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Low-Dose Ionizing Radiation Induces Neurotoxicity in the Neonate

Acute or fractionated doses and interaction with xenobiotics in mice

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Abstract

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This thesis examines the developmental neurotoxic effects of exposure to low-dose ionizing radiation (IR), alone or together with xenobiotics, during a critical period of neonatal brain development in mice.

During mammalian brain development there is a period called the brain growth spurt (BGS), which involves extensive growth and maturation of the brain. It is known that neonatal exposure during the BGS to xenobiotics can have a negative impact on neonatal brain development, resulting in impaired cognitive function in the adult mouse. In humans, the BGS starts during the third trimester of pregnancy and continues for approximately 2 years in the child.

The present thesis has identified a defined critical period, during the BGS, when IR can induce developmental neurotoxicity in mice. The observed neurotoxicity was not dependent on sex or strain and manifested as altered neurobehaviour in the adult mouse. Furthermore, fractionated dose exposures appear to be as potent as a higher acute dose. The cholinergic system can be a target system for developmental neurotoxicity of IR, since alterations in adult mouse cholinergic system susceptibility were observed. Co-exposure to IR and nicotine exacerbated the behavioural disturbances and cholinergic system dysfunction. Furthermore, co-exposure with the environmental agent paraquat has indicated that the dopaminergic system can be a potential target.

In this thesis, clinically relevant doses of IR and a sedative/anesthetic agent (ketamine) were shown to interact and exacerbate defects in adult mouse neurobehaviour, learning and memory, following neonatal exposure, at doses where the single agents did not have any impact on the measured variables. This indicates a shift in the dose-response curve for IR, towards lower doses, if exposure occurs during the neonatal brain development. In addition, co-exposed mice, showing cognitive defects, expressed elevated levels of tau protein in the cerebral cortex. Furthermore, exacerbation of neurochemical deviations were observed following co-exposure compared to irradiation alone.

Further investigations of neurotoxic effects following fractionated or acute low-dose IR, modelling the clinical situation during repeated CT scans or levels of radiation deposited in non-target tissue during radiotherapy, and possible interaction effects with xenobiotics, is of great importance in the field of radioprotection.

Keywords: Ionizing radiation, Neonatal, Neurotoxicity, Behaviour, Nicotine, Ketamine, Mouse, Cognition, Acute irradiation, Fractionated irradiation

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List of Papers

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

I Eriksson, P., **Buratovic, S**., Fredriksson, A., Stenerlöw, B., Sundell-Bergman, S. (2016) Neonatal exposure to whole body ionizing radiation induces adult neurobehavioural defects: Critical period, dose-response effects and strain and sex comparison. *Behavioural Brain Research* **304:** 11-19.

II **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 levels of tau protein in mice. *NeuroToxicology* **45:**48-55.

III **Buratovic, S**., Stenerlöw, B., Fredriksson, A., Sundell-Bergman, S., Eriksson, P. (2016) Developmental effects of fractionated low-dose exposure to gamma radiation on behaviour and susceptibility of the cholinergic system in mice. *International Journal of Radiation Biology* DOI:10.3109/09553002.2016.1164911.

IV **Buratovic, S**., Stenerlöw, B., Fredriksson, A., Sundell-Bergman, S., Eriksson, P. (2016) Developmental effects of neonatal fractionated co-exposure to low-dose gamma radiation and paraquat on behaviour in adult mice. *Manuscript*.

V **Buratovic, S**., Stenerlöw, B., Sundell-Bergman, S., Fredriksson, A., Viberg, H., Gordh, T., Eriksson, P. (2016) Ketamine interacts with low dose ionizing radiation during brain development to impair cognitive function in mice. *Submitted.*

Additional Publications

The following papers were also published during the course of my doctoral studies, but are not included in the thesis.

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- Bakshi, M., Azimzadeh, O., Barjaktarovic, Z., Kempf, S., Merl-Pham, J., Hauck, S., **Buratovic, S**., Eriksson, P., Atkinson, M., Tapio, S. (2015) Total body exposure to low-dose ionizing radiation induces long-term alterations to the liver proteome of neonatally exposed mice. *Journal of Proteome Research* **14:**366-373.

- **Buratovic, S**., Viberg, H., Fredriksson, A., Eriksson, P. (2014) Developmental exposure to the polybrominated diphenyl ether PBDE 209: Neurobehavioural and neuroprotein analysis in adult male and female mice. *Environmental Toxicology and Pharmacology* **38:**570-585.

- Kempf, S., Casciati, A., **Buratovic, S**.,Janik, D., von Toerne, C., Ueffing, M., Neff, F., Moertl, S., Stenerlöw, B., Saran, A., Atkinson, M., Eriksson, P., Pazzaglia, S., Tapio, S., (2014) The cognitive defects of neonatally irradiated mice are accompanied by changed synaptic plasticity, adult neurogenesis and neuroinflammation. *Molecular Neurodegeneration* **9:**57.

- Kempf, S., **Buratovic, S.,** von Toerne, C., Moertl, S., Stenerlöw, B., Hauck, S., Atkinson, M., Eriksson, P., Tapio, S., (2014) Ionising radiation immediately impairs synaptic plasticity-associated cytoskeletal signalling pathways in HT22 cells and in mouse brain: An *In Vitro/In Vivo* comparison study. *PLoS ONE* 9(10):e110464. Doi:10.1371/journal.pone.0110464.

- Lee, I., Eriksson, P., Fredriksson, A., **Buratovic, S.,** Viberg, H. (2015) Developmental neurotoxic effects of two pesticides: Behavior and neuroprotein studies on endosulfan and cypermethrin. *Toxicology* **335:**1-10.

- Lee, I., Eriksson, P., Fredriksson, A., **Buratovic, S.,** Viberg, H., (2015) Develpomental neurotoxic effects of two pesticides: Behavior and biomolecular studies on chlorpyrifos and carbaryl. *Toxicology and Applied Pharmacology* **3:** 429-438.

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Abbreviations

ACh	Acetylcholine
ANOVA	Analysis of variance
ADHD	Attention deficit hyperactivity disorder
BBB	Blood-brain barrier
BGS	Brain growth spurt
b.w.	Body weight
CaMKII	Calcium/calmodulin-dependent kinase II
ChAT	Choline acetyltransferase
CNS	Central nervous system
CT	Computed tomography
GAP-43	Growth associated protein 43
GLM	General linear model
GluR1	Glutamate receptor 1
Gy	Gray
IQ	Intelligence quotient
IR	Ionizing radiation
LQ	Linear-quadratic
LTP	Long-term potentiation
mAChR	Muscarinic acetylcholine receptor
MAP	Microtubule-associated protein
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRT	Multiple range test
MWM	Morris water maze
nAChR	Nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NMRI	Naval medical research institute
PND	Postnatal day
PSD95	Postsynaptic density 95
RAM	Radial arm maze
ROS	Reactive oxygen species
RT	Radiotherapy
s.c.	Subcutaneous
SD	Standard deviation

Introduction

This thesis focuses on developmental neurotoxic effects, following neonatal exposure to ionizing radiation (IR) or co-exposure to IR and different types of xenobiotics, during a defined critical period of brain growth and maturation in mice.

Brain development and vulnerable periods

The brain is generally considered as one of the least radiosensitive organs, an assumption which is applicable for the adult brain. The immature brain of a young child or toddler is suggested to be more vulnerable to radiation-induced insults, due to the high degree of proliferating cells in the central nervous system (CNS) and the incomplete establishment of functional neuronal circuits.

The brain growth spurt

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 fetal developmental period. During embryonic brain development the brain acquires its general shape and structure, accompanied with multiplication of both glial and neuronal precursor cells. Insults occurring during this time period, independent of causative factor, may result in anatomical malformations of the brain's structures. Fetal brain development is characterized by the start of formation and maturation of functional circuits in the brain. A specific period during the 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 explained by extensive myelination of neurons, synaptogenesis, dendritic arborisation and proliferation of glial cells (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, it begins at birth,

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 transmitter systems, e.g. the cholinergic system.

It has been shown that exposure to toxicants during the BGS can induce alterations in neurochemical composition, defects in neurobehaviour and altered susceptibility of the cholinergic system in a long-lasting/persistent manner (Eriksson, 1984, Eriksson et al., 1992, Fredriksson et al., 1993, Eriksson, 1997, Ankarberg et al., 2004, Viberg et al., 2008b). Interestingly, only when the toxicant was present during the peak of the BGS, were the neurotoxic manifestations apparent in the adult mouse, while acute toxic effects induced before or after the peak of the BGS appear to be of a reversible nature (Eriksson et al., 2000).

Development of the cholinergic system

The cholinergic system is known to be involved in multiple physiological processes e.g. cognition, learning, memory and attention (Karczmar, 1975, Abreu-Villaca et al., 2011). The enzyme choline acetyltransferase (ChAT) functions to catalyze 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 levels observed in mature adults (Large et al., 1986). Interestingly, 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: muscarinic acetylcholine receptors (mAChR) and nicotinic acetylcholine receptors (nAChR) (Dale, 1914). nAChRs are pentamers made up of different α (α_2-α_{10}) and β (β_2-β_4) subunits and function as ligand-gated ion channels (Gotti and Clementi, 2004). nAChR can be activated by endogenous acetylcholine as well as nicotine (Slotkin, 1998). Differentiation of nAChRs occur postnatally in mice and a distinct ontogeny of the high ($\alpha_4\beta_2$) and low (α_7) affinity nicotinic binding sites in the cerebral cortex can be observed. In rat, the high affinity nicotinic binding sites make up 100 % of the nAChRs which can be observed at birth, after which they decrease to constitute 80 % of the nAChR in the adult individual. Low affinity nicotinic binding sites have been observed on PND 17 but not on PND 5 and make up 20 % of the

nAChRs in the adult rat (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 exposure to nicotine, on PND 10-14, around the peak of the BGS. (Eriksson et al., 2000, Ankarberg et al., 2001, 2004).

Neuronal protein markers: function and ontogeny

Expression and regulation of a vast number of neuroproteins during neonatal brain development is essential to ensure proper function later in life. Only neuroproteins which differed significantly in their expression levels are discussed in this thesis.

Synaptophysin is present in high concentrations at the axonal terminals of neurons. By regulating 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 have 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 (MAP) 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 phosphorylated tau isoforms have been observed to impair normal learning and memory functions in humans and is therefore used as a diagnostic marker for 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 levels of tau peaked on PND 3-7 in the hippocampus and between PND 7-10 in the cerebral cortex and whole brain (Viberg, 2009).

Ionizing radiation

Much focus has been directed towards adverse effects following irradiation to radiotherapeutic doses and a proposed increase in cancer incidence. Less is known about non-cancer effects and how exposure to low and moderate

doses affects normal brain development, during late fetal or early postnatal life *in vivo*.

Vulnerable periods for induction of cognitive dysfunction and/or malformations of the brain's anatomy have been observed in both human and mouse, following *in utero* exposure to IR (BEIR-V, 1990, Baskar and Devi, 2000, ICRP, 2007 , Verreet et al., 2015). Epidemiological studies of atomic bomb survivors, exposed to IR *in utero* have indicated that such exposure, during early gestation and organogenesis, between week 8 and 15 of pregnancy, increases the risk for the child to suffer from anatomical malformations of the brain, microcephaly, severe mental retardation and impaired cognitive functions later in life. These severe malformations and neurotoxic manifestations are less prominent if exposure occurs between week 16 and 25 of pregnancy (Schull et al., 1990, Otake and Schull, 1998, ICRP, 2007).

Exposure to IR in the medical field, by radiological methods for imaging purposes, diagnostics and screening, and radiotherapy (RT) for tumor treatment, has come to represent the major source of such exposure in the general population (Mettler et al., 2000, Mettler et al., 2008, Bernier et al., 2012). The use of computed tomography (CT) scans has increased in general over the past decades and particularly in the fields of pediatric diagnosis and adult screening (White, 1996, Brenner and Hall, 2007). Estimations of brain doses received during CT scans show an increasing trend with decreasing age of the patient, where children under the age of 5 years are exposed to absorbed doses within the range of 50-100 mGy/scan (Brenner and Hall, 2007, Trattner et al., 2014). Furthermore, approximately 40 % of patients receiving a head CT scan had undergone previous head CTs (Mettler et al., 2000). The relatively high radiation dose delivered during a CT scan, compared to conventional X-ray, has resulted in CT scans being accountable for 40-70% 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 years have been subjected to approximately 10% 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 15% of the total collective effective dose in the general population (Mettler et al., 2000, Mettler et al., 2008). For pediatric patients, it is of great importance that the imaging physician adapts the scanning protocol to the individual patient in order to minimize the dose delivered to the target organ, e.g. the brain. Such dose reduction can be achieved by adjusting image quality to match the diagnostic goal. However, this approach is seldom applied in clinical neuroradiology (Trattner et al., 2014).

An epidemiological study has suggested that exposure to IR, to average doses of 120-150 mGy, to the brain during infancy and early postnatal brain development, for treatment of cutaneous hemangioma, may have negative impact on cognitive development during childhood and result in impaired cognitive function for the adult individual (Hall et al., 2004).

In RT, for treatment of tumors in the CNS, multiple sessions of fractionated irradiation at doses of 2 Gy/fraction are required. Due to the nature of high energy gamma- or X-rays significant levels of radiation are also deposited in non-target tissue. This has been shown to elevate the risk of developing late cognitive dysfunction in pediatric patients, as well as a progressive cognitive decline in adult patients (Pollack et al., 1995, Mulhern et al., 2004, Douw et al., 2009). In pediatric patients undergoing RT, the use of sedation/anesthesia prior to or during the procedure is often applied to relieve anxiety or to ensure safe and accurate treatment. A recent study showed that 100% of patients under 3 years of age and about 50% of patients aged 7- 8 years were either sedated or under general anesthesia during RT (McMullen et al., 2015).

Differences in neurotoxic manifestations, dependent of timing of exposure, have been observed *in vivo*. Irradiation to 1 Gy during gestation in mice, corresponding to week 7 and 8 of human pregnancy, has been found to induce morphological malformations, e.g. enlarged ventricles and reduction in normal cortical thickness, as well as alterations in signaling pathways important for LTP and synaptic plasticity (Kempf et al., 2015a, Verreet et al., 2015). On the other hand, irradiation during the BGS in mice to equivalent doses as used in the study by Verreet et al (2015), did not result in gross anatomical malformations of the brain structures (Kempf et al., 2015c). Instead, neonatal irradiation to 500 mGy has been shown to induce defects of a functional nature, affecting the adult animal's neurobehaviour and cognitive function (Eriksson et al., 2010). Furthermore, effects manifested as altered proteomic content and signaling pathways related to synaptic plasticity, dendritic function, LTP, mitochondrial function and cytoskeletal integrity in a long term/persistent manner, have been observed following neonatal irradiation to 500 mGy (Kempf et al., 2014a, Kempf et al., 2014b, Kempf et al., 2015b, Kempf et al., 2015c).

There is no common classification of radiation dose, what is considered a low-dose in RT is classified as a high dose in the field of radioprotection. Therefore, the radiation doses in this thesis have been classified as follows: Low dose $\leq$ 500 mGy; Moderate dose 501 mGy to 2 Gy; High dose > 2 Gy.

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 fetus. As a result, the most pronounced adverse effect of smoking during pregnancy is low birth weight (Ellard et al., 1996, Lambers and Clark, 1996). Cognitive

defects and lower intelligence quotient (IQ) are intimately coupled to low birth weight independent 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 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. Nicotine binds directly to the receptors and can also, via other receptors, cause an increase in ACh, serotonin, dopamine and epinephrine release into the synaptic cleft (Wonnacott et al., 1989).

In vivo 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, on PND 10-14, to 66 µg nicotine base/kg body weight (b.w.) has been shown to affect nAChR binding properties, cause behavioural disturbances, learning and memory impairments in the adult mouse (Eriksson et al., 2000, Ankarberg et al., 2001).

Paraquat

Paraquat (*N*,*N*′-dimethyl-4,4′-bipyridinium dichloride) was introduced on the market in 1962 (Sagar, 1987) and has since then become one of the most commonly used herbicides in agriculture. It is primarily classified as a pulmonary toxicant, but has also been suggested to be a neurotoxicant in mammals (Grant et al., 1980, Woolley et al., 1989, Dinis-Oliveira et al., 2008). Paraquat shares structural similarities with the known dopaminergic neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), indicating a similar mode of action. Therefore, environmental exposure to paraquat has been suggested to induce parkinsonism (McCormack et al., 2002, Dinis-Oliveira et al., 2006). It has been proposed that paraquat exerts it neurotoxic properties by inducing dopaminergic cell death, elevate levels of reactive oxygen species (ROS) and therefore alter normal mitochondrial function in the neuron (Peng et al., 2004, Castello et al., 2007, Chen et al., 2010). However, it is debated whether paraquat can cross the blood-brain barrier (BBB) and induce parkinsonism (Corasaniti et al., 1991, Naylor et al., 1995, Bartlett et al., 2009). One commonly applied hypothesis is that paraquat can cross the BBB if its permeability is altered in some way. One *in vivo* study has shown that the BBB of young or old rats is more permeable for paraquat than in middle-aged rats (Corasaniti et al., 1991). Furthermore, behavioural alterations, learning and memory impairments and an altered neurochemical state in the adult mouse striatum have been demonstrated following neonatal exposure to 0.36 mg/kg b.w. paraquat. Importantly, no sign of acute toxicity,

e.g. respiratory distress, was observed in the animals (Fredriksson et al., 1993).

Ketamine

Ketamine is used in the pediatric clinic for anesthesia during a wide variety of surgical procedures, e.g. diagnostic and interventional cardiac procedures, plastic surgery and neurosurgery (Zook et al., 1971, Singh et al., 2000, Dong and Anand, 2013). Furthermore, ketamine is also used as an effective analgesic or sedative agent in the pediatric emergency department, intensive care unit and during invasive or painful diagnostic procedures (Dial et al., 2001, Lin and Durieux, 2005, Anderson and Palmer, 2006, Dong and Anand, 2013). Dial et al. (2001) further reported that ketamine, alone or in combination with other agents, was the most frequently used sedative agent in the studied children's hospital and used in 88% of the studied cases.

Ketamine exerts its pharmacological properties by acting as an N-methyl-D-aspartate (NMDA) receptor (NMDAR) antagonist (Duchen et al., 1985, Harrison and Simmonds, 1985, Dong and Anand, 2013). The NMDAR is a tetrameric, ionotropic glutamate receptor, named after its selective binding of the agonist N-methyl-D-aspartate. The NMDAR is heteromeric, consisting of the key GluN1 subunit and one or more of the regulatory GluN2A-D subunits. The NMDAR has been shown to be involved in synaptic plasticity and to be essential for learning and memory functions, through formation of LTP (Bliss and Collingridge, 1993, D'Souza et al., 1993, Furukawa et al., 2005, Li and Tsien, 2009).

Glutamatergic signaling is important for normal mammalian brain development, where stimulation of glutamate receptors has been shown to promote synaptogenesis, neuronal migration and differentiation (Komuro and Rakic, 1993, Guerrini et al., 1995, Lujan et al., 2005). Altering expression of the NMDAR subunits, during the late fetal and early postnatal period, suggests differences in functional properties essential for normal development (Monyer et al., 1994). While the expression of GluN1 is ubiquitously present during both pre- and postnatal development, expression of the modulatory GluN2A-B follows a distinct ontogenetic pattern. In rats, GluN2B is the predominantly expressed subunit in the immature synapse during embryonic development and at birth, after which it decreases during the first two weeks of postnatal life. Simultaneously, the expression of GluN2A-containing NMDARs increases during development and is predominantly expressed in mature synapses in rat cerebral cortex and hippocampus (Takai et al., 2003, Lujan et al., 2005). This switch in expression of functional units in the NMDAR has also been described in human neonatal cortex (Jantzie et al., 2015).

Behaviour and cognition

The spontaneous behaviour in a novel home environment measures the animals' habituation capability by registering three variables: locomotion, rearing and total activity. The fundamentals of habituation involve a cognitive processing of applied stimulus, which is followed by an appropriate physiological/physical response. As the tested subject habituates, to whichever form of stimulus, there will be a decrease in innate behaviour shown over time, if no new or stronger stimulus is applied (Thompson and Spencer, 1966, Groves and Thompson, 1970, Rankin et al., 2009). Habituation is not to be confused with the concept of adaptation, which in short terms is based on exhaustion of sensory receptor output and does not call for a conscious decision or processing of sensory input of the subject being tested (Thompson and Spencer, 1966). In this study, the stimulus is represented by the novel home environment, i.e. novel home cage and novel bedding material. As the mouse is lowered into the behavioural observation cage, its innate exploratory behaviour renders it to have an initially high activity whilst searching through and exploring its novel home environment. As time progresses the mouse will retrieve already attained information, originating from the weekly cage changes, and process the new sensory input with regard to what it has already learnt about this procedure. The cognitive processing of the novel stimulus will in a mouse, with a normal spontaneous behaviour profile, result in a decrease in activity counts for the innate behaviors locomotion, rearing and total activity over the one hour observational period.

The Morris water maze (MWM) was initially described by Richard Morris, in the early 1980s, as a tool to investigate spatial learning and memory capabilities in rats (Morris, 1981). This particular maze setup has gained popularity and is probably the most commonly used water maze nowadays. Presumably due to its lack of pre-training, easy adaptation to multiple different protocols, depending on which aspect of spatial learning the investigator wants to elucidate, and the possibility to apply it to numerous different species ranging from mice to humans (Morris, 1984, Kallai et al., 2005, Eriksson et al., 2010). The general concept underlying the spatial acquisition in the MWM is that the animal must learn to navigate, in a direct path, to the position of a submerged platform by using external, fixed cues. The protocol for reversal learning can be used to measure whether or not the animal is able to learn a new, direct pathway to the platform and thus extinguish the previously acquired information about the platforms position (Vorhees and Williams, 2006) Performance in the MWM has been suggested to rely on hippocampal function which is linked to LTP, proper NMDAR function and synaptic plasticity (Morris et al., 1990, Bannerman et al., 1995). Furthermore, proper function of the cholinergic system has been shown to be crucial

for learning spatial tasks which are dependent on external cues (Sutherland et al., 1982, Whishaw, 1985, Riekkinen et al., 1990).

Another commonly used maze for investigation of spatial learning is the radial arm maze (RAM). The RAM was first described by Olton and Samuelson in the late 1970s, with the aim to investigate working memory in rats (Olton and Samuelson, 1976). The RAM requires the animal to retain memory of which arms it has already visited, in other words, an intact working memory function. Since the initial introduction of the RAM, alternative protocols have been developed and it can now be used as a tool to distinguish between different types of memory functions, e.g. working and reference memory, by baiting different sets of arms. In contrast to the MWM, the RAM requires introduction of a motivator in order for the test animal to perform the task. This is most often achieved by depriving the animal of food prior to test start and to keep it on a restricted diet throughout the testing period, meaning that the animal must be closely monitored to assure sustained health and well-being. Furthermore, from an ethical point of view, the RAM is superior to the MWM in toxicological studies when investigating agents that are suspected to impair motoric function, e.g. when modeling parkinsonism.

Objectives

The objective of this thesis was to investigate the developmental neurotoxic effects of low-dose exposure to ionizing radiation during a suggested critical period of neonatal brain development in mice. 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 and levels of essential neuroproteins.

- Whether there are sex or strain differences in susceptibility to develop neurotoxic manifestations following neonatal low-dose irradiation.

- Whether neonatal fractionated low-dose irradiation can induce similar behavioural alterations as single dose irradiation.

- Whether xenobiotics acting on the cholinergic or dopaminergic system can interact with low-dose ionizing radiation to exacerbate developmental neurotoxic effects.

- Whether neonatal co-exposure to low-dose ionizing radiation and a commonly used pharmaceutical can interact to exacerbate cognitive defects and what impact co-exposure to these agents can have on the neurochemical content in the adult mouse brain.

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. Pregnant Naval Medical Research Institute (NMRI) or C57BL/6 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

The exposure doses used in this thesis have been chosen based on previous studies where no/minor effect of the single compounds were observed on spontaneous behaviour, learning and memory and/or levels of neuroproteins (Fredriksson et al., 1993, Eriksson et al., 2000, Viberg et al., 2008b, Eriksson et al., 2010). All irradiations were performed using an external radiation source.

Table 1 shows the exposure schemes for the studies included in this thesis. Each individual exposure group was represented on each respective exposure day.

Study I: A) male NMRI mice were whole-body gamma irradiated with a single dose of 500 mGy on PND 3, 10 or 19. B) NMRI and C57BL/6 mice of both sexes were whole-body gamma irradiated with a single dose of 20, 100, 500 or 1000 mGy on PND 10. Control animals were placed in the irradiation chamber and sham irradiated.

Study II: NMRI mice of both sexes were whole-body irradiated with a single dose of 350 or 500 mGy on PND 10. Control animals were placed in the irradiation chamber and sham irradiated.

Study III: male NMRI mice were exposed to whole-body gamma irradiation 200 mGy/fraction, (-) nicotine base 66 µg/kg b.w. subcutaneously (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(total dose 400 mGy) or PND 10-12 (total dose 600 mGy), (or only nicotine on PND 10-13). Control animals received saline (10 ml/kg b.w.) s.c. and were sham irradiated.

Study IV: male C57BL/6 mice were exposed to whole-body gamma irradiation 100 or 300 mGy/fraction (total dose 200 or 600 mGy, respectively), paraquat 0.02 or 0.2 mg/kg b.w. as a single oral dose, co-exposed to 100 mGy and 0.02 or 0.2 mg mg/kg b.w. paraquat as a single oral dose or 300 mGy and 0.02 or 0.2 mg mg/kg b.w. paraquat as a single oral dose on PND 10-11. Control animals received a single oral dose of 10 ml of 20% fat emulsion vehicle/kg b.w. and were sham irradiated.

Study V: male NMRI mice were whole-body gamma irradiated with a single dose of 50, 100 or 200 mGy, exposed to ketamine 7.5 mg/kg b.w. s.c. or co-exposed to 7.5 mg/kg b.w. ketamine and 50, 100 or 200 mGy. Control animals received saline (10 ml/kg b.w.) s.c. and were sham irradiated.

Table 1. *Exposure scheme for studies I-V*

	PND 3	PND 10	PND 10-11	PND 10-12	PND 19
Study I	x	x			x
Study II		x			
Study III		x	x	x	
Study IV			x		
Study V		x			

Irradiation and exposure chemicals

In **study I, II** and **III** the 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). In **study IV** and **V** the whole-body irradiation was performed using a ^{137}Cs source, dose-rate at the surface was 0.2 Gy/min (The Rudbeck laboratory, Uppsala University, Uppsala, Sweden).

The (-)nicotine-bi-(+)tartrate in **study III** was obtained from Sigma, U.S.A. pH of (-)nicotine base was adjusted to 7.0 before s.c. injection to avoid tissue damage. The paraquat dichloride x-hydrate in **study IV** was obtained from Sigma Aldrich, Sweden. The Ketalar® in **study V** was obtained from Pfizer Inc., USA.

Behavioural tests

Spontaneous behaviour in a novel home environment

Study I: both male and female NMRI and C57BL/6 mice were observed for spontaneous behaviour at 2 and 4 months of age.
Study II: both male and female NMRI mice were observed for spontaneous behaviour at 2 and 4 months of age.
Study III: male NMRI mice were observed for spontaneous behaviour at 2 months of age.
Study IV: male C57BL/6 mice were observed for spontaneous behaviour at 2 months if age.
Study V: male NMRI mice were observed for spontaneous behaviour at 2 and 4 months of age.

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 minutes (3 consecutive 20 min periods, 0-20, 20-40 and 40-60 min) 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 counterweight connected to the test cage registered all vibrations such as movements, grooming and shaking. Total activity is included as an additional variable to detect stereotypic behaviours, e.g. excessive grooming or shaking, as this would not be detected in the locomotion or rearing variables.

Nicotine-induced behaviour

Male mice in **study III** were observed for nicotine-induced behaviour (Nordberg and Bergh, 1985, Eriksson et al., 2000). 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 (3 consecutive 20 min periods, 60-80, 80-100 and 100-120 min).

Radial arm maze (RAM)

In **study IV** control mice, mice exposed to paraquat 0.2 mg/kg b.w., irradiated with 300 mGy or co-exposed to 0.2 mg/kg b.w. paraquat and irradiated with 300 mGy on PND 10-11 were tested for learning and memory capabilities in the RAM at 3 months of age. Ten individuals/exposure group were randomly taken from 3-4 different litters. The maze was positioned 60 cm above the floor, consisted of 8 arms (8 × 35 cm, surrounded by a 2 cm boarder) radiating from a circular platform (Ø 20 cm). A food pellet (approximately 5 mg) was placed behind a low boarder 3 cm from the outer end of each arm. The mice were deprived of food 24 h before the first day of acclimatization, and kept on a restricted diet throughout the testing period, being closely monitored for weight loss, exclusion criteria were less than 95% of free feeding weight. Water was provided *ad libitum* throughout. The day before the test started each mouse was allowed to acclimatize to the maze setup for 10 min. Thereafter, the mice were tested for 10 minutes on two consecutive days (Fredriksson and Archer, 1996). The acclimatization and testing was performed during the light part of the day, between 09.00 and 15.00. Each mouse was placed on the central platform with its head facing a randomly predetermined arm and allowed to explore the maze for 10 minutes. Time to complete the task, i.e. retrieve all eight food pellets, number of total arm entries and errors were recorded. An error was defined as re-entry into an already visited arm after the food pellet had been collected or if the mouse did not retrieve the pellet.

Morris water maze (MWM)

In **study V** control mice, mice exposed to ketamine (7.5 mg/kg b.w.), irradiated with 100 or 200 mGy or co-exposed to ketamine (7.5 mg/kg b.w.) and 100 or 200 mGy on PND 10, were tested for learning and memory capabilities in the MWM (Morris, 1981), at 5 months of age. Ten to 14 individuals, from each exposure group, were randomly taken from 3-4 different litters. A gray circular container with a diameter of 103 cm was filled with water (22^{o}C) to a depth of 15 cm. A metal mesh platform, 12 cm in diameter, was submerged 1 cm beneath the water surface in the middle of the northwest quadrant. External visual cues were positioned on the north, south, east, and west wall. The animals were tested for 4 consecutive days with the platform in a fixed location to test their spatial learning ability. On the 5th day, the platform was relocated to the middle of the northeast quadrant to test their relearning ability (Eriksson et al., 2010). The position of the observer remained fixed throughout the testing period. The mouse was placed on the platform for 20 s before each trial and then released into the southwest quadrant, with its head toward the wall of the pool. Each individual was given 5 trials of 30 s/trial to locate the platform each day. After each trial the mouse rested on the platform for 20 s.

Neuroprotein analysis

Slot Blot analyses of neuroprotein levels in cerebral cortex and hippocampus were performed in:

Study II: Control mice and mice exposed to 500 mGy (n=12/exposure group) were analyzed for levels of Calcium/calmodulin-dependent kinase II (CaMKII), Growth associated protein 43 (GAP-43), synaptophysin and tau 24 h and 6 months post-irradiation.

Study V: Mice from all exposure groups (n=6/exposure group) were analyzed for levels of CaMKII, GAP-43, Glutamate receptor 1 (GluR1), Postsynaptic density 95 (PSD95), synaptophysin and tau 6 months post-exposure.

Brains were dissected out on an ice-cold glass plate, snap frozen in liquid nitrogen and stored in -80^{o}C until assayed. 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., 2008a, Viberg, 2009). Antibodies against GluR1 (Upstate Millipore,

AB1504) and PSD95 (Upstate Millipore, MABN68) were also used following evaluation of specificity with the Western blot procedure.

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 rabbit polyclonal GluR1 (1:1000) for GluR1, 5 µg and mouse monoclonal PSD95 (0.1 µg/ml) for PSD95, 3µg and mouse monoclonal synaptophysin (1:10000) for synaptophysin and 3.5 µg and mouse monoclonal tau (1:1000) for tau. A horseradish peroxidase conjugated secondary antibody against mouse (KPL 074-1806, 1:20000) or rabbit (KPL 074-1506, 1:20000) 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 III; IV** and **V**, interaction factors), recorded in the spontaneous and nicotine-induced behaviour observations, were subjected to an analysis of variance (ANOVA), balanced data, or a general linear model (GLM), unbalanced data, with split plot design and pairwise testing using a Duncan's multiple range test (MRT) in the software SAS 9.1 (Kirk, 1968, Lazic and Essioux, 2013).

Radial arm maze

In **study IV**, data of the time to complete the task, i.e. retrieve all eight food pellets, and the number of errors made, as indexed by total arm entries subtracted by the number of pellets retrieved, were subjected to a one-way ANOVA, pairwise testing using Duncan's MRT, using the software STATISTICA 10.

Morris water maze

In **study V**, the latency time to locate the submerged platform on the first four consecutive days (treatment, treatment x day, between subjects, within subjects and interaction factors) and comparisons of improvement in latency

time between trial 21 and trial 25 was subjected to a GLM with split-plot design and pairwise testing using Duncan's MRT. Improvement in latency time between trial 21 and 25, for each respective exposure group, was submitted to a Student's paired t-test. The analyses were conducted using the software SAS 9.1.

Slot Blot analysis

Study II and V: the data of protein levels from the Slot Blot analysis of CaMKII, GAP-43, synaptophysin and tau in cerebral cortex and hippocampus (GluR1 and PSD 95 were additional proteins investigated in **study V**) was normalized against controls and subjected to a one-way ANOVA, pairwise testing by Duncan's MRT using the software STATISTICA 10.

Results and Discussion

In this thesis, non-cancer effects of low-dose ionizing radiation (IR) were investigated in a neonatal animal model. The aim was to explore how exposure to low-dose IR, during a critical period of neonatal brain development, could impact behaviour and cognitive capabilities in the adult individual. Furthermore, possible target systems for radiation-induced neurotoxicity and interaction effects between IR and xenobiotics were investigated with regard to spontaneous behaviour and habituation capacity, learning and memory, cholinergic system susceptibility, as well as neurochemical composition.

The fundamentals of developmental neurotoxicity of low-dose ionizing radiation in the neonatal mouse model

In study I, an investigation of the hypothesized critical period for induction of developmental neurotoxicity, following exposure to low-dose IR, in the neonatal mouse model, was performed. Previous studies on environmental pollutants, pharmaceuticals and social drugs have indicated that exposure during a defined period in the neonatal mouse could induce not only acute effects, but also long-lasting or persistent cognitive disturbance or dysfunction.

Male NMRI mice were whole-body irradiated with a single 500 mGy dose on PND 3, 10 or 19 and observed for spontaneous behaviour in a novel home environment at 2 months of age (Fig. 1). Control mice and mice irradiated with 500 mGy on PND 19 showed a normal spontaneous behaviour and habituation capacity, at the young adult age of 2 months, by displaying a significant decrease in activity counts over the three consecutive 20 min periods. On the other hand, mice irradiated on PND 3 or 10 showed a disruption in normal spontaneous behaviour by being significantly hypoactive during the first 20 min period (0-20 min), a time period when the exploratory behaviour is expected to yield the highest activity counts. Furthermore, these mice were significantly hyperactive during the last 20 min period (40-60 min), a time period when the activity is expected to have decreased significantly if the animal has a normal ability to process and respond to the surrounding sensory stimuli, a process known as habituation. Taken together,

this deviant spontaneous behaviour is indicative of a lack of habituation and thus cognitive dysfunction (Rankin et al., 2009). Interestingly, this critical window for induction of developmental neurotoxicity, manifested as defect neurobehaviour, appears to be similar for IR and chemicals. Previous studies on developmental neurotoxicity in the neonatal mouse model have shown that persistent and non-persistent chemical agents are able to induce defects in adult mouse neurobehaviour if exposure coincides with, or if the chemical is present in the brain, during the peak of the BGS in mice (Eriksson et al., 1992, Eriksson et al., 2000, Eriksson et al., 2002).

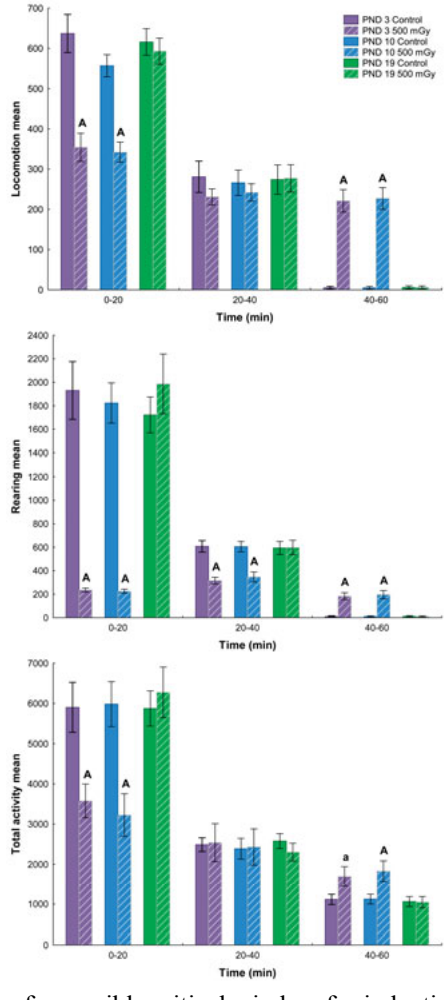

Figure 1. Investigation of a possible critical window for induction of developmental neurotoxicity in 2-month-old male NMRI mice, following a single irradiation to 500 mGy on PND 3, 10 or 19. Height of bars represent mean ± SD. The statistical differences are indicated as: A = $p \leq 0.01$ vs. control; a = $p \leq 0.05$ vs. control.

Acute effects, e.g. alterations in cholinergic receptor subpopulations, have been observed following exposure during the animal's first three weeks of life, but only when the chemical was present around PND 10 were the behavioural effects detectable in all three behavioural variables in the adult individual (Eriksson et al., 2000, Viberg et al., 2003, 2005).

The mechanism underlying the observed developmental neurotoxicity following low-dose irradiation is still not known. However, IR exposure to doses which are 10-15 times higher than used in this study, suggest a rapid increase in apoptosis and decreased proliferation in the subgranular zone of the dentate gyrus in the hippocampus (Fukuda et al., 2004, Roughton et al., 2012). Partly differing from a majority of the studies performed on chemicals, there were no differences in the observed defects in neurobehaviour between exposures on PND 3 or 10. With the knowledge that low-dose IR induces mitochondrial dysfunction and oxidative stress as an acute response (Kempf et al., 2015b), it is possible that this altered neurochemical state of the developing brain is present on PND 10 and has an impact on the brain's normal development, after exposure on PND 3. This would also give an explanation to why no defects in neurobehaviour were observed following irradiation on PND 19, since the brain in many aspects has developed to maturity at that time point. However, one cannot reject the possibility that the critical window for induction of developmental neurotoxicity is longer for IR than previously observed for chemicals or that there is another underlying mechanism, e.g. DNA-damage, genomic instability or bystander effects, resulting in the observed defect neurobehaviour.

Study I also aimed to investigate whether there are any sex differences or strain differences in neurobehavioural effects, concerning the induction of developmental neurotoxicity, following low-dose IR exposure. NMRI and

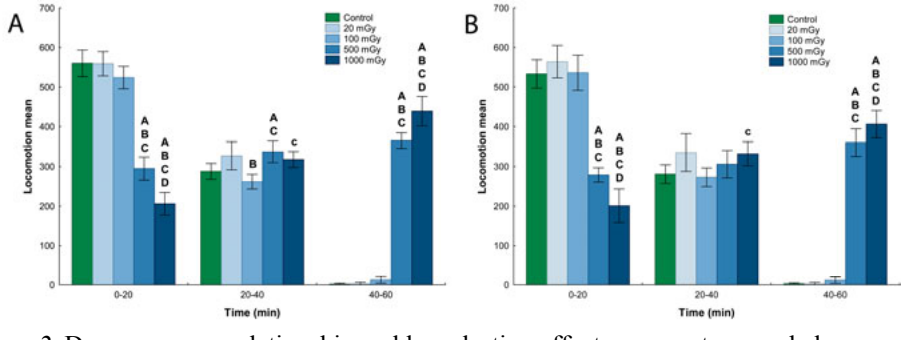

Figure 2. Dose-response relationship and long-lasting effects on spontaneous behaviour in 2-month-old (A) and 4-month-old (B) male NMRI mice, whole-body irradiated on PND 10, with 0, 20, 100, 500 or 1000 mGy. Height of bars represent mean ± SD. The statistical differences are indicated as: A = $p \leq 0.01$ vs. control; B = $p \leq 0.01$ vs. 20 mGy; C = $p \leq 0.01$ vs. 100 mGy; c = $p \leq 0.05$ vs. 100 mGy; D = $p \leq 0.01$ vs. 500 mGy.

C57BL/6 mice of both sexes were whole-body irradiated with a single dose of 20, 100, 500 or 1000 mGy on PND 10 and observed for spontaneous behaviour in a novel home environment at 2 and 4 (NMRI only) months of age (Fig. 2, only NMRI male mice shown).

No differences were observed when comparing neurobehavioural outcomes of the two strains and sexes. Mice, regardless of strain or sex, showed a significant behavioural alteration, manifested as hypoactivity during the first 20 min, hyperactivity during the last 20 min of the behavioural observation, and lack of habituation following neonatal irradiation with 500 or 1000 mGy, but not after 20 or 100 mGy, indicating that there are no differences in radiosensitivity for the tested endpoint. Furthermore, the observed effects were significantly more pronounced in mice irradiated with 1000 mGy, compared to mice irradiated with 500 mGy, suggesting that the neurotoxic manifestations are dose-response dependent.

In study II, an investigation of a possible threshold value for induction of developmental neurotoxicity was performed. Male and female NMRI mice were whole-body irradiated with a single dose of 350 or 500 mGy on PND 10 and observed for spontaneous behaviour in a novel home environment at 2 and 4 months of age (Fig. 3, only 2 months behaviour shown). In concordance with study I, mice irradiated with 500 mGy showed a lack of habituation by being significantly hypoactive in the beginning (0-20 min) of the behavioural observation and significantly hyperactive during the last 20 min (40-60 min) of the observational period, when compared to controls and mice exposed to 350 mGy. Interestingly, male and female mice irradiated with 350 mGy displayed a modified spontaneous behaviour by being significantly hypoactive, compared to controls, in the 0-20 min observational period.

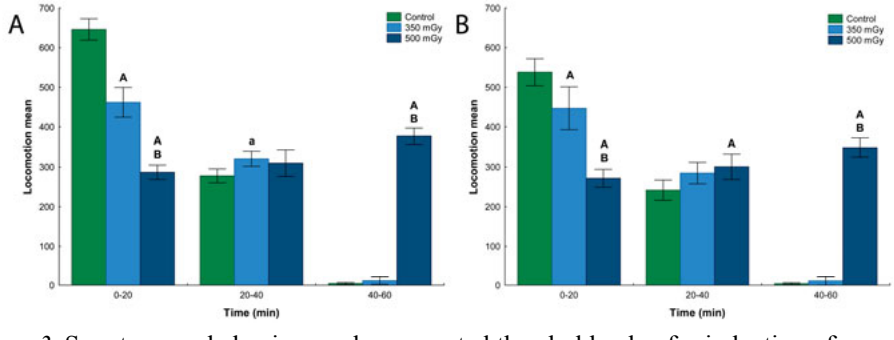

Figure 3. Spontaneous behaviour and a suggested threshold value for induction of developmental neurotoxicity in young adult male (A) and female (B) NMRI mice irradiated with 0, 350 or 500 mGy on PND 10. Height of bars represent mean ± SD. The statistical differences are indicated as: A = p≤ 0.01 vs. control; a = p≤ 0.05 vs. control; B = p≤ 0.01 vs. 350 mGy.

At the end of the observation, there was no difference in activity counts between controls and mice exposed to 350 mGy, suggesting that a single neonatal dose of 350 mGy is a potential threshold dose for induction of developmental neurotoxicity in both male and female mice.

Taken together, the results from study I and II show that there appears to be a critical window, around PND 10, for induction of long-term or persistent neurobehavioural defects, following neonatal low-dose irradiation. Furthermore, a threshold dose of 350 mGy has been observed following acute radiation exposure. These findings are well in agreement with a previous study, where a disruption in spontaneous behaviour and lack of habituation in male NMRI mice was observed, after neonatal irradiation with 500 mGy, but not after a 200 mGy dose (Eriksson et al., 2010). A vulnerable period for induction of developmental neurotoxicity following *in utero* irradiation in mice, with regard to cognitive function and alterations in brain morphology in the adult individual has been proposed by other researchers (Baskar and Devi, 2000, Verreet et al., 2015), suggesting that the most vulnerable time period of embryonic mouse development occurs around embryonic day 11, a time period characterized by extensive neurogenesis, while later fetal stages of gestation seem more radio-resistant. Nevertheless, the doses required to induce cognitive dysfunction during embryonic development are higher than the doses used in study I and II. In a study by Verreet *et al* (2015) a dose of 1 Gy during the proposed vulnerable embryonic period was required to induce developmental neurotoxic manifestations, e.g. impaired cognitive function, which may be explained by the observed malformations of the brain.

No clear difference in susceptibility, for induction of developmental neurotoxicity, following neonatal irradiation was observed between the outbred NMRI and inbred C57BL/6 mouse strain. Interestingly, neither study I nor study II showed sex-differences in susceptibility to low-dose radiation for induction of developmental neurotoxicity. In contrast, other *in vivo* studies and epidemiological data suggest that females are more susceptible than males to develop neurotoxic manifestations, following early life exposure to IR. The assumption that females are more susceptible than males to radiation-induced neurotoxicity in humans can be derived back to studies in patients being treated for tumors in or near the central nervous system. However, treatment with radiotherapy alone is rarely conducted; rather a combination of both radiotherapy and chemotherapy is applied to maximize a positive outcome of the therapy for the patient. This introduces a level of uncertainty of whether it is the radiation, the chemotherapy or an interaction effect of the two agents, that exacerbates the cognitive impairments observed in female patients (Armstrong et al., 2007). Accordingly, *in vivo* studies suggesting exacerbation of behavioural defects and impaired neurogenesis in female mice, following early life irradiation with 8 Gy on PND 14, also introduce some levels of uncertainty by anesthetizing the animals prior to irradiation, without controlling for possible interaction effects between the anes-

thetic and IR (Roughton et al., 2012, Roughton et al., 2013). Furthermore, the possibility that the observed sex-differences, in both humans and mice, are dose-dependent and may be a result of different initiating and/or underlying mechanistic events needs to be taken into consideration and further investigated.

Dose fractionation and the effect on behaviour and cognition

In study III and IV, the effect of dose fractionation on spontaneous behaviour in a novel home environment and habituation capability was investigated. Male NMRI mice were whole-body irradiated with one 200 mGy fraction/day on PND 10, 10-11 or 10-12 or with on 100 or 300 mGy fraction/day on PND 10-11 and behavioural observations were conducted at the young adult age of 2 months (Fig. 4).

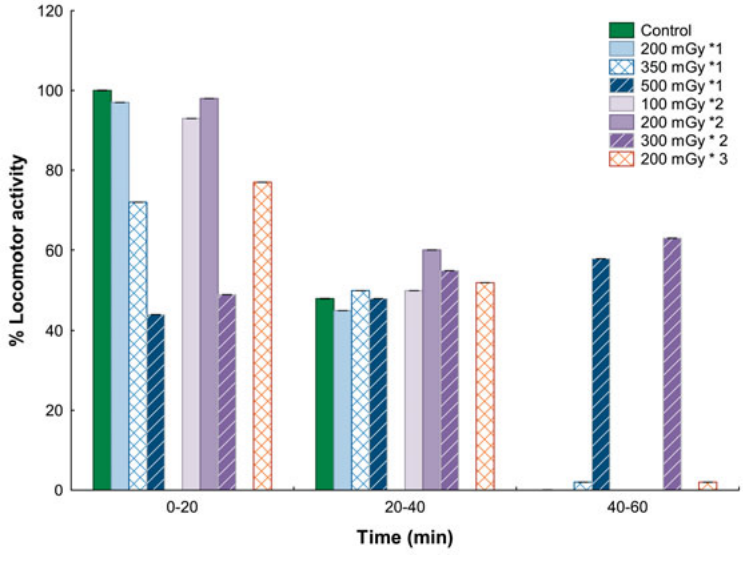

Figure 4. The effect of dose fractionation on spontaneous behaviour and habituation capability in young adult mice irradiated on PND 10, 10-11 or 10-12. The locomotor activity of control mice was set to 100 % and activity of irradiated mice normalized against controls. Height of bars represent the mean activity.

In order to be able to compare behavioural profiles, following single and fractionated dose exposures, control mouse locomotor activity was set to 100% and activity counts from irradiated mice in study II, III and IV were normalized against the controls. Mice irradiated with two fractions of 300 mGy showed a lower activity during the first 20 min (0-20 min) of the spontaneous behaviour observation and a higher activity during the last 20 min (40-60 min), when compared to controls and mice exposed to a single dose of 350 mGy. Mice irradiated with three fractions of 200 mGy displayed a lower locomotor activity during the first 20 min period of testing compared to controls, mice irradiated with one or two 200 mGy fractions as well as mice irradiated with two 100 mGy fractions.

When comparing behavioural profiles of single and fractionated exposure regimes it becomes evident that two fractions of 300 mGy (total dose 600 mGy) results in similar neurobehavioural alterations and lack of habituation as has been observed for an acute 500 mGy dose. Furthermore, mice irradiated with three 200 mGy fractions (total dose 600 mGy) show a similar modified habituation capacity as mice irradiated with a single 350 mGy dose. These findings indicate that fractionation of low-dose IR can be as potent for induction of developmental neurotoxicity as higher acute dose exposures (Fig. 4).

Determination of isoeffective doses of fractionated/protracted exposure regimes are most commonly based on the linear quadratic (LQ) model. This mechanistic approach, developed to estimate and quantify DNA double strand breaks, is indeed appropriate for estimation of risk for induction of secondary cancers, following exposure to radiation doses in the range of 2 – 18 Gy (Brenner, 2008). However, for non-cancer endpoints or low-dose exposures, there is no known model for estimation of isoeffective dose.

In an attempt to model neurocognitive function in rodents exposed during different life stages to different types, doses and/or fractions of radiation,Tome et al. (2016) suggest a conversion to equivalent dose in 2 Gy fractions (EQD_2), based on the LQ formulation:

$$EQD_2 = nd\frac{\alpha/\beta + d}{\alpha/\beta + 2}$$

(n = number of fractions, d = dose per fraction, nd = total dose. α/β ratio = 2 Gy).

The fundamental of the EQD_2 model, like the LQ model, renders it valid for IR doses of ≥ 2Gy and not appropriate for lower IR dose exposures.

However, despite the lack of knowledge about the underlying mechanisms, for the observed defects in neurobehaviour in study II, III and IV, an estimation of isoeffective dose can be made by assuming a "half-life" of one day in the target:

$$f(n) = S \sum_{i=1}^{n} \left(\frac{1}{2}\right)^{i-1}$$

(n = total absorbed dose; s = delivered dose; i = number of fractions).

Even though the assumption of a half-life of one day seems appropriate in the low-dose interval (0 – 500 mGy), on this particular behavioural endpoint, further studies using alternative fractionation schemes and, maybe more importantly, investigations of underlying mechanisms are needed in order to strengthen and validate this hypothesis.

The cholinergic system as a potential target system

In study III, male NMRI mice were exposed to 66 µg/kg b.w. nicotine base twice daily, whole-body irradiated with 200 mGy/fraction or co-exposed to both agents on PND 10, 10-11 or 10-12. At the young adult age of 2 months the animals were observed for spontaneous behaviour in a novel home environment, during 60 min, and immediately after challenged with a single s.c. injection of 80 µg/kg b.w. of nicotine and observed for another 60 min (Fig. 5, only nicotine-induced behaviour shown). During the spontaneous behaviour observation, a normal habituation capacity was observed in controls, nicotine exposed mice and mice irradiated or co-exposed on PND 10 or 10-11. Mice irradiated or co-exposed on PND 10-12 were significantly hypoactive during the first 20 min of the behavioural observation. During the last period (40-60 min), a significant increase in activity was observed in mice co-exposed to nicotine and IR on PND 10-12, while no difference in activity was evident in the irradiated (PND 10-12) mice compared to controls. This indicates that neonatal co-exposure to IR and nicotine significantly alters habituation capacity in young adult mice and results in more pronounced neurobehavioural defects compared to irradiation alone.

Directly after the spontaneous behaviour observation, the susceptibility of the animals' cholinergic system was tested by challenging the mice to a known cholinergic agonist, nicotine. A normal response to stimulation of the cholinergic system, following exposure to a cholinergic agonist, e.g. nicotine, is for the mouse to display an acute/initial increase in activity (Nordberg and Bergh, 1985, Eriksson et al., 2000).

This normal response to cholinergic system stimulation was observed in controls, nicotine exposed mice and mice irradiated or co-exposed on PND 10 or 10-11. In contrast, mice irradiated or co-exposed on PND 10-12 displayed a significantly hypoactive response to nicotine (60-80 min), where co-exposed mice showed a significant exacerbation of the defect response of the cholinergic system (Fig. 5). Moreover, mice co-exposed on PND 10-12 were significantly hyperactive during the last observational period (100-120 min), providing further strength to the earlier observation of an altered/lack habituation capacity. Noteworthy, mice irradiated on PND 10-12 were significantly hyperactive during the 100-120 min observational period, indicating a worsening of neurobehavioural defects, compared to the behavioural profile observed during the spontaneous behaviour test.

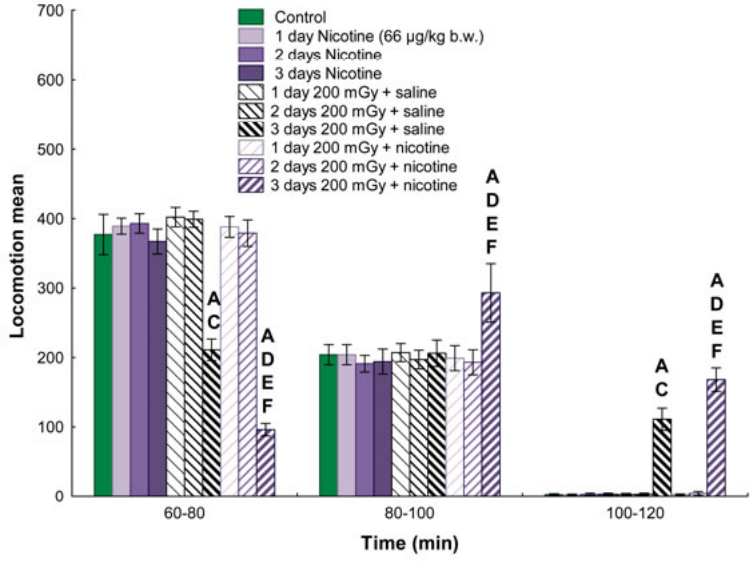

Figure 5. Nicotine induced behaviour and susceptibility of the cholinergic system in young adult NMRI mice exposed to nicotine, IR or co-exposed on PND 10, 10-11 or 10-12. Height of bars represent mean ± SD. The statistical differences are indicated as: A = p≤ 0.001 vs. control; C = p≤ 0.001 vs. 2x200 mGy; D = p≤ 0.001 vs. 3xnicotine; E = p≤ 0.001 vs. 3x200 mGy; F = p≤ 0.01 vs 2xco-exposure.

Taken together, these results indicate that exposure to low-dose IR, during a critical period of neonatal brain development in mice, could compromise or alter cholinergic system susceptibility and thus impact on cognitive function.

Earlier studies on developmental neurotoxicity, following neonatal exposure to nicotine or brominated flame retardants, showed a substantial reduction in low-affinity nicotinic binding sites in the adult mouse (Eriksson et al., 2000, Viberg et al., 2003). The observed neurochemical alterations, following neonatal nicotine exposure, were also accompanied with an altered susceptibility of the cholinergic system in these mice, rendering them to re-

spond to cholinergic system agonists in a similar way as has been observed in study III (Eriksson et al., 2000). Low-affinity nicotinic binding sites correspond to the α_7 nicotinic receptor, which has been suggested to be involved in synaptic transmission and thus learning and memory function (Levin et al., 2006). With the knowledge that the cholinergic system is undergoing a rapid developmental period, especially concerning the α_7 nicotinic receptor (Nordberg, 1993), during this critical period of brain development, a disruption of the normal ontogeny of this subpopulation of nicotinic receptors might provide an explanation to the observed neurobehavioural disturbances in study III. However, one cannot exclude disruptions in ontogeny of other nicotinic receptors, altered acetylcholine esterase activity and/or subsequent compensatory mechanisms as well as altered neuronal connectivity as the causative factor of the observed defects in neurobehaviour.

The dopaminergic system as a potential target system?

In study IV, the herbicide paraquat was co-administered with IR to investigate possible interaction effects between an agent known to affect the dopaminergic system and IR. Neonatal male C57BL/6 mice were whole-body irradiated with 100 or 300 mGy, exposed to 0.02 mg/kg b.w. or 0.2 mg/kg b.w. paraquat or co-exposed to both agents on PND 10-11. At 2 months of age, the animals were observed for spontaneous behaviour in a novel home environment (Fig. 6).

A normal spontaneous behaviour and habituation capacity was observed in controls, mice irradiated with 100 mGy and/or exposed to 0.02 mg/kg b.w. paraquat as well as mice only irradiated with 100 mGy. Mice irradiated with 300 mGy, exposed to 0.2 mg/kg b.w. paraquat, co-exposed to 100 or 300 mGy and 0.2 mg/kg paraquat or exposed to 300 mGy and 0.02 mg/kg paraquat were significantly hypoactive during the first 20 min of the behavioural observation. During the last 20 min, mice irradiated with 300 mGy or co-exposed to 300 mGy and 0.02 mg/kg paraquat displayed a significantly hyperactive behaviour compared to all other exposure groups, including controls.

This observation indicates that co-exposure to low-dose IR and paraquat can interact to exacerbate spontaneous behaviour alterations and modify habituation capacity in the adult mouse. Interestingly, mice co-exposed to 300 mGy and 0.2 mg/kg paraquat were significantly more hypoactive, compared to their irradiated internal controls, throughout the entire spontaneous behaviour observation.

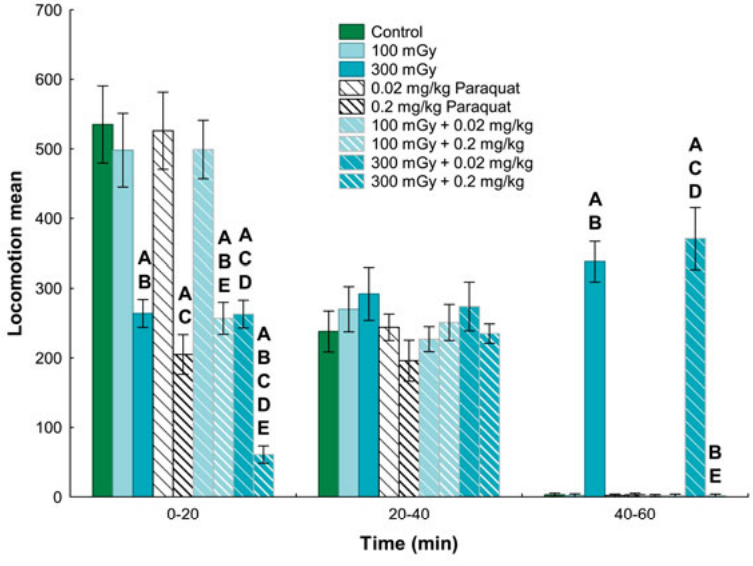

Figure 6. Interaction effects on spontaneous behaviour in 2-month old C57BL/6 mice exposed to 100 or 300 mGy, 0.02 or 0.2 mg/kg b.w. paraquat or co-exposed to both agents on PND 10-11. Height of bars represent mean ± SD. The statistical difference is indicated as: A = significantly different vs. control; B = significantly different vs. internal radiation control; C = significantly different vs. internal paraquat control; D = significantly different vs.100 mGy+ equivalent paraquat dose; E = significantly different vs equivalent radiation dose + 0.02 mg/kg paraquat. $p \leq 0.01$.

In order to investigate whether this aberrant behavioural pattern, observed in mice co-exposed to 300 mGy and 0.2 mg/kg b.w. paraquat, was predominately attributed to a motoric dysfunction or to a cognitive defect, the mice and their corresponding controls were tested for learning and memory capabilities in a RAM at 3 months of age (Table 2).

No significant weight loss was observed during the testing period. No difference in time to complete the task was observed between the different exposure groups. However, mice co-exposed to IR and paraquat made significantly more incorrect arm entries compared to controls and mice only exposed to a single agent.

Adult paraquat exposure, to doses 10-50 times higher than used in study IV, has been shown to reduce spontaneous home cage activity in mice (Litteljohn et al., 2009, Chen et al., 2010). Furthermore, impairments in learning and memory faculties, a reduction in striatal dopamine content and increased levels of ROS in the hippocampus has been observed following prolonged adult paraquat exposure (Litteljohn et al., 2009, Chen et al., 2010).

Table 2. *Learning and memory faculties in controls, mice irradiated with 300 mGy, exposed to 0.2 mg/kg b.w. paraquat or co-exposed to both agents on PND 10-11, tested in a radial arm maze.*

	Time (s)	Error (% of control)
Control	530 ±58	100 ± 26
300 mGy	498 ± 68	118 ± 26
0.2 mg/kg Paraquat	516 ± 52	114 ± 19
300 mGy + 0.2 mg/kg	587 ± 18	172 ± 35 ($p \leq 0.05$)

Exposure to paraquat in the neonatal mouse model, to doses in the same order of magnitude as in study IV, has also been shown to reduce spontaneous behaviour activity and to alter both dopamine and dopamine metabolite concentration in the adult mouse striatum (Fredriksson et al., 1993). These alterations in behaviour and neurotransmitter content have been suggested to result from increased levels of ROS (Chen et al., 2010). With the knowledge that both IR and paraquat can increase levels of ROS in the CNS, it is not unlikely that co-exposure to the two agents could interact in an additive or synergistic manner, to induce the defects in neurobehaviour, learning and memory faculties observed in the present study. However, further studies on the dopaminergic system as a potential target system for induction of developmental neurotoxicity are needed.

Interaction effects with anesthetics and shift in dose-response curve for ionizing radiation

Study V aimed to explore possible developmental neurotoxicity following exposure to clinically relevant doses of IR and a commonly used anesthetic/sedative agent ketamine. Neonatal NMRI male mice were irradiated with a single whole-body IR dose of 50, 100 or 200 mGy, exposed to ketamine (7.5 mg/kg b.w.) or co-exposed to both agents on PND 10. Control mice were injected with saline and sham irradiated. Behavioural tests were conducted at 2, 4 and 5 months of age. At 6 months of age, the animals were euthanized and the brains analyzed for neuroprotein levels.

Results from the spontaneous behaviour observations at 2 and 4 months show that control mice, mice exposed to a single agent and mice co-exposed to 50 mGy and ketamine displayed normal behaviour and a normal habituation capacity (Fig. 7). At 2 months of age (Fig. 7A), mice co-exposed to ketamine and 100 or 200 mGy showed a significantly hypoactive behaviour in the beginning and a hyperactive behaviour towards the end of the one hour observational period, compared to controls, mice exposed to a single agent and mice co-exposed to 50 mGy and ketamine. Furthermore, mice co-exposed to 200 mGy and ketamine showed a significant increase in activity

during the 40-60 min period compared to animals co-exposed to 100 mGy and ketamine.

The observed alterations in activity, at a young adult age, indicate that co-exposure to 100 or 200 mGy and ketamine can induce developmental neurotoxicity, manifested as alterations in neurobehaviour and lack of habituation, at doses where the individual agents do not have an impact on the tested endpoints.

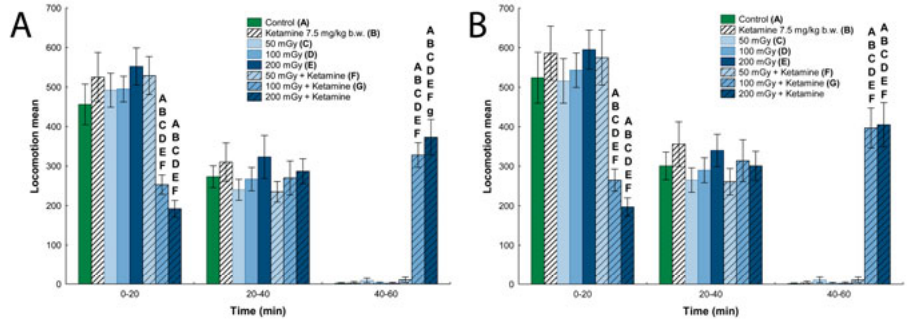

Figure 7. Interaction effects on spontaneous behaviour in 2-month old (A) and 4-mont-old (B) male NMRI mice exposed to 50, 100 or 200 mGy, 7.5 mg/kg b.w. ketamine or co-exposed to both agents on PND 10. Height of bars represent mean ± SD. The statistical differences are indicated as: A = $p \leq 0.001$ vs. control; B = $p \leq 0.001$ vs. ketamine; C = $p \leq 0.001$ vs. 50 mGy; D = $p \leq 0.001$ vs. 100 mGy; E = $p \leq 0.001$ vs. 200 mGy; F = $p \leq 0.001$ vs. 50 mGy and ketamine; g = $p \leq 0.01$ vs. 100mGy and ketamine.

To test whether the effects observed in the 2-month-old spontaneous behaviour observation were of a persistent or long-lasting nature, the mice were again tested at 4 months of age (Fig. 7B). At 4 months of age, a significantly defect neurobehaviour and lack of habituation, in concordance with the previously observed behavioural defects, were observed in mice co-exposed to 100 or 200 mGy and ketamine. In addition, no difference between mice co-exposed to 100 or 200 mGy and ketamine was evident at 4 months of age. This is indicative of a persistent or long-lasting effect on neurobehaviour and habituation capacity, induced by early life exposure to clinically relevant doses of IR and ketamine. Interestingly, exposure to a single agent did not affect the measured variables, strongly indicating an interaction effect between these two agents which are commonly used in pediatric patients.

At 5 months of age, mice irradiated with 100 or 200 mGy, exposed to ketamine or co-exposed to 100 or 200 mGy and ketamine, as well as controls, were tested for learning and memory capabilities in the MWM (Fig. 8). These exposure groups were chosen for further learning and memory testing based on the defect neurobehaviour in the spontaneous behaviour in a novel home environment observation. During the spatial acquisition period (first 4 days, trial 1-20) all mice significantly improved their latency time to find the

submerged platform. Mice co-exposed to 100 or 200 mGy and ketamine showed significantly longer average latency times to find the submerged platform, on the third and fourth day of testing, compared to control mice or mice exposed to a single agent.

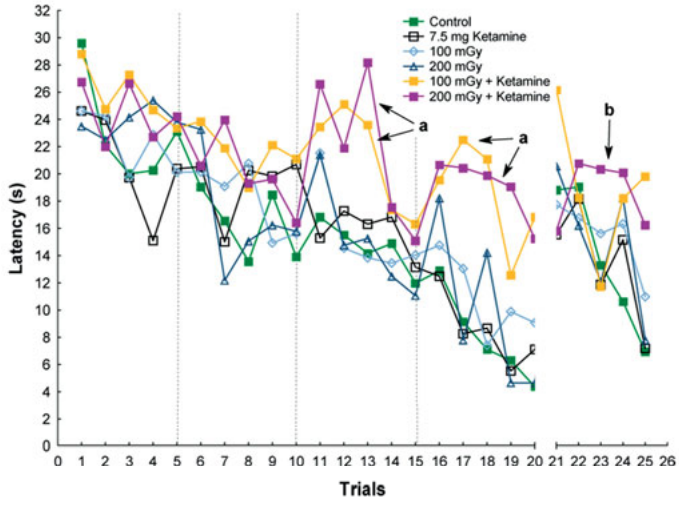

Figure 8. Learning and memory faculties of 5-month-old male NMRI mice, exposed to 0, 100 or 200 mGy, 7.5 mg/kg b.w. ketamine or co-exposed to both agents on PND 10, were tested in the MWM. The statistical differences are indicated as: a = $p \leq 0.05$ vs. control, ketamine exposed or mice irradiated with 100 or 200 mGy; b = $p \leq 0.05$ vs. control.

On the fifth day (trial 21-25) the platform was relocated to the northeast quadrant of the pool and the animals' relearning capabilities were tested in five trials. A normal relearning behaviour for a mouse is to spend longer time searching for the platform in the previous target quadrant (northwest) (Eriksson et al., 2010, Buratovic et al., 2014). Improved latency time, in trials 21-25, was observed in control mice and mice exposed to one single agent and thus showing a normal relearning behaviour. Mice co-exposed to 100 or 200 mGy and ketamine failed to improve their latency time to find the submerged platform. Furthermore, mice co-exposed to 200 mGy and ketamine were significantly different compared to controls. These observations show that co-exposure to clinically relevant doses of IR and ketamine can interact to exacerbate neurotoxic manifestations, not only on habituation capacity, but also on spatial reference memory and spatial working memory.

Quantification of neuroprotein levels in cerebral cortex and hippocampus was performed in study II and V. In study II both acute (24 h post-irradiation) and late (in 6-month-old mice) effects on neuroprotein levels, following neonatal irradiation to 500 mGy, were investigated. In study II the cerebral cortex appeared to be more sensitive to radiation-induced injury by expressing significantly elevated levels of tau protein, both as an acute and

late effect, while alterations in hippocampal neuroprotein levels seemed to be of a reversible nature. Additionally, an elevation of synaptophysin was observed in cerebral cortex 24 h post-irradiation, but not at the adult age if 6 months. The neuroprotein analysis, performed in study V, of adult male mouse cerebral cortex revealed a significant elevation of tau protein 6 months post-irradiation in mice neonatally co-exposed to 200 mGy and ketamine. Interestingly, these co-exposed mice also expressed a significant elevation of synaptophysin in the cerebral cortex at 6 months of age. This finding indicates that co-exposure to clinically relevant doses of IR and ketamine can induce long-lasting/persistent alterations in cerebrocortical synaptophysin levels, while the observed alteration in synaptophysin level following irradiation only was of a reversible nature. Acute, but not persistent, changes in synaptophysin levels have also been observed following co-exposure to different types of anesthetics (Nikizad et al., 2007). Elevated levels of tau protein in cerebrospinal fluid is commonly used as a diagnostic marker for Alzheimer's disease in human health care (Ikeda et al., 2013). However, whether the observed elevations of tau protein in cerebral cortex can provide a mechanistic explanation to the observed cognitive deficits still needs to be further investigated. Taken together, study II and V indicate that the cerebral cortex might be more susceptible to radiation-induced neurotoxicity, manifested as elevations in cerebrocortical tau protein levels 6 months post-irradiation, than the hippocampus. Furthermore, it is of special relevance to note that co-exposure to IR and ketamine seems to shift the dose-response curve from 500 mGy (IR only) to 200 mGy for induction of this type of developmental neurotoxicity, manifested as impaired cognitive function and alterations in levels of neuroproteins.

Ketamine is recognized as a developmental neurotoxicant and early life exposure has been shown to induce apoptotic neurodegeneration in many different *in vivo* models, e.g. rodents and non-human primates (Fredriksson and Archer, 2004, Slikker et al., 2007). Furthermore, late effects, such as impaired neurobehaviour and neurocognitive skills, can be observed in the adult individual following neo-/perinatal ketamine exposure (Fredriksson and Archer, 2003, 2004, Paule et al., 2011, Lecointre et al., 2015). In a previous study, impaired cognitive function and a hyperactive phenotype was observed in the adult mouse, following neonatal exposure to ketamine (50 mg/kg b.w.) (Fredriksson and Archer, 2003, 2004). Interestingly, Fredriksson and Archer (2003) reversed this aberrant phenotype by administering d-amphetamine to the hyperactive individuals. Early life exposure to general anesthesia has been proposed to increase the risk of developing attention deficit hyperactivity disorder (ADHD) in children (Sprung et al., 2012). Furthermore, stimulants such as d-amphetamine or methylphenidate hydrochloride are often used as part of the treatment for ADHD symptoms, in both pediatric and adult patients (Solanto, 1998). Noteworthy, pretreatment with clonidine has been shown to ameliorate the neurotoxic manifestations on

neurobehaviour and apoptotic neurodegeneration following neonatal exposure to ketamine (Ponten et al., 2012).

Taken together, study V shows that low-doses of IR can interact with clinically relevant doses of ketamine to induce developmental neurotoxicity in the neonatal mouse brain, which might have consequences for induction of neurological or neurodegenerative diseases/disorders.

General considerations for risk-assessment

A majority of studies conducted up to date, with the aim to investigate detrimental effects of early life exposure to IR and late impact on cognitive function, have been performed in either atomic bomb survivors or pediatric patients being treated for tumors in the CNS. These studies have shed light on how the developing CNS can be affected by embryonic or high dose IR exposure and have developed models to facilitate the prediction and prevention of secondary cancers or cognitive dysfunction, as a direct result of medical or anthropogenic/environmental exposure to radiation. The present thesis aimed to investigate how the vulnerable neonatal brain could be affected by exposure to low doses of IR and what impact this exposure could have on neurobehaviour, cognitive function and neuroprotein content. Furthermore, this thesis aimed to investigate the effect of fractionated dosing regimens, compared to single dose exposures, and if co-exposure to IR and xenobiotics could exacerbate developmental neurotoxic manifestations by altering the susceptibility of the developing CNS or to induce impairments or alterations in cognitive processes and functions.

A particularly vulnerable period of neonatal brain development has been established in mice. Exposure to different types of xenobiotics during this period has been shown to alter neurobehaviour, learning and memory, cholinergic system function and nicotinic receptor composition as well as neuroprotein content (Eriksson, 1997, Eriksson et al., 2000, Eriksson et al., 2002, Buratovic et al., 2014). This thesis shows that the proposed vulnerable period, around PND 10 in mice, is also relevant for induction of persistent or long-lasting radiation-induced developmental neurotoxicity. It has been proposed that females are more susceptible than males to develop cognitive dysfunction following radiotherapy (Armstrong et al., 2007). Furthermore, *in vivo* studies on developmental neurotoxicity following 8 Gy acute irradiation have also suggested that female mice are more susceptible than male mice to develop late manifestations of neurotoxicity (Roughton et al., 2012, Roughton et al., 2013). Interestingly, in this thesis no difference was observed between male and female mice to develop an altered/impaired neurobehaviour following neonatal irradiation to a low to moderate dose of 350 - 1000 mGy. These findings are in concordance with proteomic studies on neonatally irradiated C57BL/6 female and NMRI male mice (Kempf et al., 2014a, Kempf

et al., 2014b, Kempf et al., 2015c). In these studies, no clear differences were observed between the male NMRI and female C57BL/6 mouse, 24 h or 6-7 months post irradiation. Both strains and sexes displayed overlapping alterations in deregulated signaling pathways and proteins following neonatal irradiation to moderate doses between 500 and 1000 mGy. The deregulated signaling pathways were primarily involved in synaptic plasticity and maintenance of a normal dendritic morphology while the deregulated proteins were coupled to LTP. Furthermore, the observed proteomic alterations were prominent following irradiation to 500 mGy, but not 100 mGy, which is well in agreement with the observed defects in neurobehaviour in this thesis. Moreover, no gross alterations in the brain's morphology or state of myelination was observed in female mice neonatally irradiated to 100, 500 or 2000 mGy, while irradiation to 8 Gy on PND 14 significantly impaired white matter growth in the adult female mouse (Roughton et al., 2013, Kempf et al., 2015c).

The dose delivered during a single CT scan is dependent on many different factors, such as scanning time and size of the patient (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 scan during the study period (Pearce et al., 2012a). Late cognitive effects resulting from irradiation of 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 human-like phantoms provides 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 LQ 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 2-18 Gy/fraction (Brenner, 2008, Tome et al., 2016). The LQ model is preferably related to tumor control and normal tissue complications i.e., acute effects which are due to cell killing, which may not necessarily be the causative factors underlying the behavioural observations seen in this thesis. Possible explanations to the cognitive disruption and altered neurochemical composition observed in this thesis might be derived to genomic instability or bystander effects. In this thesis the radiations doses are well below 2 Gy/fraction rendering a high

degree of uncertainty in estimations of isoeffective dose, using the LQ model. However, when comparing the neurobehavioural pattern observed in the present thesis, it appears that three 200 mGy fractions (total dose of 600 mGy) are as potent as an acute dose of 350 mGy and two fractions of 300 mGy (total dose 600 mGy) are as potent as an acute 500 mGy dose, for induction of persistent developmental neurotoxicity in mice, which would suggest a retention of 50% of the radiation-induced damage over 24 h.

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 an anesthetic/sedative agent occurs. Worth noting is that the behavioural alterations, as well as the observed altered levels of neuroproteins, are expressed following co-exposure to clinically relevant ketamine doses and IR doses in the range of a repeated head CT scan. Whether the alterations in neurochemical composition and/or function may alter/impact the susceptibility to pharmaceuticals or possibly accelerate neurodegenerative processes is not to be neglected and calls for further studies. 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.

Concluding Remarks

This thesis has shown that exposure to low doses of IR, during a defined critical period of neonatal brain development in mice, can result in persistent behavioural defects, cognitive disruption and alterations of essential neuroproteins. Moreover, no major difference between sexes or two commonly used laboratory mouse strains, NMRI and C57BL/6, was evident in the low-dose range for the tested endpoint.

This thesis has also shown that a fractionated irradiation scheme can be as potent as higher acute dose exposures. When comparing neurobehavioural output between single and fractionated doses, an assumption of 50 % retention of the induced effect/damage over 24 h may provide an estimation of isoeffective dose following low-dose fractionation.

It has also been shown that IR can interact with different types of xenobiotics, e.g. commonly used social drugs affecting the cholinergic system agents affecting the dopaminergic system, and pharmaceuticals affecting the GABA-ergic system, to exacerbate neurotoxic manifestations in mice. It is plausible that a toxic agent or pharmaceutical with a targeted mode of action, e.g. binding to specific receptors in the brain or interference with the redox state of the CNS, will alter the neonatal susceptibility of the target and render it more vulnerable to the general neurotoxic effect/impact of IR.

Late alterations in susceptibility of the CNS, induced by early life exposure to low-dose IR and/or xenobiotics, has emerged as a novel risk-factor which needs to be taken into consideration. This thesis has shown that early life exposure to low doses of IR, alone or in combination with nicotine, resulted in an altered adult susceptibility of the cholinergic system.

In the present thesis, an interaction between IR and the commonly used sedative/anesthetic agent ketamine was seen, causing a shift in the dose-response for IR towards lower doses for both neurobehavioural and neurochemical endpoints.

There is an increased use of radiological methods, in diagnostics, imaging and therapy, primarily in the pediatric clinic where patients are suggested to have an increased vulnerability to insults. This increased vulnerability can be explained by their not fully matured brain and often more frequent requirement of analgesic/sedative agents during painful or invasive procedures, compared to adult patients. This calls for further studies in order to develop and refine existing medical procedures and minimize the risk for this vulnerable group of patients to develop late detrimental effects on cognitive func-

tion or to show an increased incidence in neuropsychiatric symptoms or neurodegenerative disorders.

Summary in Swedish

Utvecklingsneurotoxikologiska effekter av lågdos joniserande strålning och interaktionseffekter med kemikalier

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 ämnen, vilka verkar på olika av hjärnans signalsystem, under samma utvecklingsperiod hos mus.

Vi utsätts dagligen 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. datortomografi 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 eller narkosmedel, tidigt i livet har föreslagits. Även neurodegenerativa sjukdomar som Alzheimers sjukdom eller Parkinsons sjukdom 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 relevant för kemikalier, samt att inga könsskillnader verkar föreligga. Exponering för lågdos joniserande strålning under denna kritiska period av hjärnans utveckling resulterade hos det vuxna djuret i försämrad kognitiv förmåga och förhöjda nivåer av neuroproteinet tau, som inom humanvården kopplas till Alzheimers sjukdom. Vid samexponering för kliniskt relevanta doser jonise-

rande strålning och narkosmedel kunde stråldosen minskas med 60 % och ändå orsaka förhöjningar av proteinet tau samt orsaka störningar i den vuxna musens kognitiva förmåga. Dessa förändringar i kognitiv förmåga och nivåer av proteinet tau kunde inte observeras hos möss som enbart exponerats för strålning eller sövningsmedel.

Fraktionering av stråldos är vanligt inom humanvården, för att försöka minska de neurotoxiska effekter vilka påvisats efter strålterapi riktad mot huvudet, vid behandling av tumörer i eller i närheten av hjärnan. Denna fraktionering har visat sig minska risken för att patienten utvecklar sekundära tumörer, vilka föreslås vara orsakade av DNA-skada tillfogad av strålterapin. Vid planering av strålterapi finns det således matematiska modeller vilka kan appliceras för att beräkna det mest optimala antalet fraktioner och storlek av stråldoser för att minimera risken för negativa biverkningar, orsakade av strålterapin. Något verktyg för att försöka förutsäga och förebygga kognitiva skador, förorsakade av exponering för lågdos strålning saknas. Denna avhandling indikerar en ackumulering av strålskada, efter exponering för fraktionerad lågdos strålning under nyföddhetsperioden, där ungefär hälften av den tillfogade skadan kvarstår efter ett dygn. Detta innebär att upprepad exponering för lågdos strålning kan orsaka liknande neurotoxiska effekter som högre enkeldoser.

Samexponering för lågdos joniserande strålning och nikotin, under nyföddhetsperioden hos mus, resulterade i försämrad kognitiv förmåga i den vuxna individen, samt förändringar i känsligheten hos det kolinerga systemet, vilket är kopplat till kognition, beteende, inlärning och minne. Dessa neurotoxiska effekter av samexponering observerades vid doser där exponering för enbart nikotin inte hade någon effekt. Exponering för fraktionerad lågdos joniserande strålning, under nyföddhetsperioden hos mus, orsakade liknande förändringar i känsligheten hos den vuxna musens kolinerga system som observerats efter samexponering för lågdos joniserande strålning och nikotin. Detta visar att det kolinerga systemet kan vara ett målorgan för utvecklingsneurotoxicitet orsakad av lågdos joniserande strålning. Ytterligare indikationer, efter samexponering för lågdos joniserande strålning och ett ämne känt för att verka på det dopaminerga systemet, tyder på att även detta signalsystem kan påverkas.

Vetskap om att känsligheten hos dessa viktiga signalsystem i hjärnan kan förändras och få följder för den vuxna individen, som exponerats under nyföddhetsperioden, är viktig eftersom den hos människa kan innebära att vissa patienter inte reagerar på avsett vis vid behandling med läkemedel för lindring av symptom vid t.ex. Alzheimers sjukdom eller Parkinsons sjukdom.

I denna avhandling har det visats att lågdos joniserande strålning kan samverka med kemikalier och läkemedel, för att förvärra neurotoxiska effekter t.ex. störningar i kognitiva funktioner, inlärning och minne samt känsligheten hos viktiga transmittorsystem i den vuxna musen, ifall exponering sker under nyföddhetsperioden. Det är av största vikt att fortsätta studera dessa

samverkanseffekter för att kunna utveckla behandlingsregimer vilka resulterar i en minimal negativ påverkan på den unga hjärnan. Detta kan göras genom att specificera de underliggande cellulära mekanismerna, vilka orsakar kognitiva störningar efter exponering för lågdos strålning. Dessutom är det viktigt att fortsätta undersöka hur exponering för lågdos strålning, enbart eller i kombination med kemikalier, under kritiska perioder av hjärnans utveckling kan påverka och förändra känsligheten hos den vuxna hjärnan för både toxiska agens och läkemedel. Vidare bör effekter av samexponering för lågdos strålning och vanligt använda läkemedel, för exempelvis smärtlindring, sedering eller anestesi, undersökas för att i den mån det är möjligt skydda känsliga populationer, såsom barn.

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