al., 1996, Lambers and Clark, 1996). Cognitive defects and lower IQ are intimately coupled to low birth weight independently of causative agent (Corbett and Drewett, 2004, Viggedal et al., 2004). Furthermore, smoking during pregnancy can result in spontaneous abortion, Sudden Infant Death Syndrome and an increased risk for the child to suffer from learning impairments and neuropsychiatric disorders (Bell and Lau, 1995, DiFranza and Lew, 1995, Tran et al., 2013).

Another important use for nicotine is as an insecticide. When applied to pest-infested crops, nicotine acts on nicotinic receptors in motor nerves where it causes over-stimulation which further leads to blockage of synapses (James and Nordberg, 1995). Nicotine binds directly to the receptors and can also, via other receptors, cause an increase in acetylcholine (ACh), serotonin, dopamine and epinephrine release into the synaptic cleft (Wonnacott et al., 1989).

Animal studies have shown that prenatal exposure to 6mg/kg/day can result in hyperactivity in the offspring at an adult age (Tizabi et al., 1997). Neonatal exposure to 66 µg nicotine base/kg b.w. has been shown to affect nicotinic acetylcholine receptor (nAChR) binding properties and behavioural disturbances and learning and memory impairments in the adult mouse (Eriksson et al., 2000, Ankarberg et al., 2001).

The developing brain and vulnerable periods

Development of the mammalian CNS is a delicate interplay between multiple essential processes meaning that even the slightest perturbation of an

important event may have great impact for the maturing individual. Mammalian gestation can roughly be divided into the embryonic and foetal developmental period. During embryonic brain development the brain acquires its general shape and structure accompanied with multiplication of both glial cells and neurons. Insults occurring during this time period, independent of causative factor, may result in anatomical malformations of the brain's structures. Embryonic brain development is followed by the foetal developmental period. Foetal brain development is characterized by formation and maturation of functional circuits in the brain. A specifically vulnerable period during the foetal brain development is called the brain growth spurt (BGS) (Davison and Dobbing, 1968). During the BGS a marked growth in brain size is noticeable. This expansion in brain weight and volume is attributed to extensive myelinisation of neurons, synaptogenesis, dendritic aborisation and gliogenesis (Dobbing and Sands, 1979, Kolb and Whishaw, 1989). Duration and time of onset of the BGS differs between mammalian species. In humans the BGS starts during the third trimester of pregnancy, peaks around birth and continues for approximately two years of the child's life. In mouse and rat the BGS is postnatal, peaks around postnatal day (PND) 10 and continues for approximately 4 weeks in the pup (Davison and Dobbing, 1968). The extensive changes in cellular composition of the neonatal mouse brain entail further biochemical changes, resulting in novel motor and sensory faculties accompanied by a peak in spontaneous behaviour (Bolles and Woods, 1964, Campbell et al., 1969), as well as a rapid development of the cholinergic system.

Development of the cholinergic system and neuronal protein markers

The cholinergic system is known to be involved in multiple physiological processes e.g. cognition, learning and memory and attention (Karczmar, 1975, Abreu-Villaca et al., 2011). The enzyme choline acetyltransferase (ChAT) functions to catalyse biosynthesis of acetylcholine (ACh) and is commonly used as a marker for the developing cholinergic system (Nachmansohn and Machado, 1943). The first appearance of ChAT immunoreactive cells has been observed around embryonic day 14 and 17 in mouse forebrain (Schambra et al., 1989). ChAT activity increases after birth with a marked elevation of activity around PND 10 in the cerebral cortex and hippocampus in rat. Around PND 21 the levels of ChAT activity reaches the ones observed in mature adults (Large et al., 1986). This time period of rapid cholinergic system development also coincides with the peak of the BGS. Accompanying the increase in ChAT activity is the development of cholinergic receptors. These receptors can be divided into two classes: mus-

carinic acetylcholine receptors (mAChR) and nicotinic acetylcholine receptors (nAChR). (Dale, 1914). nAChR are pentamers made up of different α (α_2-α_{10}) and β (β_2-β_4) subunits and functions as ligand-gated ion channels (Gotti and Clementi, 2004). nAChR can be activated both by endogenous acetylcholine as well as nicotine (Slotkin, 1998). Differentiation of nAChR occurs postnatally in mice and a distinct ontogeny of the high ($\alpha_4\beta_2$) and low (α_7) affinity nicotinic binding sites can be observed. In rat the high affinity nicotinic binding sites can be observed at birth after which they gradually decrease over the lifespan of the animal. Low affinity nicotinic binding sites have been observed on PND 17 but not on PND 5 (Nordberg, 1993). Previous studies have observed alterations in adult mouse cholinergic receptor populations, learning and memory faculties as well as altered susceptibility of the cholinergic system to cholinergic agents following neonatal exposure to nicotine (Eriksson et al., 2000, Ankarberg et al., 2001, 2004).

Expression and regulation of a vast number of neuroproteins during neonatal brain development is essential to ensure proper function later in life.

When investigating mammalian neuronal tissue one of the most commonly found protein kinases is the calcium/calmodulin dependent protein kinase II (CaMKII) (Erondu and Kennedy, 1985). CaMKII is a serine/threonine specific kinase which is thought to play a crucial role in the process of dendritic aborisation, long-term potentiation, learning and memory (Lisman and Goldring, 1988, Lisman et al., 2002, Yamauchi, 2005). The activated CaMKII stimulates glutamatergic NMDA receptor transmission as well as enhancing the signal via glutamatergic AMPA receptors (Lisman et al., 2012). During mouse neonatal brain development levels of CaMKII continuously increase in cerebral cortex, hippocampus and whole brain. 28 days after birth the measured levels are 28 times higher than on PND 1 with the highest increase rate occurring between PND 10-PND 14 (Viberg et al., 2008).

Growth associated protein-43 (GAP-43) is found in the growth cone of axons where it guides the sprouting cell. Extensive expression of GAP-43 is intimately coupled to development of the nervous system, hence making it an excellent biomarker for axonal growth and sprouting (Oestreicher et al., 1997). It has also been proposed that GAP-43 plays a crucial role in long-term potentiation by acting as a protein kinase C substrate (Benowitz and Routtenberg, 1997). Ontogeny of GAP-43 in postnatal mice revealed a peak in hippocampal protein level around PND 7 and around PND 10 for cortex and whole brain. An unwavering decline in protein levels was observed after the peak, which by PND 28 had reduced the GAP-43 levels to lower than observed on PND 1 in hippocampus and whole brain. GAP-43 levels were still slightly higher in cortex on PND 28 compared to PND 1(Viberg et al., 2008).

To date the exact function of synaptophysin is not fully understood. It is present in high concentrations at the axonal terminal of neurons. By regulat-

ing the cycling and to some extent formation, of synaptic vesicles synaptophysin plays an important part in neuronal plasticity (Sarnat and Born, 1999). By assuring faithful signal propagation between neurons the process of long term potentiation (LTP), which is intimately coupled to learning and memory, is made possible (Lynch, 2004). Neonatal ontogeny of synaptophysin in mouse hippocampus, cortex and whole brain has been studied by Viberg (2009), who observed a pronounced increase of synaptophysin during the animals first four weeks of life with up to a 45-fold increase in protein levels at the end of this time period, when compared to levels on PND 1. The fastest rate of protein level increase was observed on PND 7-10 (Viberg, 2009).

Tau is a member of the microtubule-associated protein family which functions to stabilize and maintain a normal morphology of neurons, establish polarity and support the outgrowth of neural processes (Wang and Liu, 2008). Elevated levels of the phosphorylated tau isoform have been observed to impair normal learning and memory functions in humans and this is therefore used as a diagnostic marker for diagnosing Alzheimer's disease in the clinic. Levels of tau fluctuate during normal development of the mouse brain. During the first days after birth increasing levels were observed which then decreased during the rest of the observational period. As a result, tau levels observed on PND 28 were below the levels observed on PND 1. The amount of tau peaked on PND 3-7 in the hippocampus and between PND 7-10 in the cerebral cortex and whole brain (Viberg, 2009).

Materials and methods

More detailed descriptions of the materials and methods are presented in the individual papers.

Animals

Experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), after approval from the local ethics committees (Uppsala University and the Agricultural Research Council) and by the Swedish Committee for Ethical Experiments on Laboratory Animals, approval number C347/10. Pregnant Naval Medical Research Institute (NMRI) mice were purchased from Scanbur, Sollentuna, Sweden. The animals were housed individually in macrolon cages (42 x 26 x 15 cm) in a room for females only with an ambient temperature of 22°C and a 12/12 h constant light/dark cycle. The animals were supplied with standardized pellet food (Lactamin, Stockholm, Sweden) and tap water *ad libitum*. Females were checked for birth twice daily (08.00 and 18.00 h) and the day of birth was designated day 0. Within the first 48 h after birth, litter sizes were adjusted and standardized to 10-12 pups of both sexes by euthanizing excess pups (Irvine and Timiras, 1966). At approximately 4 weeks of age, male and female offspring were separated by sex and raised in sibling groups of 3-7 individuals in separate male or female rooms under the same conditions as stated above.

Exposure

Study I: NMRI mice of both sexes were whole-body gamma irradiated with a single dose of 350 mGy or 500 mGy on PND 10. Control animals were placed in the ionization chamber and sham irradiated.
Study II: male NMRI mice were exposed to whole-body gamma irradiation 200 mGy/fraction, (-) nicotine base 66 µg/kg b.w. s.c. twice daily or co-exposed to 200 mGy whole-body irradiation + 66 µg/kg b.w. (-) nicotine base s.c. twice daily on PND 10, PND 10-11 or PND 10-12 (or only nicotine on PND 10-13). Control animals received saline (10 mg/kg b.w.) s.c. and were sham irradiated.

Irradiation and exposure chemicals

Whole-body irradiation was performed using a ^{60}Co source, dose-rate at the surface was 0.02 Gy/min (The Svedberg laboratory, Uppsala University, Uppsala, Sweden).
(-)nicotine-bi-(+)tartrate was obtained from Sigma, U.S.A. pH of (-)nicotine base was adjusted to 7.0 before s.c. injection to avoid tissue damage.

Behavioural tests

Spontaneous behaviour in a novel home environment

Study I: both male and female NMRI mice were observed for spontaneous behaviour at 2 and 4 months of age.
Study II: male NMRI mice were observed for spontaneous behaviour at age 2 months.

Observations took place between 08.00 and 13.00 under the same light and temperature conditions in which the animals were housed. A total of 10-12 individuals from each exposure group, 3-4 individuals taken randomly from at least 3 different litters, were observed. Recordings were made in 12 macrolon cages (42 × 26 × 15 cm) equipped with two series (high and low) of infrared beams (Rat-O-Matic, ADEA Elektronik AB, Uppsala, Sweden) (Fredriksson, 1994). During 60 consecutive minutes an automated system recorded the motoric activity of the animals and recordings of the variables locomotion, rearing and total activity were made.

Locomotion: Movements made in the horizontal plane were registered by the low level (10 mm above the bedding material) infrared beams.

Rearing: Movements made in the vertical plane were registered by the high level (80 mm above the bedding material) infrared beam.

Total activity: A needle mounted on a horizontal arm with a counter-weight connected to the test cage registered all vibrations such as movements, grooming and shaking.

Nicotine-induced behaviour

Male mice in study II were observed for nicotine-induced behaviour. Directly after the spontaneous behaviour observation all mice received an s.c. injection of (-)nicotine base (80 µg/kg b.w.) and were observed for variables locomotion, rearing and total activity as described for spontaneous behaviour. The nicotine-induced behaviour test lasted for an additional 60 min time period.

Neuroprotein analysis

In study I the cerebral cortex and hippocampus were dissected out, snap frozen in liquid nitrogen and used for neuroprotein analysis (n=12/exposure group). The protein levels of CaMKII, GAP-43, synaptophysin and tau from control mice and mice irradiated to 500 mGy were analysed using the Slot Blot analysis. All samples were homogenized and the total protein content was determined using the Pierce BCA Protein assay method. Viberg and co-workers have previously evaluated the specificity of antibodies CaMKII (Upstate Millpore, 05-552), GAP-43 (Chemicon Millipore, AB5220), synaptophysin (Calbiochem, 573822) and tau (Santa Cruz, 32274) by Western blot procedure with adequate results (Viberg et al., 2008, Viberg, 2009).

The total amount of protein loaded and the amount of antibody used in the Slot Blot assay was: 4 µg and mouse monoclonal CaMKII (1:5000) for CaMKII, 4 µg and rabbit polyclonal GAP-43 (1:10000) for GAP-43, 3µg and mouse monoclonal synaptophysin (1:10000) for synaptophysin and 3.5 µg and mouse monoclonal tau (1:1000) for tau. A horseradish peroxidase conjugate secondary antibody against mouse (KPL 074-1806, 1:20000) or rabbit (KPL 074-1506) was used to detect immunoreactivity. Immunoreactive bands were traced using an enhanced chemiluminescent substrate (Pierce, Super Signal West Dura) and imaged on a LAS-1000 (Fuji Film, Tokyo, Japan). Band intensity was quantified using IR-LAS 1000 Pro (Fuji Film). The protein levels of control animals were normalized to 100% and protein levels of exposed animals expressed as percentage of controls.

Statistical analysis

Spontaneous and nicotine-induced behaviour

The data from the variables locomotion, rearing and total activity (treatment, time, treatment x time, between subjects, within subjects and, in study II, interaction factors) recorded in the spontaneous and nicotine-induced behaviour observations were subjected to an ANOVA (analysis of variance) with split plot design and pairwise testing using a Duncan's MRT (multiple range test) in the software SAS 9.1 (Kirk, 1968, Festing, 2006, Lazic and Essioux, 2013).

Slot Blot analysis

Study I: the data of protein levels from the Slot Blot analysis of CaMKII, GAP-43, synaptophysin and tau in cerebral cortex and hippocampus was subjected to a one-way ANOVA, pairwise testing Duncan's MRT using the software STATISTICA 10.

Results and discussion

The objective of this thesis was to investigate whether low-dose exposure to IR, during a critical period of neonatal brain development in mice, can induce similar behavioural defects and lack of habituation as has previously been observed for environmental pollutants. Furthermore, the thesis aims to investigate sex differences between male and female mice in susceptibility to low-dose irradiation as well as to compare single dose exposures with fractionated exposure regimes and possible interaction effects with nicotine, manifested as impaired habituation capacity, a trait which may be used as an indicator of cognitive dysfunction and altered susceptibility of the cholinergic system.

Effects on spontaneous behaviour following neonatal low-dose irradiation

In study I male and female mice were whole-body irradiated with 0, 350 or 500 mGy on PND 10 and observed for spontaneous behaviour in a novel home environment at 2 and 4 months of age (Figure 1 & 2). A normal spontaneous behaviour in mice, which was displayed by the control animals in this study, includes having an initially high activity when confronted with a novel home environment, as the animal explores its new surroundings. As the novelty of the test chamber diminishes, the animals display less activity over time and habituate to the novel home environment (Fredriksson, 1994, Eriksson et al., 2010a). Male and female mice neonatally irradiated to 500 mGy displayed a significantly deranged spontaneous behaviour at 2-months of age. When compared to control animals these mice were significantly hypoactive during the first 20 min of spontaneous behaviour testing and significantly hyperactive during the last 20 min observational period for the observed variables locomotion, rearing and total activity. Male and female mice neonatally irradiated to 350 mGy displayed a significantly altered activity during the first 20 min test period by being significantly hypoactive when compared to controls for the observed variables locomotion, rearing and total activity. By the end of the 60 min test period the irradiated animals showed no significant difference in activity when compared to control animals for the previously stated variables.

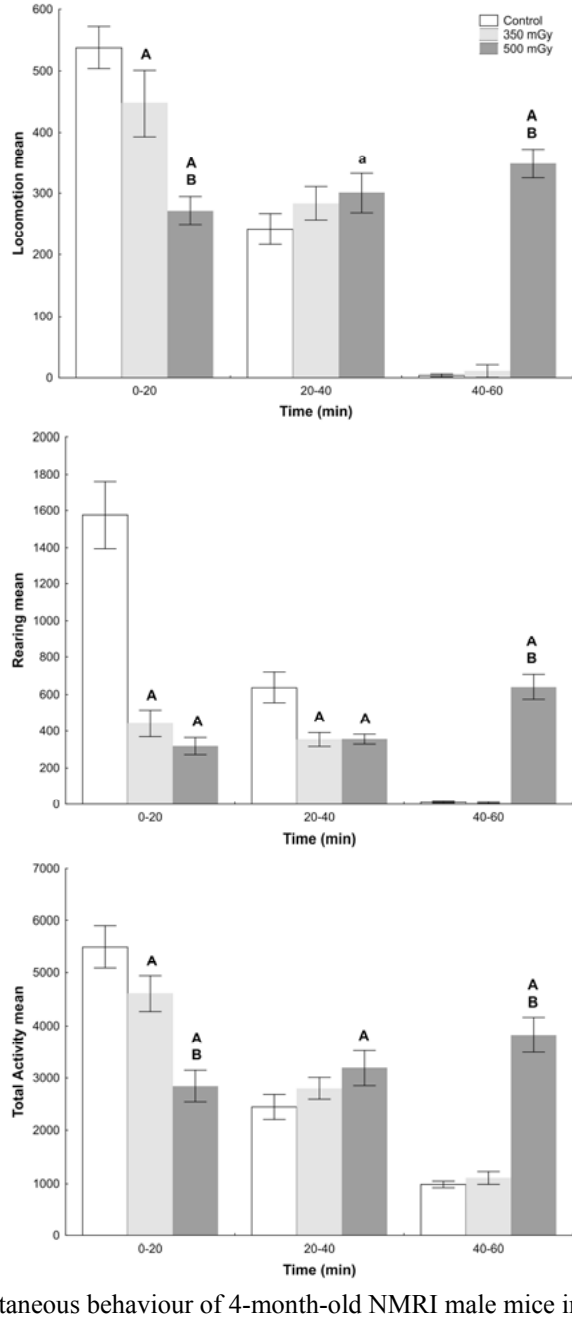

Figure 1. Spontaneous behaviour of 4-month-old NMRI male mice irradiated with 350 mGy, 500 mGy or sham irradiated on PND 10. The statistical differences are indicated as: (A) significantly different vs. control, $p \leq 0.01$; (a) significantly different vs. control, $p \leq 0.05$; (B) significantly different vs. 350 mGy, $p \leq 0.01$. Height of bars represents mean ± SD.

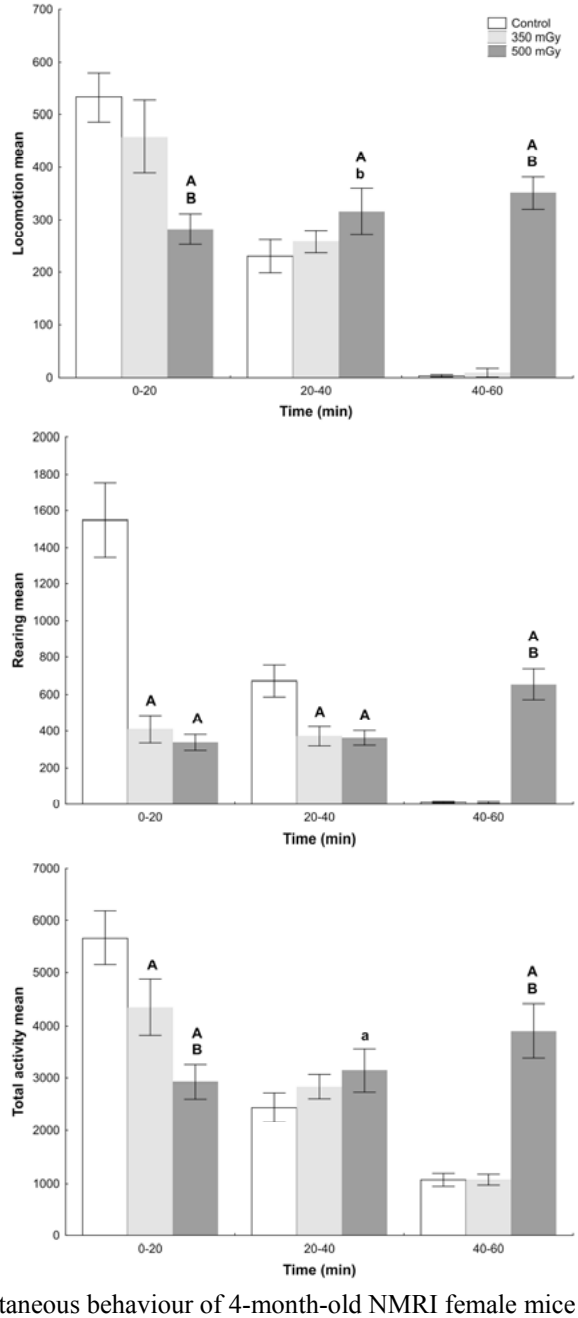

Figure 2. Spontaneous behaviour of 4-month-old NMRI female mice irradiated with 350 mGy, 500 mGy or sham irradiated on PND 10. The statistical differences are indicated as: (A) significantly different vs. control, $p \leq 0.01$; (a) significantly different vs. control, $p \leq 0.05$; (B) significantly different vs. 350 mGy, $p \leq 0.01$; (b) significantly different vs. 350 mGy, $p \leq 0.05$. Height of bars represents mean ± SD.

At 4 months of age male and female mice were again observed for spontaneous behaviour in a novel home environment. The alterations in spontaneous behaviour and habituation capacity observed in 4-month-old male and female mice was in concordance with the observations made at 2 months of age, with no additional significant changes being observed. This indicates that the aberrant spontaneous behaviour and lack of habituation can be a persistent neurotoxic effect induced by neonatal irradiation.

In study II neonatal male mice were whole-body irradiated with one 200 mGy fraction per day on PND 10, 10-11 or 10-12 and observed for spontaneous behaviour in a novel home environment at 2 months of age (Figure 3). Control animals were sham irradiated. Control animals displayed a normal spontaneous behaviour and habituation capacity with initially high activity which decreased over the 60 min observational time period (Fredriksson, 1994, Eriksson et al., 2010a). Male mice irradiated to 200 mGy fractions on PND 10-12 showed an aberrant spontaneous behaviour during the first 20 min test period by being significantly hypoactive when compared to control animals and also animals irradiated on PND 10 and PND 10-11. This hypoactivity was observed for the variables locomotion, rearing and total activity. By the end of the 60 min test period no significant deviations from the normal behaviour displayed by control animals was observed in the irradiated mice.

During the spontaneous behaviour observations, integration of sensoric input into an appropriate motoric output is needed in order for the animal to be able to habituate. Habituation is frequently called the "simplest form of learning" and can therefore be used as a measurement of cognitive function (Daenen et al., 2001, Rankin et al., 2009). In the present studies habituation is defined as a decrease in measured counts for the variables locomotion, rearing and total activity during the 60 min observational period. Habituation by this definition was evident in control animals while mice irradiated to 500 mGy (study I) showed a lack of habituation by displaying a significantly deviant behaviour compared to controls. A modified habituation capacity was observed in mice irradiated to a single dose of 350 mGy (study I), this indicates that 350 mGy could be a possible threshold dose for induction of developmental neurotoxic effects. Furthermore, in study II fractionated irradiation to 200 mGy on PND 10-12 resulted in the same behaviour profile alterations and modification of habituation capacity as a single dose of 350 mGy, indicating that a low-dose fractionated irradiation scheme can be as potent in inducing developmental neurotoxic effects as higher single dose irradiations. In a previous study Eriksson and co-workers (2010) observed the same aberrant spontaneous behaviour and lack of habituation when male mice were irradiated to 500 mGy as a single dose. Also, in that study no behavioural alterations were observed following a single dose irradiation to 200 mGy. This is well in concordance with the behavioural alterations observed in this thesis where mice displayed a lack of habituation capacity

when irradiated to 500 mGy (study I) but no aberrant behaviour when irradiated to 200 mGy on PND 10 (study II). Studies investigating embryonic irradiation to 500 mGy in mice have reported persistent alterations in locomotor activity in the open field test accompanied with learning and memory impairments at an adult age (Hossain and Devi, 2000, 2001). Moreover, multiple studies aiming to explore neurotoxic effects following internal uranium exposure in the adult rat have shown that exposure to enriched uranium resulted in anxiety-like behaviour, altered sleeping patterns and impairments in spatial working memory. Exposure to depleted uranium, with no essential radiation component, did not affect the spatial working memory or the anxiety like behaviour, as was seen in the animals exposed to enriched uranium, but did affect sleep-wake cycles in the rats (Houpert et al., 2005, Lestaevel et al., 2005a, Lestaevel et al., 2005b). Houpert and co-workers therefore suggest that the radiation component of enriched uranium is the causative agent of the neurotoxicological disturbances (Houpert et al., 2005). An additional explanation to the observed differences in neurotoxicity following exposure to enriched or depleted uranium could be an interaction effect between IR and the heavy metal (uranium).

In study I adult female mice displayed similar behavioural disturbances, as was seen in age matched male mice, following neonatal irradiation to 350 or 500 mGy, manifested as an aberrant spontaneous behaviour and modified habituation capacity. This finding indicates that females can be as susceptible as males to neurotoxic insults caused by low-dose neonatal irradiation. Other studies have shown that acute irradiation to 8 Gy on PND 14 renders female mice to present more pronounced neurotoxicity, manifested as impaired hippocampal neurogenesis and white matter growth as well as anxiety-like behaviour at an adult age when compared to male mice (Roughton et al., 2012, Roughton et al., 2013). It remains to be investigated whether there are sex differences in mechanisms underlying the observed neurotoxicity of high dose irradiation compared to low-dose irradiation. In the studies by Roughton and co-workers both male and female mice were anaesthetized prior to irradiation (Roughton et al., 2012, Roughton et al., 2013). As the animals were exposed to both radiation and an anaesthetic agent, one cannot rule out a possible interaction effect as the causative factor for the sex differences observed by Roughton and co-workers.

Effects on essential neuroproteins following neonatal low-dose irradiation

One of the objectives in study I was to elucidate whether low-dose irradiation during neonatal brain development in mice can impact on levels of essential neuroproteins in the neonatal as well as adult brain. Male mice irradi-

ated to 500 mGy on PND 10 were chosen for analysis since they expressed the most pronounced spontaneous behaviour alterations and lack of habituation. Cerebral cortex and hippocampus from 11-day-old and 6-month-old male mice irradiated to 0 or 500 mGy were collected and analysed for levels of proteins CaMKII, GAP-43, synaptophysin and tau using the Slot Blot technique. Protein levels of control animals were normalized to 100% and protein levels from irradiated mice expressed as percentage of control animal levels (Table. 1). In both 11-day-old (24 h post irradiation) and 6-month-old male mouse cerebral cortex a significant elevation of tau protein (118% and 105% respectively) was observed following irradiation to 500 mGy on PND 10. Cerebrocortical levels of synaptophysin in 11-day-old male mice were also significantly elevated with 54% compared to controls. Moreover, a significant reduction (33%) in hippocampal levels of tau was observed in 11-day-old irradiated mice when compared to controls.

Table 1. Protein levels of CaMKII, GAP-43, synaptophysin and tau in male mice exposed to 500 mGy on postnatal day 10[1].

	11-day old		**6-month-old**	
	Cerebral cortex	**Hippocampus**	**Cerebral cortex**	**Hippocampus**
Control	100	100	100	100
CaMKII	124±18	102±15	114±24	105±9
GAP-43	108±6	94±8	100±10	98±8
Synaptophysin	154±19 **	109±13	114±10	106±9
Tau	218±14 ***	67±14 **	205±17 ***	106±8

[1]Animals were sacrificed as neonatals 24 hours post-irradiation or at the adult age of 6 months. The control value was set to 100% (± SD) and the statistical difference between control and 500 Gy exposed mice is indicated with ** for $p \leq 0.01$ or *** for $p \leq 0.001$.

During neonatal brain development in mice a distinct ontogeny of the investigated neuroproteins CaMKII, GAP-43, synaptophysin and tau has been observed (Viberg et al., 2008, Viberg, 2009). Furthermore, Viberg and coworkers observed the most pronounced elevation in levels of the previously mentioned proteins around PND 7-14, a time period which also coincides with the BGS and extensive cholinergic system development as well as an observed peak in spontaneous behaviour in rodents (Bolles and Woods, 1964, Davison and Dobbing, 1968, Abreu-Villaca et al., 2011). The observed elevation of synaptophysin and tau in cerebral cortex and the reduction in tau protein in the hippocampus in the irradiated 11-day-old mouse brain indicates that the normal brain development occurring at this developmental time period is altered. A clinical study performed on human patients showed that prophylactic cranial irradiation in adults induces an elevation in cerebrospinal fluid levels of tau protein during the subacute phase after treatment. This elevation was reversible and not present 12 months post irra-

diation (Kalm et al., 2014). Whether the observed elevation of tau protein in the present study of neonatal mouse cerebral cortex is a persistent trait which is present during the animals' full lifespan or if the levels fluctuate over time remains to be investigated. Furthermore, the possibility of different mechanisms for induction of neurotoxicity in the young brain compared to the mature adult one needs to be taken into consideration. Whether disruption in neonatal mouse neuroprotein levels is a persistent trait which can be observed in the adult animal has been shown to be highly influenced by at which time point during the BGS exposure occurs (Eriksson et al., 2000). However, the observed alteration in spontaneous behaviour and lack of habituation capacity at 2 and 4 months of age, accompanied by elevation of tau protein at 6 months of age indicate that persistent developmental neurotoxicity can be induced following a single irradiation to 500 mGy. Neurotoxicity induced by radiation and manifested as altered levels of essential neuroproteins has previously been observed. Irradiation at the adult age of 45 days to 500 mGy of x-rays was observed to reduce levels of CaMKII in male mice but not in females (Silasi et al., 2004). In a study by Goutan and co-workers (1999) a decrease in cerebellar GAP-43 levels but no alteration in synaptophysin was observed following acute 2 Gy gamma irradiation on PND 1 in rat. The observed decrease in cerebellar GAP-43 levels was not present 48 h post-irradiation which may indicate a plasticity in the cerebellum that enables recovery of protein levels back to normal following irradiation during postnatal brain development (Goutan et al., 1999). However, having in mind the distinct ontogeny of the neuroproteins investigated in study I, it is reasonable to assume that the magnitude and persistency of neuroprotein alterations vary highly, depending on at which time-point during brain development the irradiation occurs.

Effects on spontaneous behaviour and susceptibility of the cholinergic system following neonatal co-exposure to nicotine and irradiation

In study II male mice were whole-body irradiated to 200 mGy fractions of gamma radiation on PND 10, 10-11 or 10-12 and/or exposed to 66 µg/kg b.w nicotine base s.c. twice daily on PND 10, 10-11 or 10-12. One group of mice was also exposed to only nicotine on PND 10-13. At 2 months of age the animals were first observed for spontaneous behaviour (Figure 3), performed as described above, in a novel home environment and immediately after the 60 min observational period the mice were injected with 80 µg/kg b.w s.c. and observed for nicotine-induced behaviour for an additional 60 min time period (Figure 4). This study was conducted in order to investigate whether nicotine and IR could interact to induce more pronounced manifes-

tations of developmental neurotoxicity on spontaneous behaviour and habituation capability. Furthermore, by challenging the cholinergic system to a known cholinergic agent, such as nicotine, one can investigate whether the cholinergic system can be a target system for neurotoxicity induced by IR.

During the spontaneous behaviour observation a normal spontaneous behaviour and habituation capability was observed in the control animals, with initially high activity at the beginning of the observational period which decreased during the 60 min test period. Mice co-exposed to IR and nicotine on PND 10 or PND 10-11 did not display any significantly aberrant spontaneous behaviour and habituation capacity when comparted to controls. Male mice co-exposed to IR and nicotine on PND 10-12 showed significantly decreased activity for the variables locomotion, rearing and total activity during the first 20 min observational period when compared to controls and animals co-exposed on PND 10 and 10-11. During the last 20 min of the spontaneous behaviour mice co-exposed on PND 10-12 displayed significantly increased activity for the above-mentioned variables when compared to controls and animals co-exposed on PND 10 and PND 10-11.

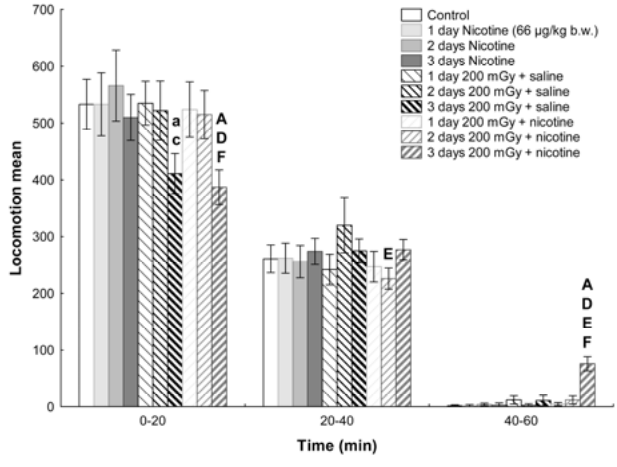

Figure 3. Spontaneous behaviour of 2-month-old NMRI male control mice, mice exposed to 200 mGy gamma radiation, mice exposed to nicotine (66 µg/kg b.w.) and mice co-exposed to 200 mGy gamma radiation and to nicotine (66 µg/kg b.w.) on postnatal day 10, 10-11or 10-12. Height of bars represents mean ± SD. The statistical differences are indicated as: (A) significantly different vs. control, $p \leq 0.01$; (a) significantly different vs. control, $p \leq 0.05$; (c) significantly different vs. 200 mGy, $p \leq 0.05$; (D) significantly different vs. 66 µg/kg b.w nicotine on PND 10-12, $p \leq 0.01$; (E) significantly different vs. 200 mGy on PND 10-12, $p \leq 0.01$; (F) significantly different vs. nicotine 66 µg/kg b.w. and 200 mGy on PND 10-11, $p \leq 0.01$.

Immediately after the 60 min spontaneous behaviour all mice were injected s.c. with 80 µg/kg b.w. nicotine base and observed for an additional 60 min time period. A normal response in mice when challenged to nicotine is to display an increase in activity for the variables locomotion, rearing and total activity (Nordberg and Bergh, 1985, Eriksson et al., 2000). Control mice responded in a normal way to the nicotine injection with an increase in activity for the above-mentioned variables, when compared to the activity counts registered during the last time period of spontaneous behaviour observation. This observed elevation in activity gradually decreased over the 60 min (60-120 min) observational period. Mice neonatally exposed only to nicotine on PND 10, 10-11 and 10-12 as well as mice neonatally irradiated on PND 10 and 10-11 also displayed a normal behavioural response to nicotine. Mice neonatally exposed only to radiation on PND 10-12 were significantly hypoactive during the first 20 min of the nicotine-induced behavioural observation and significantly hyperactive towards the end of the observational period when compared to controls and to mice irradiated on PND 10 and 10-11.

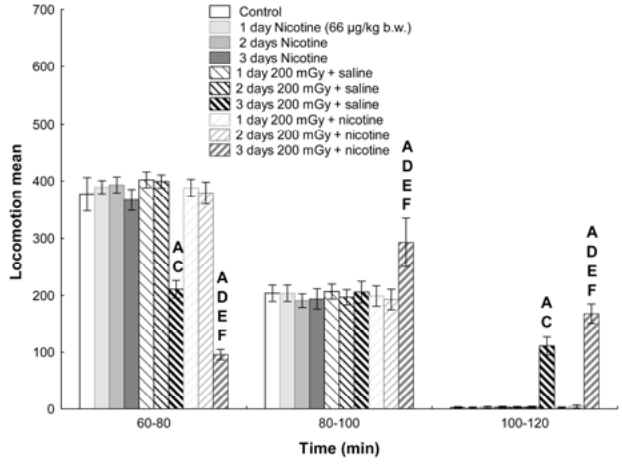

Figure 4. Nicotine-induced behaviour of 2-month-old NMRI male control mice, mice exposed to 200 mGy gamma radiation, mice exposed to nicotine (66 µg/kg b.w.) and mice co-exposed to 200 mGy gamma radiation and to nicotine (66 µg/kg b.w.) on postnatal day 10, 10-11or 10-12. Immediately after the initial 60 min time period of spontaneous behaviour observation (see fig. 3), all tested animals were injected with a challenge dose of 80 µg/kg b.w. nicotine base s.c. and observed for variables locomotion, rearing and total activity for an additional 60 min time period (fig. 4). Height of bars represents mean ± SD. The statistical differences are indicated as: (A) significantly different vs. control, $p \leq 0.01$; (C) significantly different vs. 200 mGy, $p \leq 0.01$; (D) significantly different vs. 66 µg/kg b.w nicotine on PND 10-12, $p \leq 0.01$; (E) significantly different vs. 200 mGy on PND 10-12, $p \leq 0.01$; (F) significantly different vs. nicotine 66 µg/kg b.w. and 200 mGy on PND 10-11, $p \leq 0.01$.

Mice neonatally co-exposed to nicotine and IR on PND 10 or 10-11 displayed a normal behavioural response when being challenged to nicotine while mice neonatally co-exposed to nicotine and IR on PND 10-12 were significantly hypoactive in the beginning of the observational period and significantly hyperactive towards the end, also when compared to controls. The behavioural alterations observed in mice co-exposed on PND 10-12 were also more pronounced when compared to mice only exposed to IR on PND 10-12. This suggests that an agent acting on the cholinergic system can interact with IR to exacerbate developmental neurotoxic effects, manifested as a lack of habituation and reduced cognitive function, as well as cholinergic system dysfunction in the adult mouse. Furthermore, it has been shown that IR can interact with environmental pollutants. In a study by Eriksson and co-workers (2010a) a similar disturbance in spontaneous behaviour and habituation capacity was observed following co-exposure to MeHg (0.4 mg/kg b.w.) and IR (200 mGy) as observed here in mice co-exposed to nicotine and IR on PND 10-12 in study II. In the study by Eriksson and co-workers (2010a) the disruptions in spontaneous behaviour and habituation capacity were not present in mice only exposed to a single agent.

In addition to investigating the effect of fractionated irradiation and co-exposure to nicotine and IR, on spontaneous behaviour and susceptibility of the cholinergic system, a part of study II was performed to explore if there is a dose-response relationship following neonatal nicotine exposure. No alterations in spontaneous behaviour and habituation capacity were observed in the mice only exposed to nicotine. However, in the nicotine-induced behaviour test, mice exposed to nicotine on PND 10-13 showed a significantly hypoactive response to the nicotine injection when compared to controls and all other nicotine exposure groups, which displayed a normal increase in activity when challenged to the cholinergic agent. This is in line with a previous study by Eriksson and co-workers (2000) where an altered response to nicotine at 2-months of age, following nicotine exposure on PND 10-14 was observed. No spontaneous behaviour alterations or aberrant habituation capacity were observed (Eriksson et al., 2000).

General discussion

Taken together these studies show that developmental neurotoxic defects, manifested as disrupted spontaneous behaviour, impaired or modified habituation capacity, altered levels of essential neuroproteins and cholinergic system dysfunction can be induced in a dose-response related manner following neonatal exposure to low-dose IR. Furthermore, IR can interact with chemicals such as nicotine to exacerbate behavioural disturbances and cholinergic system dysfunction.

A particularly vulnerable period for induction of persistent neurotoxicity, expressed as aberrant spontaneous behaviour, learning and memory defects, alterations in cholinergic system receptor populations and/or altered levels of neuroproteins has been seen at around PND 10 in mice for a wide range of chemicals and environmental pollutants (Eriksson et al., 1984, Eriksson et al., 2000, Ankarberg et al., 2001, Viberg et al., 2008, Johansson et al., 2009, Viberg, 2009, Eriksson et al., 2010b). In this thesis we show that the vulnerable period of the BGS, around PND 10 in mice, is also applicable when studying induction of neurotoxicity as a result of exposure to low-dose IR.

Here we show that a single dose of 350 mGy is sufficient to induce persistent neurotoxicity. Worth noting is that the behavioural alterations as well as the observed altered levels of neuroproteins are prominently expressed following higher acute radiation exposure than will be received during a conventional CT scan. The dose delivered during a single CT scan is dependent on a wide range of factors such as scanning time, size of the patient, degree of overlapping adjacent CT slices and tube voltage (Brenner and Hall, 2007) but has been estimated to range between 21-153 mGy/scan (Leitz and Almén, 2010, Pearce et al., 2012b). Noteworthy, around 30% of patients younger than 22 years old underwent more than one CT during the years 1993-2002 in Great Britain (Pearce et al., 2012a). Late cognitive effects resulting from irradiation to non-target tissue in radiotherapy in children (Mulhern et al., 2004) is also an exposure route which needs to be taken into consideration when executing risk-benefit estimations of dosimetry in the clinic.

Another challenge when investigating non-cancer effects following exposure to low-dose IR is to quantify the specific organ dose and the corresponding biological effect. Organs and tissues differ in their radiosensitivity and the age of the patient is an important factor to consider, since children by nature are more radiosensitive than adults. The use of humanlike phantoms can provide a tool for measuring and calculating organ doses which are translated into a CT dose index. However, the CT dose index will not relate the measured dose to organ risk, rather just provide a tool for quality assurance (Brenner and Hall, 2007). Furthermore, estimations of isoeffective doses when comparing acute irradiation and fractionated/protracted irradiation schemes are most often based on the linear-quadratic model. This way of modelling isoeffective doses is based on calculations of radiotherapy schemes and has thus only been proven to be accurate in the range of 2-15 Gy/fraction (Brenner, 2008). The linear-quadratic model is developed to estimate elevated risks coupled to cancer incidence e.g. DNA single and double strand breaks and chromosomal aberrations which may not necessarily be the causative factors underlying the behavioural observations seen in this thesis. In this thesis the radiation doses are well below 2 Gy/fraction rendering a high degree of uncertainty in estimations of isoeffective dose using the linear-quadratic model. However, when comparing the behavioural

output observed in study I and II it appears that three 200mGy fractions are as potent as an acute dose of 350 mGy for induction of persistent developmental neurotoxicity in mice. The underlying mechanisms behind these radiation-induced behavioural disturbances are not known but recent proteomic data suggest that acute radiation exposure on PND 10 leads to rapid dendritic spine and synapse morphology alterations via aberrant cytoskeletal signalling and processing (Kempf et al., 2014). Further, neonatal exposure to ionizing radiation, although at doses 10 times higher than in this thesis, have shown reduced proliferation and increased apoptosis in the subventricular zone (SVZ) and the granular cell layer (GCL) of the dentate gyrus within 24 hours post irradiation (Fukuda et al., 2004). Because several processes, including cell death, reduced proliferation and morphological changes, might be involved in the acute radiation-response in the neonatal brain, no known model can be applied to predict the outcome of repeated fractions of low doses. However, the results in our study, showing similar effects of 200 mGy/day (3 days) and 350 mGy in a single fraction (Buratovic et al., 2014), may suggest a "half-life" of one day ($f(n) = S \sum_{i=1}^{n} (1/2)^{i-1}$) in the target (n= total absorbed dose; s=delivered dose; i= number of fractions).

The findings in this thesis, together with previous findings, also suggest a shift of the dose-response curve for IR towards lower doses, when co-exposure to different types of environmental agents occurs. It is of special interest and importance to continue investigating if interaction effects can be observed following exposure to IR and medical drugs which may have implications for risk-benefit estimations in vulnerable populations such as children.

Summary in Swedish

Utvecklingsneurotoxikologiska effekter av lågdos joniserande strålning och interaktionseffekter med nikotin

Denna avhandling syftar till att undersöka neurotoxiska effekter orsakade av exponering för lågdosstrålning under en känslig period i hjärnutvecklingen under nyföddhetsperioden hos mus. Vidare undersöks även samexponering för lågdosstrålning och nikotin under samma utvecklingsperiod hos mus.

Vi utsätts vardagligen för olika typer av strålning genom vår miljö men även vid medicinska undersökningar eller behandlingar. I takt med teknikens framsteg blir de medicinska undersökningsinstrumenten t.ex. CT-scan mer lättillgängliga och även billigare att använda. Detta har resulterat i en markant ökning av olika typer av röntgenundersökningar som utförs på patienter, där barn utgör en betydande del av patientgruppen.

Nyföddhetsperioden hos många däggdjur, inklusive människa, karaktäriseras av snabb tillväxt och utveckling av hjärnan. Hos människa påbörjas denna tillväxt under den sista trimestern av graviditeten och fortsätter under barnets första levnadsår. Hos mus och råtta sträcker sig denna period från födseln och 3-4 veckor därefter. Många studier har visat att hjärnan är mycket känslig för exponering för olika kemikalier under denna utvecklingsperiod. En studie har visat att barn som exponerats för joniserande strålning, i medicinskt syfte, under nyföddhetsperioden hade reducerad kognitiv förmåga i vuxen ålder. Kopplingar mellan neuropsykiatriska åkommor som ADHD eller autism och exponering för olika kemikalier t.ex. nikotin tidigt i livet har föreslagits. Även neurodegenerativa sjukdomar som Alzheimer misstänks vara beroende av både genetisk predisponering och levnadsmiljön.

Studierna i denna avhandling visar att den outvecklade hjärnan är känslig för strålning under samma kritiska period som tidigare har visats för kemikalier. Exponering för lågdos joniserande strålning under denna kritiska period av hjärnans utveckling resulterade i försämrad kognitiv förmåga och förhöjda nivåer av neuroprotein, som kopplas till Alzheimer, hos det vuxna djuret. Den försämrade kognitiva förmågan som observerades verkar inte vara könsbunden.

Samexponering för lågdos joniserande strålning och nikotin under nyföddhetsperioden resulterade i försämrad kognitiv förmåga samt förändring-

ar i det kolinerga systemet, vilket är kopplat till kognition, beteende, inlärning och minne, i den vuxna individen. Vidare observerades dessa neurotoxiska effekter av samexponering vid doser där exponering för enbart strålning eller nikotin inte hade någon effekt.

Vetskapen att lågdos joniserande strålning kan samverka med kemikalier för att förvärra neurotoxiska effekter gör att fokus bör riktas mot möjliga samverkanseffekter mellan joniserande strålning och läkemedel eller miljöföroreningar.

Acknowledgements

This thesis was carried out at the Department of Environmental Toxicology, Uppsala University, Sweden. I wish to express my sincerest gratitude to those who have helped me through and contributed to this work:

Per Eriksson: my excellent supervisor. Thank you for all the support, laughs and understanding you have given me through the years. You have helped me a good bit on the hard road of becoming an independent researcher by always letting me try my ideas (even the ones you knew beforehand wouldn't work out good). It has been and will continue to be a privilege to be a part of your group and awesome work.

Anders Fredriksson: my co-supervisor and "mouse whisperer". I have never met anyone who knows as much as you do about mouse behaviour and I am grateful that you have taken me under your wings and passed down the legacy.

Henrik Viberg: my co-supervisor and "research Wikipedia". Thanks to you I have learnt how to attack a problem from different angles when the most common solutions haven't worked out.

Bo Stenerlöw: my co-supervisor and radiation expert. Thank you for guiding me through the world of radiation and for all the fun scientific as well as non-scientific discussions we have had.

Synnöve Sundell-Bergman: you have taught me to look beyond the graphs and statistical significances and see the true application of my work. For this I am forever grateful!

Iwa Lee: my treasured partner in crime. Thank you for all the fun times in lab, all the not so fun times in lab and all other times too. Together we are one brain!

My family: For your unconditional love. As well as for all the stress relief and dog sitting!

Miljötox, with all its past and present members.

This work was financially supported by the European Community's Seventh Framework Program (EURATOM) [CEREBRAD; grant number 29552] and the Swedish Radiation Safety Authority.

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