Behavior and cytogenesis following irradiation or isoflurane exposure to the developing brain

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Abstract

In this thesis, the effects of an anesthetic agent, isoflurane, on the young brain and resulting behavioral effects were investigated. Anesthesia is commonly used in young children during surgery or other procedures associated with pain or discomfort. Animal studies have demonstrated serious effects on the brain from exposure to anesthesia and recent human studies have found indications of learning impairments following exposure to anesthesia. It is known from animal studies that anesthetic agents can affect proliferation as well as differentiation and can lead to learning impairments. In addition, the effects of irradiation on the young brain were investigated. Cancer is one of the most common causes of death in children and radiotherapy is commonly used to treat cancer (together with surgery and/or chemotherapy). During the last decades, improvements in treatment protocols have lead to more and more children surviving their cancers. This has however also resulted in more children experiencing long-term side effects, particularly resulting from radiotherapy. These side effects include impaired intelligence and memory as well as attention deficits. From animal models, it is known that irradiation cause cell death and a long term reduction in cell proliferation in the young brain that can result in impairment on some memory tasks.

In these experiments, we have used one model of repeated isoflurane exposure and one model for radiotherapy. The animals' behavior was investigated using the IntelliCage system, as well as other behavioral tests, followed by immunohistochemical analysis of the hippocampus. Isoflurane was found to cause a reduction in cell proliferation, accompanied by a reduction in neural stem cells. No evidence of cell death was seen, and the reason behind the reduction is therefore unknown. In addition, less neuronal differentiation was seen following isoflurane exposure, accompanied by an increase in astrocyte differentiation. These effects were especially clear when the young brain was exposed. Animals that were exposed to isoflurane at a young age later developed severe and progressive memory impairments. Following irradiation, a decrease in cell proliferation in the dentate gyrus of the hippocampus was seen. The irradiated animals displayed learning and relearning deficits judged by the IntelliCage analysis, but neither open field nor trace fear conditioning tests could detect impairments.

In summary, we found irradiation-induced changes in the hippocampus and saw changes in behavior, using the IntelliCage system, that were not detectable using other methods like open field and fear conditioning. We also found isoflurane-induced changes that suggest that the young brain is particularly sensitive to anesthetic agents like isoflurane and that isoflurane-anesthesia should be used with caution, especially in pediatric patients.

Keywords: Radiotherapy, isoflurane, anesthesia, dentate gyrus, neurogenesis, memory, learning, IntelliCage, trace fear conditioning, open field, object recognition.
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Populärvetenskaplig sammanfattning på svenska

Hjärnan är ett känsligt organ, speciellt hos barn, hos vilka hjärnan genomgår en omfattande tillväxt. I denna avhandling har effekterna av ett vanligt narkosmedel och strålning på den unga hjärnan undersökts. Det är sedan tidigare känt att barn som blivit utsatta för strålning som en del av cancerbehandling kan drabbas av biverkningar senare i livet. Dessa biverkningar utgörs bland annat av inlärnings- och minnessvårigheter, men även hyperaktivitet, depression och en påverkan på det sociala livet kan ses. En förklaring till detta kan vara att strålningen påverkar delande celler i hippocampus, som är viktig för hjärnans minnesprocesser. Det är känt att det i en del av hippocampus, gyrus dentatus, finns stamceller och andra celler som fortsätter att dela sig och bilda nya nervceller under hela livet, och dessa celler kan påverkas negativt och dö av strålning. Detta kan förklara inlärnings- och minnessvårigheter efter strålbehandling.

De senaste åren har också användandet av narkosmedel fått mer uppmärksamhet på grund av möjliga biverkningar av sövningen. Nyligen publicerades studier där isofluran, ett narkosmedel, kopplades till inlärningssvårigheter. Även från djurstudier är det känt att isofluran kan leda till mindre celldelning i gyrus dentatus men även att isofluran kan leda till celldöd i hjärnan.

I denna avhandling har en modell för strålning och en modell för isofluran-sövning använts. För att utvärdera effekter på beteende har ett relativt nytt system som heter IntelliCage använts. IntelliCage är en metod för att utvärdera mössens beteende i en mer naturlig och social miljö. Utöver detta har även andra beteendemetoder använts. Efter beteendetesterna analyserades hjärnorna med immunohistokemi.

Strålning ledde till klara inlärnings- och minnessvårigheter i IntelliCage, men ingen skillnad sågs med andra beteendeanalyser. Strålning ledde också till färre delande celler i hjärnan. Efter isofluran-sövning sågs inlärnings- och minnessvårigheter i IntelliCage när testet gjordes svårare, och minnessvårigheter var också tydliga i ett annat beteendetest. Effekterna på beteende sågs bara när unga djur sövdes. Sövningen ledde också till lägre celldelning i hippocampus, färre stamceller och färre nervceller och effekten var tydligast hos yngre djur. Sammanfattningsvis så är IntelliCage en lämplig metod för att utvärdera effekterna av strålning på beteende, även när skillnader inte kan detekteras med andra metoder. Sövning med isofluran hade negativa effekter på hjärnan, något som bör tänkas på vid upprepad sövning, speciellt hos barn.

List of original papers

This thesis is based on the following papers or manuscripts:

I. Barlind, A., Karlsson, N., Björk-Eriksson, T., Isgaard, J., and Blomgren, K. **Decreased cytogenesis in the granule cell layer of the hippocampus and impaired place learning after irradiation of the young mouse brain evaluated using the IntelliCage platform.** *Experimental Brain Research, (2010) 201, 781-787*

II. Zhu, C., Gao, J., Karlsson, N., Li, Q., Zhang, Y., Huang, Z., Li, H., Kuhn, H.G., and Blomgren, K. **Isoflurane anesthesia induced persistent, progressive memory impairment, caused a loss of neural stem cells, and reduced neurogenesis in young, but not adult, rodents.** *Journal of Cerebral Blood Flow & Metabolism, (2010) 30, 1017-1030*

III. Karlsson N, Kalm M, Nilsson MKL, Mallard C, Björk-Eriksson T, Blomgren K. **Learning and activity after irradiation of the young mouse brain analyzed in adulthood using unbiased monitoring in a home cage environment.** *(manuscript)*

Additional papers not included in the thesis:

- Barlind, A., Karlsson, N., Berg, N.D., Björk-Eriksson, T., Blomgren, K., and Isgaard, J. **The growth hormone secretagogue hexarelin increases cell proliferation in neurogenic regions of the mouse hippocampus.** *Growth Hormone & IGF Research, (2010) 20, 49-54.*

- Zhu, C., Huang, Z., Gao, J., Zhang, Y., Wang, X., Karlsson, N., Li, Q., Lannering, B., Björk-Eriksson, T., Georg Kuhn, H., and Blomgren, K. **Irradiation to the immature brain attenuates neurogenesis and exacerbates subsequent hypoxic-ischemic brain injury in the adult.** *Journal of Neurochemistry, (2009) 111, 1447-1456.*

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Abbreviations

AIF	Apoptosis-inducing factor
ANOVA	Analysis of variance
BrdU	Bromodeoxyuridine
CNS	Central nervous system
DAB	3-3’-diaminobenzidine tetrahydrochloride
DCX	Doublecortin
DNA	Deoxyribonucleic acid
FBDP	Fodrin breakdown product
GABA	gamma aminobutyric acid
GCL	Granule cell layer (in reference to the dentate gyrus)
GEE	Generalized estimating equations
GFAP	Glial fibrillary acidic protein
Gy	Gray (SI unit, absorbed radiation dose)
LC3	Microtubule-associated protein 1, light chain 3
LED	Light-emitting diode
NeuN	Neuronal nuclear protein (marker of neurons)
NMDA	N-methyl-D-aspartic-acid
P	Postnatal day
PBS	Phosphate-buffered saline
RFID	Radio-frequency identification
RMS	Rostral migratory stream
SGZ	Subgranular zone (in reference to the dentate gyrus)
SSC	Saline-sodium citrate
SVZ	Subventricular zone
TBS	Tris-buffered saline
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

Background

Children and anesthesia

Anesthesia is commonly used in young children during surgery or during procedures that are associated with pain or discomfort for the child. Attention to the possible side effects of surgical anesthesia has increased as animal studies have revealed sometimes serious side effects of anesthesia. However, little is known about the consequences of anesthesia exposure in children and there are too few clinical studies to draw sufficient conclusions about the side effects in children. Recently, cohort studies presented findings that according to the authors indicate that anesthesia could cause impairment later in life. Wilder and colleagues (2009) found indications that repeated exposure to isoflurane was associated with increased disability in children. Similar indications, although non-significant, were seen by Kalkman and colleagues (2009). Neither of these studies provides sufficient evidence to verify clinical effects, as they were not controlled for confounding factors. For example, a child might require surgery, and therefore anesthesia, for a condition that itself is the cause of the disability. In neither of these studies is it possible to differentiate between effects of anesthesia and effects of the underlying condition requiring the anesthesia. Future clinical studies are needed to elucidate if there are significant clinical complications after anesthesia and what these complications are; these will have to be controlled for effects of the underlying condition requiring the anesthesia.

Children and cancer

In 2007 there was a total of more than 50 000 diagnosed cases of cancer in Sweden, of these, 231 cases were in children under the age of 15 (Cancerfonden and Socialstyrelsen, 2009). In adults, prostate cancer and breast cancer are the most common forms of cancer. In children however, the two most common forms of cancer are leukemia and brain tumors. The cancer is often treated with

radiotherapy, chemotherapy and/or surgery. More than 75 % of the diagnosed children survive their cancer and since the 70s, the mortality has decreased, but it still remains one of the most common causes of death in children under the age of 15 (Cancerfonden and Socialstyrelsen, 2009; Socialstyrelsen, 2010). The increase in survival has resulted in more children experiencing the long-term side effects of the treatment, particularly radiotherapy, and has lead to a greater understanding of these effects. As many as 96 % of brain tumor survivors suffer from late-occurring side effects and treatment with radiotherapy seems to lead to more late side effects (Han et al., 2009). It is known that chemotherapy leads to fewer side effects compared to radiotherapy (Spiegler et al., 2006). The side effects after irradiation include impaired intelligence, information processing and memory but also include attention deficits and depression. Some of these side effects might result from white matter injury and females seem to be more sensitive (reviewed in Butler and Haser, 2006; Byrne, 2005; Spiegler et al., 2004). In addition, irradiation in childhood can lead to growth hormone deficiency and short stature (Lannering et al., 1990; Mulder et al., 2009) that may take several years to develop (Rohrer et al., 2009).

In a recent study, the authors looked at the long-term outcome after childhood cancer in Sweden. Surviving a tumor in the central nervous system was associated with a lower degree of education later in life, higher unemployment and lower salary (Boman et al., 2010). It is unclear if this is a result from the tumor itself or the treatment, but based on the known side effects from radiotherapy; at least part of it is likely due to irradiation. A lower degree of education and higher unemployment has also been shown in other studies (reviewed in Gurney et al., 2009). Previous studies have also demonstrated effects on the social life of patients. Children that have been irradiated are for instance less likely to get married and start a family (Gurney et al., 2009; Lannering et al., 1990).

Cellular effects

Anesthesia

For general anesthesia a combination of NMDA (N-methyl-D-aspartic-acid) antagonists (inhibit NMDA receptors) and GABA (gamma aminobutyric acid) agonists (stimulate $GABA_A$ receptors) are typically used. NMDA antagonists include for instance nitrous oxide (N_2O,"laughing gas") and ketamine while GABA agonists include halothane, propofol, barbiturates (for instance thiopental and phenobarbital) and benzodiazepines (such as diazepam and midazolam) (for reviews, see Olney, 2002; Olney et al., 2000; Patel and Sun, 2009). During development, when the major growth of the brain occurs, the brain is especially sensitive to excessive activation or inhibition of NMDA and $GABA_A$ receptors as this can cause neurodegeneration and apoptosis (Ikonomidou et al., 1999; reviewed in Olney, 2002; Patel and Sun, 2009). NMDA antagonists and GABA agonists, commonly used as anesthetics, therefore have the potential to cause neurodegeneration and apoptosis in the developing brain (reviewed in Olney et al., 2000). In addition, there is evidence from animal studies that also the adult and aging brain can be sensitive to anesthesia and that females may be more sensitive (Jevtovic-Todorovic et al., 2001). Isoflurane, an inhalable anesthetic that was used in this thesis, can act both as a NMDA antagonist and as a GABA agonist (Ranft et al., 2004). Although the use of isoflurane in humans has declined, it is still common for veterinary purposes and related agents, e.g. sevoflurane, are in clinical use, also for children.

Irradiation

Radiotherapy is commonly used to treat malignancies in humans. This type of therapy relies on malignant cells being more sensitive to oxidative stress and not having the same DNA repair capabilities as healthy cell. There are two major ways that irradiation can affect cells. When the irradiation interacts with the water

molecules in the cells, free electrons and highly reactive free radicals will be produced. One of these free radicals is an uncharged hydroxyl (·OH) radical. This radical has an unpaired electron that makes it very reactive. If oxygen is present, there will also be further production of other reactive products. The radicals cause oxidative stress and can react with proteins, lipids as well as DNA and cause damage. Another way that irradiation can affect the cells is by directly interacting with the DNA and changing it. The irradiation may cause damage directly to bases of the nucleotides, the essential building blocks of DNA. In addition, the irradiation can cause strand breaks that can be either single stranded or double stranded. The survival of cells where the DNA has been damaged by irradiation is dependent on the cells' intrinsic ability to repair the damage and the extent of the injury. Although irradiation in many cases can force cancer into remission, it also carries with it a risk of secondary cancers due to DNA damage that is improperly repaired (for review, see Bhatia and Sklar, 2002).

Proliferation, survival and neurogenesis

In humans, the major growth of the brain occurs perinatally (the time surrounding the birth), in rats on the other hand, the major growth occurs postnatally (Bayer et al., 1993; Dobbing and Sands, 1979). This is also true for the hippocampus in rats, where the majority of cells are formed during the first three weeks after birth (Bayer, 1980). In humans, this structure (Fig. 1A) continues to form for 8 months after birth (Seress et al., 2001). Although the majority of cells in the brain are formed early, there are two structures that are recognized as having a life-long potential for cell proliferation and neurogenesis. These are the subgranular zone (SGZ) of the dentate gyrus in the hippocampus and the subventricular zone (SVZ) of the lateral ventricle (Fig. 1B-D). These areas are thought to contain stem cells that can divide and give birth to new neurons. The work in this thesis has focused on the dentate gyrus where the stem cells are believed to be radial glia-like cells that in addition to nestin, also express GFAP. As these cells stop expressing GFAP,

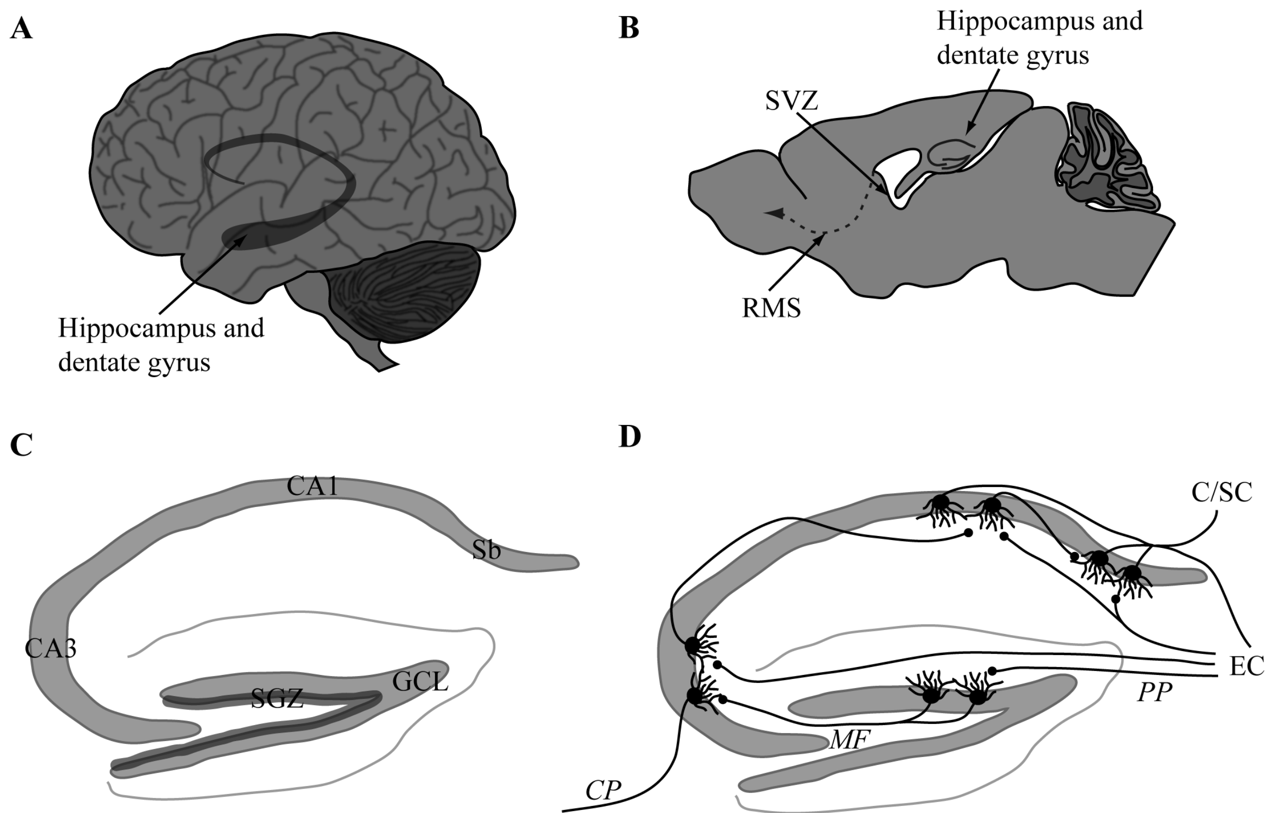

Fig. 1 (A) Representative picture of a human brain showing the position of the hippocampus. **(B)** Representative picture of a rodent brain showing the hippocampus with the dentate gyrus, the lateral ventricle, the SVZ and the rostral migratory stream (RMS) projecting to the olfactory bulb. **(C)** Illustration of the dentate gyrus with the granule cell layer (GCL), the subgranular zone (SGZ), CA3, CA1 and the subiculum (Sb). **(D)** Simplified illustration of the connections within the hippocampus. The GCL receives input from the entorhinal cortex (EC) through the perforant pathway (PP). CA3 receives input from the GCL through the mossy fibers (MF), but also from the EC. CA3 sends signals through the commissural projections (CP) to the contralateral hippocampus and to the CA1 region. CA1 connects to the subiculum and both regions receive input from the EC. Both CA1 and subiculum project to the entorhinal cortex and to other cortical (C) and subcortical regions (SC). An extensive review of the connections in the rat hippocampus can be found in The Rat Nervous System (Paxinos, 2004).

they become neuronally committed and initiate expression of DCX, an early marker for neurons and neuronally committed cells. This is then followed by down regulation of DCX and instead the cells start expressing the mature neuronal marker NeuN as well as calretinin and calbindin (Brown et al., 2003b; Kempermann et al., 2004). In addition to GFAP, the radial-glia-like cells also express SOX2 (Cavallaro et al., 2008; Ferri et al., 2004). Not all of the dividing cells will however become neuronally committed; some instead take on a glial fate and become astrocytes. It has been estimated that under normal environmental conditions, up to 50% of the newly generated cells in the adult dentate gyrus die within a month after birth through apoptosis (Dayer et al., 2003). Of those surviving cells in the adult dentate gyrus, roughly 85-90% become neurons (Brown et al., 2003a; Brown et al., 2003b). The cells that do adopt a neuronal fate will gradually migrate into the granular cell layer and become functionally integrated (Fig. 1D). The proliferation and differentiation will result in approximately 240 000 (Kempermann et al., 1997a) and 1.2 million (West et al., 1991) neurons in the dentate gyrus granule cell layer of an adult mouse (C57BL/6) and rat (Wistar), respectively. In contrast, the cells that are formed in the subventricular zone migrate along the rostral migratory stream towards the olfactory bulb (Fig. 1B) where they are then able to differentiate into mature neurons and become functionally integrated (Winner et al., 2002).

The microenvironment of the adult dentate gyrus is highly permissive to the birth of new cells and plays an important role in proliferation and differentiation of these cells. Furthermore, this microenvironment can be regulated in response to many different types of stimuli. The cell proliferation in the dentate gyrus appears to occur in close proximity to blood vessels (Palmer et al., 2000) and this vascular niche could be an important factor behind this proliferation. In addition, there are several different factors that can alter proliferation in the dentate gyrus. Stress and corticosterone (a hormone involved in stress) is known to reduce proliferation and

neurogenesis (Gould et al., 1998; Mirescu and Gould, 2006; Wong and Herbert, 2006). In contrast, voluntary running and enriched environment can increase proliferation, neurogenesis and survival (Brown et al., 2003a; Clark et al., 2008; Clark et al., 2010; Kempermann et al., 1997b; Meshi et al., 2006; Naylor et al., 2005). Interestingly, running has also been shown to increase vascular density in the dentate gyrus (Clark et al., 2009). Extended running has however also been shown to reduce proliferation (Naylor et al., 2005) and although enriched environment can increase proliferation and neurogenesis, new cells are not necessarily required for improvements in learning and memory (Meshi et al., 2006). In addition to these factors, adult neurogenesis also has a negative correlation with age (an extensive review on factors affecting neurogenesis can be found in Taupin, 2005). Although the dentate gyrus and SVZ have been considered to be exclusive sites for adult neurogenesis, cell proliferation and neuronal differentiation has been found in the adult mouse hypothalamus (Kokoeva et al., 2007) and with conflicting reports about the substantia nigra (Frielingsdorf et al., 2004; Zhao et al., 2003). In humans, both adult neurogenesis in the hippocampus and the existence of a rostral migratory stream have been demonstrated (Curtis et al., 2007; Eriksson et al., 1998).

Detecting proliferating cells

BrdU is commonly used in neuroscience to label proliferating cells in the brain. BrdU is a synthetic nucleoside (DNA "building block", a nucleobase connected to a deoxyribose molecule) that is integrated into the cells' DNA during replication. The molecule is similar to thymidine (one of the four building blocks of DNA), but one of its hydrogen ions have been replaced with a bromide ion (Fig. 2A-B). During DNA replication, the BrdU molecule can take the place of thymidine (Fig. 2C). By using an antibody against BrdU, the cells that were dividing at the time of injection can be labeled and by using other markers e.g. for nerve cells, one can determine how many cells were proliferating at that specific time and how many of those cells

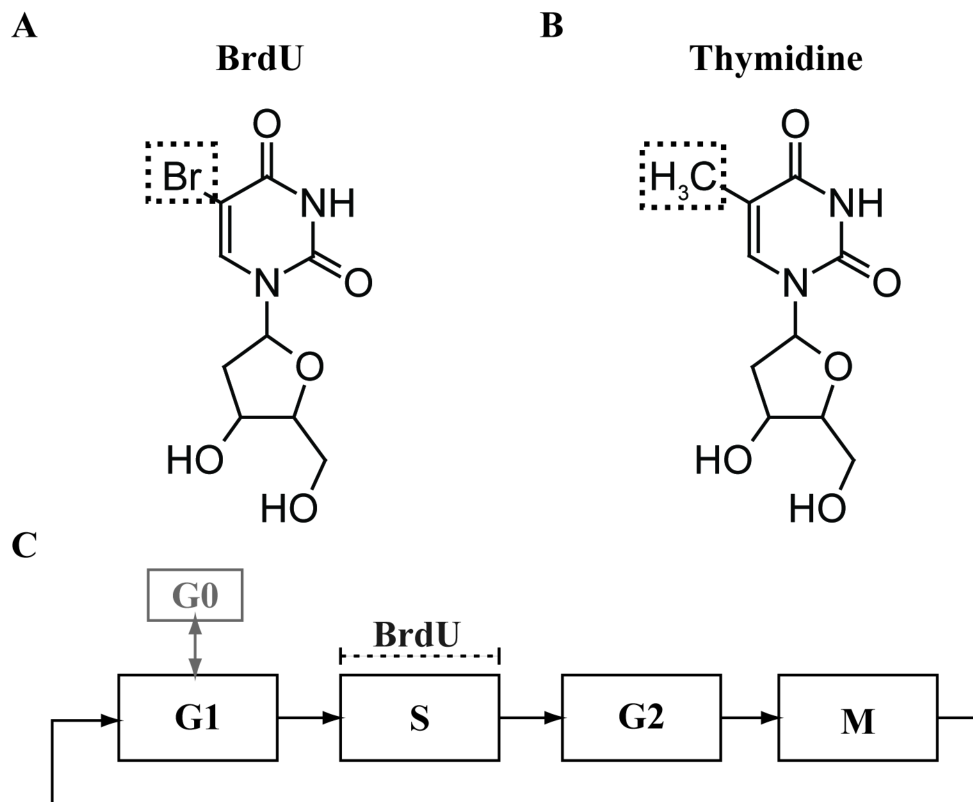

Fig. 2 (A) Structure of the BrdU molecule. **(B)** Structure of thymidine molecule. **(C)** In the cell cycle, BrdU is taken up by the cell during the synthesis phase (S), when the DNA is replicated.

later become neuronal cells (or any other type of cell depending on what markers that are used). As BrdU takes the place of thymidine, there is a risk for mutations and toxicity at high doses due to the much bigger bromide ion in BrdU compared to the hydrogen ion in thymidine. Embryonic neuronal and proliferative sensitivity to thymidine analogs in rodents has been demonstrated *in vivo* (Biggers et al., 1987; Kolb et al., 1999; Kuwagata et al., 2007; Sekerkova et al., 2004) and *in vitro* (Caldwell et al., 2005; Ross et al., 2008) using varying doses of BrdU. A common problem with the *in vitro* studies is the time of BrdU exposure. In the mentioned studies, the cells were exposed to BrdU for 24 hours. In contrast, a single injection only exposes the brain cells to BrdU for up to two hours (Cameron and McKay, 2001). The S-phase of proliferating cells in the dentate gyrus is around 6 hours

(Burns and Kuan, 2005). Therefore, cells that are exposed for 24 hours have the potential to take up more BrdU and hence show more signs of toxicity. In the adult rat hippocampus, BrdU doses up to 480 mg/kg (Hancock et al., 2009) and 600 mg/kg (Cameron and McKay, 2001) seem to be tolerated without any apparent toxic effects. Although different doses have been used in the publications mentioned, making it difficult to draw conclusions, the data indicate a developmental sensitivity to BrdU. This is supported by a less developed blood brain barrier in embryos and neonates (reviewed in Taupin, 2007). If an experiment requires multiple BrdU injections per day or long *in vitro* exposures, effects on proliferation and differentiation should be a concern. One other concern is that injured cells might take up BrdU (as a part of DNA repair) and thus be labeled as proliferating cells, but BrdU does not appear to be incorporated to a great extent during DNA repair or in dying postmitotic neurons (Bauer and Patterson, 2005; Cooper-Kuhn and Kuhn, 2002; Kuan et al., 2004). Interestingly, the combination of hypoxia and ischemia can induce DNA synthesis (through reentry into the S phase) and BrdU incorporation in adult rodent neurons (Kuan et al., 2004).

Effects of isoflurane

In vitro studies have demonstrated impaired growth of progenitor cells, isolated from the young rat hippocampus, when exposed to isoflurane (Sall et al., 2009). Similarly, isoflurane reduced the number of proliferating cells when rats were exposed just after birth or at postnatal day 7, resulting in fewer neurons in adulthood and a smaller hippocampus (Rothstein et al., 2008; Stratmann et al., 2009b). In young adult rats, isoflurane has been shown to decrease the proliferation only to later result in an increase in proliferation (Stratmann et al., 2009b). There seems to be a developmental difference in the sensitivity to isoflurane and newborn male rats appear more sensitive to isoflurane compared to females (Rothstein et al., 2008). Isoflurane has been reported to induce a higher neuronal differentiation and reduce SOX2 mRNA *in vitro* (Sall et al., 2009). A higher neuronal differentiation

has also been seen in young adult rats after isoflurane exposure, this was however not seen in rats at postnatal day 7 (Stratmann et al., 2009b). In addition to effects on proliferation and differentiation, isoflurane has been demonstrated to cause neuronal cell death and neurodegeneration in hippocampus of P7 rats. This occurred in a dose-dependent manner and when the exposure times were at least two hours (Stratmann et al., 2009a:Jevtovic-Todorovic, 2003 #335). Another study has however demonstrated that P10 and P14 rats do not show neurodegeneration following isoflurane exposure (Yon et al., 2005). Although cellular death has been demonstrated in organotypic hippocampal slice cultures (Wise-Faberowski et al., 2005), another *in vitro* study failed to show this (Sall et al., 2009). Interestingly, isoflurane and the related anesthesia desflurane may both have neuroprotective properties after ischemia (Bickler et al., 2003; Kurth et al., 2001; Loepke et al., 2002). Other anesthetic agents, such as propofol (Vutskits et al., 2005), phenobarbital (Rothstein et al., 2008), ketamine (Jevtovic-Todorovic et al., 2001; Wang et al., 2006; Young et al., 2005) and nitrous oxide (Jevtovic-Todorovic et al., 2001), have also been reported to cause cell death and neuronal degeneration and ketamine, like isoflurane, has been shown to do so in an age- and gender-dependent manner (Jevtovic-Todorovic et al., 2001; Slikker et al., 2007).

Effects of irradiation

Irradiation can have detrimental effects on cells and tissues. In animal models, irradiation is known to induce cell death and reduce proliferation in the GCL and SVZ and lead to lower neurogenesis both at young and old age (Barlind et al., 2010b; Ben Abdallah et al., 2007; Fukuda et al., 2005a; Fukuda et al., 2004; Hellström et al., 2009; Monje et al., 2002; Naylor et al., 2008; Raber et al., 2004a; Rola et al., 2008; Rola et al., 2004; Tada et al., 2000). If the dose used is high enough, the injury on the proliferative pool of cells may be permanent. Fukuda and colleagues (2004) demonstrated a reduction in proliferation 7 days post-irradiation in young rats and Tada and colleagues (2000) showed less proliferation in adult rats

even after 120 days. Our own experiments also indicate a long-term reduction in proliferation after 6-8 Gy to the young brain (Fukuda et al., 2005b; Hellström et al., 2009) that is still apparent after one year (unpublished results). However, a lower dose, 4 Gy, may only lead to temporary changes in proliferation and neurogenesis (Ben Abdallah et al., 2007).

Irradiation to the brain causes an acute increase in the number of microglia and activated microglia (Kalm et al., 2009b; Monje et al., 2002 ; Rola et al., 2008). The number of microglia then declines after a few days and show signs of cell death (Kalm et al., 2009b). It is unclear if these microglia cells are endogenous to the brain or if they are recruited from outside the brain or a combination of the two. Microglia proliferate and become active as a part of an immune response and inflammation is known to occur in the brain after irradiation (Kalm et al., 2009a; Monje et al., 2003). These changes are dose-dependent as lower doses of irradiation do not seem to lead to detectable microglia activation (Ben Abdallah et al., 2007). Interestingly, inflammatory blockades using anti-inflammatory drugs in conjunction with irradiation can lead to partial restoration of neurogenesis and a reduction in the number of activated microglia (Monje et al., 2003). From these experiments it is obvious that there is, in addition to a direct effect on the proliferation, a change in the microenvironment after irradiation that affects the cells and their differentiation. A change in the microenvironment is further substantiated by increased oxidative stress (Fukuda et al., 2004; Raber et al., 2009; Zhu et al., 2007), less neuronal differentiation of cells transplanted into an irradiated brain (Monje et al., 2002), less clustering of proliferating cells around blood vessels (Monje et al., 2002) and recent data showing that newly formed neurons are unable to integrate properly following irradiation (Naylor et al., 2008). Besides a microglia response, astrocytes also become active, increase in numbers and display a hypertrophic morphology (Kalm et al., 2009a; Rola et al., 2008). In addition, less myelinization is known to occur after irradiation (Fukuda et al.,

2005a; Fukuda et al., 2004) and high doses can cause demyelination (Panagiotakos et al., 2007), something that can greatly affect the signaling efficiency of the neurons.

Memory

Memory can be divided into long-term memory and working memory (with short-term memory as a part of the broader term working memory). A short-term memory typically lasts seconds to hours while a long-term memory lasts days to weeks (Nader, 2003). Long-term memory can further be divided into declarative memory and non-declarative memory (Purves, 2008). Declarative memory includes semantic memory (memory of facts) and episodic memory (memory of events) while non-declarative memory includes procedural memory (e.g. memory of skills). The hippocampal structure plays an essential role in declarative memory, the cerebellum and neostriatum are important in procedural memory while the cerebral cortex is important in working memory, decision making and long term declarative memory (reviewed in Diamond et al., 2007; Eichenbaum, 2000; Squire et al., 2004). Different parts of the hippocampus also seem to be involved in different tasks, while the dorsal hippocampus is involved in spatial memory (a part of the declarative memory, Squire et al., 2004), the ventral part seems not (Bannerman et al., 1999; Moser et al., 1993; Pothuizen et al., 2004). Interestingly, stress is known to modulate the hippocampus and may exert both a positive and negative influence on memory (Diamond et al., 2007; Hölscher, 1999; Okuda et al., 2004; Yuen et al., 2009).

Role of neurogenesis

The role of neurogenesis in the hippocampus and its direct correlation to memory has been hotly debated and there is evidence both for and against a need for adult neurogenesis in learning (Leuner et al., 2006). Jaholkowski and colleagues (2009)

found that adult neurogenesis was not necessary for learning. In the same study, it was demonstrated that animals without neurogenesis in fact performed better in hippocampal-dependent trace fear conditioning (where animals have to learn an association between a sound and an electric shock). Saxe and colleagues (2007) even suggest that less neurogenesis may in fact enhance working memory. This is however in contradiction to the studies by Shors and colleagues (2001), who found that new neurons were involved in the same conditioning test, as well as to Dupret and colleagues (2008), where mice without adult neurogenesis showed spatial memory deficits. Others have found that neurons that are 4-28 days old at time of training are required for learning (Snyder et al., 2005) and that apoptosis in some newborn cells is an important step in the spatial memory (Dupret et al., 2007). It is however possible that neurogenesis is not required for all types of hippocampal-dependent learning (Shors et al., 2002).

Using computational models (Becker, 2005; Becker et al., 2009), it’s been suggested that neurogenesis may be involved in the creation of distinct memory traces for memories that are very similar and that neurogenesis may reduce interference between memories. It has also been suggested that neurogenesis may be an adaptation for future learning and not necessary for immediate learning (Snyder et al., 2005) and that it’s needed for the clearance of old memories from the hippocampus (Feng et al., 2001). Although the importance of neurogenesis in memory and learning can be debated, several studies have shown the importance of an intact hippocampal structure for memory and learning. Rats suffering from lesions in the hippocampus are impaired in hippocampal-dependent tests such as Morris water maze and certain hippocampal-dependent fear conditioning tests like contextual fear conditioning (Bangasser et al., 2006; Hernandez-Rabaza et al., 2008; Phillips and LeDoux, 1992; Whishaw and Tomie, 1997) .

Effects of isoflurane

Isoflurane exposure in rats immediately after birth, leads to impairment in adulthood in hippocampal-dependent tests such as Morris water maze and radial arm maze (Rothstein et al., 2008). These effects were especially apparent in males. When rats were exposed at postnatal day 7, an age with extensive brain growth, they later developed spatial memory deficits and impairment in fear conditioning (Stratmann et al., 2009b). These deficits were only present when the animals had been treated with isoflurane for four hours; shorter exposures (one and two hours) did not result in memory deficits (Stratmann et al., 2009a). In comparison, exposure to isoflurane in two month old animals can instead lead to improved spatial memory (Stratmann et al., 2009b). Similar results had already been demonstrated by Culley and colleagues (2003) but using a combination of isoflurane and laughing gas and older animals (six months). Isoflurane and laughing gas in combination with midazolam has also been shown to result in memory deficits when rats were exposed at postnatal day 7 and this combination can also affect synaptic functions (Jevtovic-Todorovic et al., 2003).

Effects of irradiation

As with the effects of neurogenesis on memory, the effect of irradiation on memory is less than clear. While some researchers found some impairments in hippocampal-dependent spatial learning and memory using Morris Water Maze tests (Snyder et al., 2005; Wojtowicz et al., 2008), others have not found a difference (Meshi et al., 2006; Raber et al., 2004a; Raber et al., 2004b; Saxe et al., 2006) and some have found only memory retention or task learning problems (Raber et al., 2009; Rola et al., 2004). With Barnes Maze, another hippocampal-dependent test, the situation is similar as there are conflicting results regarding the effects of irradiation (Raber et al., 2004b; Rola et al., 2004). It has even been reported that irradiation improves hippocampal-dependent working memory (Saxe et al., 2007). In contextual fear conditioning, a hippocampal-dependent test used to evaluate mice and rats,

irradiation in adulthood has been shown to impair animals in their performance (Hernandez-Rabaza et al., 2009; Raber et al., 2009; Saxe et al., 2006; Winocur et al., 2006; Wojtowicz et al., 2008). Although, in novel object recognition, a test where the hippocampus is important (Broadbent et al., 2004), no differences were found (Rola et al., 2004). Irradiation may have an impact on signal transmission between neurons in the hippocampus without affecting synaptic plasticity in CA1 (Saxe et al., 2006) and irradiation can also disrupt neuronal activity associated with memory (by disrupting Arc expression, Rosi et al., 2008). Different animal strains, irradiation procedures and differences in the behavior protocols are likely to explain the discrepancies between the different studies and it cannot be excluded that certain hippocampal-dependent behavior tests are more sensitive to irradiation than others.

General aim

The aim of this project was to investigate if the IntelliCage system is suitable for investigating behavioral effects resulting from treatment with irradiation and isoflurane, to investigate the cellular effects in the hippocampus, and to attempt to correlate cellular effects with behavioral effects. This was done to further our knowledge of the effects of irradiation and isoflurane.

Specific aims

I. To use the IntelliCage system to investigate behavioral effects following irradiation to the young mouse brain, characterize these and compare with other behavior tests.

II. To characterize the cellular effects of isoflurane exposure and to investigate the behavioral effects of this exposure.

Materials and Methods

Animals

In papers I-III, C57BL/6 mice were used. These animals were ordered in litters with 5-6 male pups per litter from Charles River Laboratories (Sulzfeld, Germany). In paper II, male Wistar rats were used. These animals were ordered from B&K Universal (Solna, Sweden). Rat litters were delivered with 10 male pups per litter. All animals were kept on a 12-hour light cycle and food and water was available *ad libitum*. After weaning, the mice were kept in groups up to 10 animals per cage and rats were kept in groups with 4 animals per cage. At the time of weaning or thereafter, the mice were subcutaneously injected with RFID (Radio Frequency Identification) microtransponders (Datamars, Petlink, Youngstown, USA) to enable identification in the IntelliCages. All animal experiments were approved by the local committee of the Swedish Animal Welfare Agency (Djurskyddsmyndigheten) or Swedish Board of Agriculture (Jordbruksverket, 180-2005, 212-2005, 46-2007, 47-2007 and 326-2009).

Comment: In these papers, mice and rats were chosen as a model for irradiation and isoflurane exposure. Cell cultures can be very useful tools, but animal models are required to investigate systemic effects of a treatment and their effects on behavior.

Irradiation model

A linear accelerator (Varian Clinac 600 D, Radiation Oncology Systems LLC, San Diego, CA, USA) with 4 MV nominal photon energy was used to irradiate the animals in papers I and III. In paper I, the animals were irradiated on P10 (postnatal day 10) with 6 Gy and in paper III, 8 Gy on P14. For the irradiation procedure, the animals were anaesthetized with intraperitoneal injections of 50 mg/kg

tribromoethanol (Sigma, Stockholm, Sweden). The animals were then placed on a polystyrene bed with the head covered with 1 cm tissue equivalent material. The radiation field was 2 × 2 cm and the source to skin distant was 99.5 cm. The dose was administered in one fraction with a rate of 2.3 Gy/min and the dose variation in the target was estimated to 5%. After the irradiation procedure was completed, the animals were kept on a warm bed (36°C) and then returned to the dams in their home cages. The warm bed is especially important as the animals at this age are unable to maintain their body temperature and a lower body temperature has been shown to reduce the irradiation injury (Fukuda et al., 2005a).

Comment: The LQ model can be used to estimate the equivalent doses in 2 Gy fractions (Fowler, 1989). Although it is uncertain for doses with only one fraction (like in these experiments), 6 and 8 Gy is (using a α/β value of 3) roughly equivalent to 12 and 18 Gy respectively, given in repeated 2 Gy fractions. (Fukuda et al., 2004; Naylor et al., 2008), 18 Gy is used as prophylactic cranial irradiation in some cases of childhood acute lymphatic leukemia, and therefore the 8 Gy dose represents a clinically relevant dose. Doses for treating tumors are higher and can be as much as 55 Gy.

In paper I, we irradiated mice at P10. When looking at brain growth, a rat at P7-8 corresponds approximately to a human brain at birth (Dobbing and Sands, 1979). As irradiation in newborns is uncommon, we instead used P14 in paper III to better reflect a clinical setting. The comparison between rodent age and human age is far from easy and depending on what parameters are looked at, the result will differ (Clancy et al., 2007; Quinn, 2005)

Isoflurane model

Rats (P14 or P60) and mice (P14) were randomly assigned to either isoflurane treatment or control treatment in paper II. Animals in the isoflurane group were placed in a plastic chamber that was continuously flushed with 1.7% isoflurane (Isoba, Schering-Plough Corporation, Kenilworth, NJ, USA) in an air-oxygen mixture (1:1) for 35 minutes on one day or on four consecutive days. Control animals received the same treatment but with no isoflurane in the air-oxygen mixture. A heating pad set to 37°C was used to control the temperature on the chamber floor. The isoflurane treatment did not induce visible signs of discomfort or neurological symptoms and no mortality was observed during or directly after treatment.

Comment: Previously published work on anesthesia indicated learning disabilities after repeated anesthesia. In our experiments, the animals were anesthetized for 35 minutes on four sequential days as a model of repeated isoflurane exposure. A time span of 35 minutes in rats and mice is presumably equivalent to a longer time span in humans.

Labeling of proliferating cells

In papers I-II, the animals were injected intraperitoneally with BrdU (Roche Diagnostics GmbH, Mannheim, Germany) dissolved in 0.9% NaCl to label cells that were synthesizing DNA and dividing at the time of injection and to detect differences resulting from the treatments. In paper I, the animals were injected when they were 27, 29 and 31 days old with a dose of 50 mg/kg per injection. In paper II, the animals were injected on four consecutive days with 50 mg/kg after each treatment.

Comment: BrdU is a permanent marker, but if the cells have a high proliferation rate or if the time between injection and sacrifice is long, the BrdU can become diluted in cells. Depending on the goal of the labeling, different strategies might be used when injecting BrdU. If the goal is to investigate the acute effects of a treatment, the BrdU injection should take place right after the treatment, but for long term effects, the BrdU should be injected at a later time point (days, weeks or months) depending on the expected long term effects of the treatment. Also, the number of injections will affect the results as more injections give more labeled cells. The number of labeled cells will also depend on what BrdU dose is used. In our experiments, we have used 50 mg/kg, a dose that we have found works well in our experiments. There are however conflicting reports regarding how many cells that are labeled at different doses, part of which might be explained by different species, strain and age. Cameron and McKay (2001) found that 50 mg/kg labels just under half of the cells compared to 300 mg/kg in adult rats, data that are supported by Hancock and colleagues (2009) but others have shown that 50 mg/kg is enough to label at least 90% of the proliferating cells in adult mice (Burns and Kuan, 2005). It is important to know this labeling frequency when the aim of the research is to estimate the true total number of dividing cells at a certain time point as this otherwise might lead to misleading numbers. It is less important when the aim is to compare treatment groups with a control group to look at possible changes in proliferation induced by a treatment.

Blood pressure and blood gas analysis

A Samba Preclin catheter (Samba Sensor, AB, Gothenburg, Sweden.), inserted in the left carotid artery during isoflurane anesthesia, was used to measure blood pressure in paper II. The surgical procedure took approximately 10 minutes and the blood pressure could then be recorded in real time with high resolution. In one group with P14 rats, the blood pressure was measured continuously during a 35 minute exposure. In a second group, the P14 rats were exposed to three 35 minute

isoflurane exposures on three consecutive days and then had their blood pressure measured during the fourth exposure on the fourth day. In addition, a third group with P60 rats had their blood pressure measured during one 35 minute exposure. After blood pressure had been measured, animals were immediately decapitated and mixed venous/arterial blood was collected from the neck vessels and analyzed using a blood analyzer (ABL 725, Radiometer A/S, Copenhagen, Denmark). The following parameters were measured: pH, pCO_2, pO_2 and concentration of glucose, lactate and bicarbonate.

Behavior

IntelliCage

The IntelliCage system was used in paper I-III to assess the animals' ability to learn a task in a social home cage environment. Each IntelliCage consist of a plastic cage (610 mm x 435 mm) with one conditioning corner in each corner (fig. 3A). Each conditioning corner consists of a small space that can only be entered by passing a circular antenna that registers implanted RFID chips. Each conditioning corner is equipped with two water bottles to which access can be limited by motorized doors. In addition, each corner contains a temperature sensor that registers a raise in temperature when an animal enters ("presence"), a sensor that detects attempts to drink ("nose pokes"), a lick sensor, three LED lights above each door and an air valve that can be used for negative reinforcement. Several cages can be connected in a series and then connected to a computer. When a mouse enters a corner, the antenna registers the mouse's unique RFID and transmits this information to the computer. The computer can then, depending on the programming, respond by opening or closing a door, turning on or off a LED or administer an air puff. For example, the computer can be programmed to open a door covering the water bottle when a mouse performs a nose poke in the corresponding correct corner, but to ignore a nose poke in an incorrect corner. The computer will register how long a

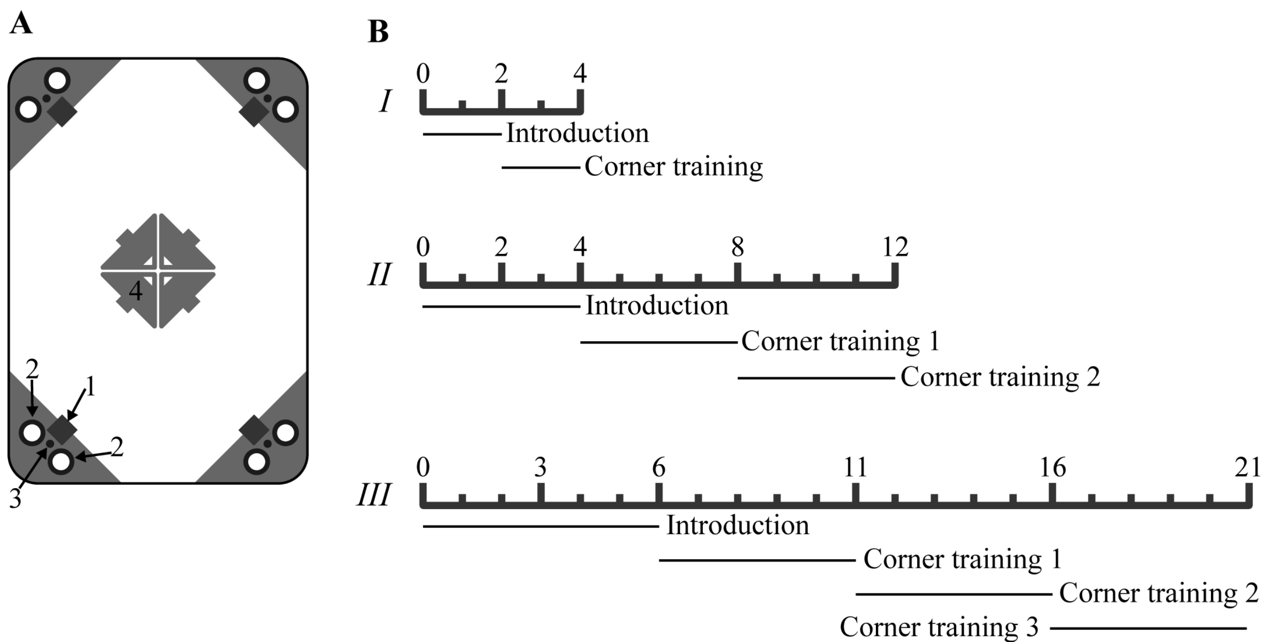

Fig. 3 (**A)** Illustration of an IntelliCage. Each corner has two water bottles (2) that can be accessed by entering the corner through a circular antenna (1). Each corner is equipped with an air valve (3) for administering air puffs. Four red plastic houses (4) in the cage provide shelter and access to the food grid. **(B)** Overview of IntelliCage experiments in papers I-III.

mouse stays in the corner and what sensors become active. Each IntelliCage has wood chips on the floor and four red plastic houses that provide shelter and easy access to the food grid. Animals that lost or had a malfunctioning microchip or did not learn how to drink were excluded from the experiment.

Each experiment started with an acclimatization period ("introduction") where the animals had to learn to visit the corners to be able to drink and to perform nose pokes to gain access to water bottles. This was followed by assigning each animal to a single corner ("corner training"). The animal was only allowed to drink from this corner and the other corners were programmed as being incorrect (the animals were able to enter the corner, but a nose poke did not give them access to the water bottle).

Before the start of the corner training, the animals were assigned to the least visited corner (paper II), randomized to any of the four corners (paper I) or randomized to one of the three least visited corners (paper III). A second corner training was used in paper II and in paper III both a second and third corner training period was used. Before the start of each corner training period, the animals were again assigned or randomized to a new corner. In paper III, care was taken not to randomize the animals to a corner that they had previously been assigned to. The acclimatization period lasted for 2 (paper I), 4 (paper II) or 6 (paper III) days and each corner training period lasted for 2 (paper I), 4 (paper II) or 5 (paper III) days. The IntelliCage experiments in paper I-III are summarized in figure 3B. Air puffs were used as negative reinforcement in paper II and in a separate experiment in paper III. In addition, another experiment where green LEDs were turned on when the mice entered the correct corner was performed in paper III. In both paper II and III, one water bottle was supplemented with fructose as positive reinforcement and to measure preferences.

The IntelliCage data was divided into day time bins. In paper I and II, these bins consisted of the data from 24 hours, both the active and inactive period of the mice day cycle. In paper III, the data from the inactive part of the day was excluded. All data was processed in Microsoft Excel and then analyzed using statistical software from SPSS (SPSS Inc., Chicago, USA).

SocialBoxes were used in paper III to attempt to assess differences in social preference. One SocialBox consists of a smaller plastic cage (26.7 × 20.7 × 14.0 cm) connected to the IntelliCage through a plastic cylinder. The cylinder is equipped with two antennas that read the RFID chip implanted in the mice. The antennas register when a mouse enters and leaves the SocialBox but also time spent in the small cage and which mice were there at the same time. Three SocialBoxes were connected to each IntelliCage.

Comment: The IntelliCage system has the advantage of being a home cage behavior analysis system. This minimizes animal handling, stress and reduces variation introduced by the experimenter during the behavior test. The disadvantage is that the system is expensive to purchase, is more prone to technical problems due to the mechanics and requires more space for a longer time period compared to normal cages. At the time of our initial experiments, IntelliCage was only commercially available for mice, but recently it has also become available for rats. IntelliCage performance has been shown to be reproducible between different labs (Krackow et al., 2010).

Open field

The motor activity patterns of irradiated and control animals were analyzed using open field and video tracking. Each animal was introduced into an unfamiliar open field arena and then immediately videotaped for 50 minutes. Each arena (46 × 33 × 35 cm) was indirectly illuminated and four arenas were positioned by each other for simultaneous videotaping. The floor of each arena was covered with gray gravel that had been previously exposed to other mice. Video tracking was performed using EthoVision 3.1 (Noldus Information Technologies bv. Wageningen, the Netherlands) and a sampling frequency of 12.5 Hz. The software analyzed distance moved, number of stops, spatial variability in movement path, exploratory rearing and time spent in the middle of the arena and summarized the data into 10 minute bins. Open field using this experimental setup has previously been described by Nilsson and colleagues (2006).

Trace fear conditioning

Trace fear conditioning was used in paper III to investigate the effects of irradiation nine and 15 weeks after the animals were subjected to irradiation. Each animal was tested individually in an observation chamber (Modified automatic reflex conditioner, Ugo Basile, Comerio VA, Italy). The floor of the chamber consists of

stainless steel rods that were wired to a power source for administering a shock. A speaker, used to deliver a tone, was mounted on the side of the chamber. On the first day, each mouse was placed in the chamber for 2 minutes to measure baseline freezing. This was followed by a tone (80 dB, 670 Hz) for 30 seconds, a 20 second pause and then a 2 second foot shock (0.5 mA). On the second day, baseline freezing and freezing after a tone was measured for two minutes each. Experiments were videotaped to facilitate analysis.

Object recognition

Rats in paper II were subjected to object recognition (Bertaina-Anglade et al., 2006). Each animal was placed individually in a plastic arena (65 × 48 × 28 cm) on day one for a five minute habituation. On day two, each animal was placed in the arena again, this time with two identical objects that they were allowed to explore for five minutes. On day three, one of the previous objects was replaced with a new, novel object and each animal was allowed to explore for five minutes. Each animal was videotaped both during day two and three and the time spent exploring each object was measured. Exploration was defined as touching the object with the nose, sniffing the object at a distance closer than two centimeters or rearing on the object. From this, a recognition memory index was calculated by dividing the difference between the time spent exploring the novel object and the familiar object, with the total time spent exploring both objects.

Olfactory test

To assess the animals' ability to smell, an olfactory test was used on the mice in paper III. Animals were exposed to small pieces of cheese in their normal home cage environment before the experiment and they were also habituated to the test cage on the day before the test. The test cage consisted of a standard mouse cage (42 × 27 × 16 cm) filled with four-five centimeters of wood chips. On the day of the testing, a 1.5 ml Eppendorf tube drilled with tiny holes was filled with cheese

and buried in the bedding material in the middle of the cage. Animals were judged as having passed the test if they within ten minutes from being placed in the cage, found and uncovered the hidden cheese-filled tube.

Tissue analysis

Tissue preparation

The animals were deeply anesthetized using sodium pentobarbital followed by transcardial perfusion with Histofix (HistoLab, Göteborg, Sweden) or 4% formaldehyde in 0.1M phosphate buffer. The brains were removed and immersion fixed in the same solution for 24 hours at 4°C. In paper I and II, one hemisphere was then transferred to 30% sucrose for at least 3 days before being cryosectioned on a sliding microtome (free floating sections). The brains were cut in 30µm sections in series of twelve and the sections was stored in a cryo-protection solution (one part glycerol, one part ethylene glycol and two parts 0.1M phosphate buffer) at -20°C or 4°C until staining. In paper II, the other hemisphere was dehydrated, embedded in paraffin and then cut into 5µm coronal sections.

Immunohistochemistry

Depending on the antibodies and the sections (free floating or paraffin) used, the staining protocol was slightly different. A list of the antibodies, dilutions and manufacturer used can be found in table 1. In both paper I and II, BrdU staining was performed on free floating sections. Sections were rinsed in tris-buffered saline (TBS, 0.08M Trizma-HCL, 0.016M Trizma-Base, 0.15M NaCl, pH 7.5) and then incubated in 0.6% H_2O_2 for 30 minutes to block endogenous peroxidases. This was followed by TBS washing and incubation in 2M HCl at 37°C for 30 minutes to denature the DNA and expose the incorporated BrdU. Sections were then further incubated in 0.1M borate buffer (pH 8.5), for 10 minutes, followed by additional TBS washing. To block unspecific binding, the sections were incubated in a

Antibody	Dilution	Company
rat anti-BrdU	1:500	Nordic Biosite
rat anti-BrdU	1:100	Oxford Biotechnology Ltd
rabbit anti-phospho-histone H3	1:200	Upstate
mouse anti-NeuN	1:200	Chemicon
rabbit anti-S-100β	1:1000	Swant
goat anti-SOX2	1:200	Santa Cruz Biotechnology
mouse anti-GFAP	1:500	Chemicon
rabbit anti-active caspase 3	1:100	BD Pharmingen
rabbit anti-FBDP	1:50	Cell Signaling
rabbit anti-LC3	1:300	Cell Signaling
goat anti-AIF	1:100	Santa Cruz Biotechnology
goat anti-synapsin 1 a/b	1:150	Santa Cruz Biotechnology

Table 1 List of primary antibodies

blocking solution (3% donkey serum and 0.1% Triton X-100 in TBS) for 30 minutes at room temperature and then incubated with rat anti-BrdU in blocking solution over night at 4°C. After rinsing in TBS, sections were incubated with a biotinylated donkey anti-Rat IgG for 1-2 hours in room temperature. This was followed by TBS rinsing and incubation in a biotin-avidin solution (Vectastain ABC elite kit, Burlingame, CA, USA) for 1 hour at room temperature. After further rinsing, the staining was developed using 3-3'-diaminobenzidine tetrahydrochloride (DAB, Saveen Werner AB, Malmö, Sweden) in a TBS solution containing NiCl and H_2O_2. In paper I, the sections were incubated for two hours at 65°C in a formamide/SSC (saline-sodium citrate) solution followed by 5 minutes in SSC before the HCl to further expose the BrdU in the DNA. Staining for phospho-histone H3 (Paper II), was performed in a similar way, but instead of HCl and borate buffer, a pre-treatment step consisting of incubation in 10mM sodium citrate (pH 9) for 30 minutes at 80°C was used.

In paper II, triple immunofluorescence labeling was used to investigate the phenotypes of the BrdU containing cells. NeuN was used as a marker of mature neurons while S100β was used as a marker for mature astrocytes. The DNA was denatured as previously described with 2M HCl at 37°C for 30 minutes. After blocking, the sections were incubated with rat anti-BrdU, mouse anti-NeuN and rabbit anti-S100β in blocking solution for 1 hour in room temperature. After rinsing with TBS, the sections were then incubated with donkey anti-rat IgG Alexa 488, donkey anti-mouse IgG Alexa 555 and donkey anti-rabbit IgG Alexa 647 in TBS for 1 hour in room temperature. For double labeling of SOX-2 and GFAP cells, sections were incubated with goat anti-SOX-2 and mouse anti-GFAP in blocking solution over night, followed by incubation with donkey anti-goat Alexa 488 and donkey anti-mouse Alexa 555 for two hours in room temperature. Sections were mounted and coversliped using ProLong Gold (for fluorescence, with or without DAPI (4',6-diamidino-2-phenylindole), Invitrogen, Invitrogen Corporation, Carlsbad, CA, USA) or mounted, dried and then coversliped with Neomount (Merck, Merck & Co., Inc, NJ, USA).

Paraffin sections were used to stain for markers involved in cell death: AIF (apoptosis inducing factor), active caspase-3, LC3 (microtubule-associated protein 1, light chain 3) and FBDP (fodrin breakdown product). Paraffin sections were also used to detect DNA strand breaks through TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) and synapsin I. The sections were deparaffinized and hydrated through an alcohol series, followed by boiling the sections in 10mM citric acid (pH 6) for 10 minutes. Non-specific binding was blocked by incubation with 4% goat or horse serum (depending on the secondary antibody) in phosphate-buffered saline (PBS) for 30 minutes in room temperature, followed by 60 minutes incubation in room temperature with rabbit anti-active capsase-3, rabbit anti-LC3, rabbit anti-FBDP, goat anti-AIF or goat anti-synapsin I a/b. This was followed by incubation with biotinylated goat anti-rabbit or horse

anti-goat for 60 minutes in room temperature. Endogenous peroxidases were blocked using 3% H_2O_2 in methanol for 5 minutes, followed by incubation with biotin-avidin solution (Vectastain ABC elite kit, Burlingame, CA, USA) for 60 minutes. After further rinsing, the staining was developed using 3-3'-diaminobenzidine tetrahydrochloride (DAB, Saveen Werner AB, Malmö, Sweden) in a PBS solution containing ammonium nickel sulfate, β-D glucose and β-D glucose oxidase (all from Sigma, Stockholm, Sweden). Between the different steps, the sections were rinsed with PBS. The TUNEL staining was performed according to instructions from the manufacturer (Roche Applied Science, Penzberg, Germany). Staining was performed as previously described for paraffin sections up until the blocking step. Sections for TUNEL staining was blocked with 3% bovine albumin serum in 0.1M Tris-HCl (pH 7.5) for 30 minutes and then incubated with a TUNEL reaction mixture (deoxynucleotidyl transferase, fluorescein-2'-deozyuridine and deoxynucleotide triphosphate) for 60 minutes. After rinsing, endogenous peroxidases were blocked with 0.3% H_2O_2 in methanol for 10 minutes and then followed by further incubation in 3% bovine albumin serum in 0.1M Tris-HCl (pH 7.5) for 30 minutes. After incubation with anti-flourescein at 37° for 30 minutes, the staining was developed using the same 3-3'-diaminobenzidine tetrahydrochloride solution described above. Paraffin sections were dehydrated and coversliped with Neomount.

Quantification of cells

In paper I and II, the GCL and/or CA1 of the hippocampus was outlined using a microscope with a camera connected to a computer running StereoInvestigator (Microbrightfield Inc., Magdeburg, Germany). With the same software, the stained cells were counted. The person doing the counting was blinded to the treatments. For BrdU and phospho-histone-H3 every twelfth (Paper II) or sixth sections (Paper I) was analyzed. On each section, the area of interest was measured and the number of cells was counted. From this, the total volume was estimated by multiplying the

area with section thickness and the sampling frequencies (twelve or six). The total amount of cells was estimated by multiplying the counted number of cells with the sampling frequencies. Based on this, a density can be calculated by dividing the estimated number of cells with the estimated volume. To investigate the phenotypes of the BrdU containing cells, at least 50 cells were investigated for co-labeling with NeuN or S100β using a confocal laser scanning microscope (Leica TCS SP, Heidelberg, Germany). From this, a ratio of NeuN-BrdU and S100β-S100β was calculated. From this ratio, the total number of newborn neurons (NeuN-BrdU) and astrocytes (S100β-BrdU) were calculated from the total number of BrdU containing cells. SOX-2 and GFAP double-labeled cells were counted in the entire granular cell layer. Total neuronal cell numbers was estimated by counting DAPI positive cells in the granular cell layer. For this purpose, a fractionator was used. The fractionator overlays a grid system and selects counting frames in the traced area. Cells within the counting frame and cells touching two of the four borders are counted and based on this; the total number of cells in the section can be estimated by StereoInvestigator. Synapsin 1 optical density was analyzed in the dentate gyrus and CA3 using Image Gauge (Fujifilm, Tokyo, Japan).

Statistical analyses

Statistical test were performed using SPSS, Microsoft Excel or GraphPad Prism (GraphPad Software Inc., La Jolla, USA) and $p < 0.05$ was considered statistically significant. Student's t test was used to compare differences in number of cells, synapsin I density, cell death markers, blood gas parameters in adult rats and body weights. Blood gas and blood pressure parameters in young rats were analyzed using an ANOVA with Bonfferroni/Dunn post hoc test. Mann-Whitney U test was used to compare differences in object recognition, time to visit the first corner in IntelliCage and water preference. Trace fear conditioning and social preference was analyzed using Mann-Whitney U test and/or Wilcoxon signed rank. Differences in

olfaction and exclusion resulting from air puffs in IntelliCage were analyzed using Fischer's exact test.

After consulting a statistician, generalized estimating equations (GEE) was used to analyze IntelliCage and open field parameters. GEE estimates and compares the average responses of the population for the different parameters that are investigated. For integer values (nose pokes, visits and rearings), a Poisson-model was used while a binominal model was used to calculate differences in ratios. For all other values, a normal log model was used. Data was first analyzed for a time × treatment interaction and if no significance was seen, time and treatment was analyzed separately. Differences between the groups were calculated from the obtained β-values.

Results and discussion

Isoflurane reduces the number of neural stem cells, proliferation and neurogenesis

There has been growing concern regarding the effects of the anesthetics used in patients. In 2009, two studies (Kalkman et al., 2009; Wilder et al., 2009) were published where the authors found indications that repeated exposure to isoflurane, an inhaled anesthetic, could lead to increased disability later in life. Anesthesia usually involves a combination of different drugs that are either NMDA antagonists or $GABA_A$ agonists. As the brain is sensitive to both too much activation and too much inhibition of the NMDA and $GABA_A$ receptors, the usage of these anesthetic agents has the potential to cause neurodegeneration and apoptosis (Ikonomidou et al., 1999; Olney, 2002; Olney et al., 2000). In our studies, we have investigated the effects of repeated isoflurane exposure in both young and adult rodents. Isoflurane has the potential to act both as a NMDA antagonist and as a $GABA_A$ agonist (Ranft et al., 2004). Both *in vitro* and *in vivo* studies have previously demonstrated less proliferation in the hippocampus following isoflurane exposure (Rothstein et al., 2008; Sall et al., 2009; Stratmann et al., 2009b). We used a protocol that involved 35 minutes of isoflurane exposure on four consecutive days starting either at P14 (young) or P60 (adult). We found that the isoflurane immediately reduced the proliferation in the dentate gyrus with 21% in P14 rats (evaluated at P18) and four weeks after isoflurane exposure, the treated animals had 71% less proliferation compared to control animals. This data is in agreement with the studies by Stratmann and colleagues (2009b) where the young P7 rat brain showed less proliferation during and after isoflurane exposure. In P60 rats they also saw a decrease in proliferation during isoflurane treatment, but after four days, an increase was instead seen. In contrast, in our experiments, we saw no change in proliferation immediately after isoflurane exposure and no changes in proliferation in this age group four weeks after exposure. Differences in rat strains (strain was

not indicated in the Stratmann paper), BrdU injection protocols and isoflurane treatment protocols could also explain this discrepancy. It is possible that there is a transient increase in proliferation days after isoflurane exposure in the P60 group that later normalizes and that could not be detected using our BrdU protocols. We also investigated the effect of isoflurane on the neural stem cells by looking at radial glia-like cells that co-expressed SOX2 and GFAP (Naylor et al., 2008). In animals treated at P60, there was no difference in the number of cells after 4 weeks, but in the group treated at P14, there was a 43% reduction in these cells. In agreement, a previous study has demonstrated a reduction in SOX2 mRNA *in vitro* following isoflurane exposure (Sall et al., 2009). A reduction in the number of neural stem cells could be the cause of the less proliferation seen.

In our studies, we also saw less neuronal differentiation (by counting BrdU and NeuN double positive cells) in both age groups that was followed by more astrocyte differentiation. The reduction in neuronal differentiation was largest in the P14 group (48% compared to 26% in P60) while the increase in astrocyte differentiation was slightly larger in the P60 group. A reduction in neuronal differentiation was also seen by Stratmann and colleagues (2009b) when rats were treated with isoflurane at P60 for four hours and then administered BrdU 4-7 days after (and evaluated four weeks later). This difference was however not seen in P7 rats using the same protocol. Interestingly, BrdU injection before the four hour isoflurane treatment instead revealed an increase in neuronal differentiation (BrdU and NeuroD co-labeling) immediately after treatment in the P60 group but not in the P7 group (Stratmann et al., 2009b). An increase in neuronal differentiation has also been demonstrated *in vitro* when cells were evaluated four days after isoflurane treatment (Sall et al., 2009). It is possible that there is a temporary increase in neuronal differentiation following isoflurane treatment, something that we did not look for in our study. We were unable to show a reduction in total granule neurons in the dentate gyrus following isoflurane in the P60 group in our

studies. Ten weeks following isoflurane treatment, there was however a reduction in the group that was treated at P14, a difference that was not seen four weeks post-treatment in the same age group. Rats that have been treated with isoflurane as newborns have previously been shown to have fewer neurons in the hippocampus (Rothstein et al., 2008). It appears that it takes several weeks for a difference in total number of neurons to develop between isoflurane treated and control animals. This would explain why we were unable to find a difference four weeks post-treatment and why Stratmann and colleagues (2009b) did not find a difference in their P7 group. From our results and the above mentioned studies, it is evident that there are developmental differences in the effects of isoflurane. While a rat exposed at young age several weeks later had fewer neurons in the dentate gyrus, adult rats were unaffected even though both young and adult rats had less neuronal differentiation. Hypercarbia (high levels of CO_2) has previously been suggested as a possible cause of the effects seen by anesthetic agents. We did however not find any signs indicating hypercarbia in our experiments. The blood pressure, pCO_2, pO_2 and body temperature of our animals remained stable during the anesthesia but a slight respiratory acidosis and hyperglycemia was seen.

Isoflurane does not cause cell death or change synaptic properties in the dentate gyrus

A possible explanation for the reduced proliferation and neural stem cells seen after isoflurane treatment could be due to cell death. Several other anesthetic agents have been reported to cause cell death and neuronal degeneration. Cell death and neurodegeneration after isoflurane exposure has been shown in P7 rats (Stratmann et al., 2009a:Jevtovic-Todorovic, 2003 #335) and in organotypic hippocampal slice cultures (Wise-Faberowski et al., 2005). One *in vitro* study did not find evidence for cell death in hippocampal progenitor cells (Sall et al., 2009) and Yon and colleagues (2005) found no differences in P10 and P14 rats after isoflurane

exposure. Perhaps it is therefore not surprising that we were unable to find any evidence of cell death in the dentate gyrus and CA1 of P14 and P60 rats after isoflurane exposure. We looked at markers for necrosis, DNA strand breaks, autophagy, caspase-dependent apoptosis and caspase-independent apoptosis without finding any differences. Based on this, one possibility is that there is a developmental sensitivity to cell death induced by isoflurane. This could be explained by reduced proliferation due to cell death in the very young brain (< P10). While in the slightly older animals, P10-14, isoflurane might instead affect proliferation directly without causing cell death and in the P60 animals, the cells are overall less sensitive to the effects of isoflurane. The sensitivity in the very young brain would correspond to a time when brain growth is peaking or is about to. The slightly older (P10-14) would correspond to a time right after the brain growth peak and the P60 brain when the major brain growth has long been completed (Dobbing and Sands, 1979). In addition to investigating cell death, we investigated synapse density in the dentate gyrus and CA3 10 weeks after isoflurane exposure. We did not find an effect on synapse density in the hippocampus after isoflurane exposure in P14 rats. A previous study on anesthetic agents did not find effects of isoflurane on synaptic function either, but a combination of isoflurane, nitrous oxide and midazolam did affect synaptic function (Jevtovic-Todorovic et al., 2003).

Isoflurane treatment results in memory impairments

Rats exposed to isoflurane as newborns or at P7 have been shown to later develop impairments on hippocampal-dependent behavior tests (Rothstein et al., 2008; Stratmann et al., 2009a; Stratmann et al., 2009b). To investigate the behavioral effects of the repeated isoflurane exposure in our study we used object recognition to evaluate the rats four and ten weeks after isoflurane exposure. P14 rats showed impaired object recognition 4 weeks after isoflurane exposure. This difference was further aggravated ten weeks after the exposure. In contrast, rats exposed to

isoflurane at P60 showed no impairment at either time points. Object recognition is considered a behavioral test for working memory (Buccafusco, 2009) and areas such as the perirhinal cortex has been found to be important for this test (Barker et al., 2007). The hippocampus has however also been found to be important for this type of test (Broadbent et al., 2004).

In a separate study, we exposed mice to isoflurane. These animals were then placed in the IntelliCage two weeks after the last isoflurane exposure. In the IntelliCage, isoflurane treated animals were equally able to find the correct assigned corner (one out of four corners) as control animals. After the assigned corner was changed and the animals had to relearn the task ("reversal learning"), isoflurane treated animals were however unable to learn the correct corner. Random visits to the corners in the IntelliCage will yield an incorrect visit ratio of 75%. While the control animals quickly dropped below an incorrect visit ratio of 75%, the isoflurane treated animals only seemed to make random visits without learning and never went below 75%, indicating dramatically impaired reversal learning. During the reversal learning the isoflurane treated animals made 49% less correct visits during the four day period. Both control and isoflurane treated animals spent more and more time in the correct corners during reversal learning, but isoflurane spent an average of 58% less time in the correct corner. Both groups made more and more nose pokes in the correct corners and more correct nose pokes per correct visit, but isoflurane treated animals made 73% less correct nose pokes and 71% less nose pokes per correct visit during reversal learning. This together indicates that the isoflurane treated animals had problems unlearning the old task and learn the new one (a new corner). Isoflurane treated animals appear to have an inability to replace one declarative memory with a new and modified one (reversal learning). The learning and relearning in the IntelliCage is similar to the Morris water maze that contains both a declarative part (finding the correct quadrant in the pool) and a procedural part (swimming to find the platform) and where the reversal learning requires

replacement of one declarative with the new modified one while maintaining the procedural memory of the task (Rossato et al., 2006). As the isoflurane treated mice improve over time, the procedural memory seems intact while the declarative memory seems affected. Declarative memory includes spatial memory and is linked to the hippocampus (Manns and Eichenbaum, 2006). In the IntelliCage, we used short air puffs as negative reinforcement. Negative reinforcement can be stressful for the animals and the amygdala has been implicated in regulating the effects of stress on memory (Roozendaal et al., 2009). Our histological investigations were focused on to the dentate gyrus and we cannot exclude that other regions, such as amygdala, are also affected and take part in this type of learning task.

Our behavior results from the IntelliCage and object recognition are in agreement with other studies in the field showing impaired learning after isoflurane exposure. We were able to detect isoflurane-induced impairments in learning when animals were exposed at P14 and others have previously demonstrated learning impairments after exposure in younger ages (Rothstein et al., 2008; Stratmann et al., 2009a; Stratmann et al., 2009b). We were however unable to demonstrate learning impairments in animals that had been exposed to isoflurane in adulthood (P60), further indicating developmental differences in sensitivity to this anesthetic agent.

Irradiated mice have less proliferation in the dentate gyrus

Several studies have shown that proliferation in the dentate gyrus can be severely reduced following irradiation towards the hippocampus (Ben Abdallah et al., 2007; Fukuda et al., 2005a; Fukuda et al., 2004; Hellström et al., 2009; Monje et al., 2002; Naylor et al., 2008; Raber et al., 2004a; Rola et al., 2008; Rola et al., 2004; Tada et al., 2000). If the dose is high enough, a permanent reduction in proliferation may be seen. When irradiating mice at P10 with 6 Gy, we saw an approximate 50%

reduction in proliferation and survival (evaluated seven weeks after irradiation). This data is in agreement with the previously mentioned studies that have shown reduction in proliferation both in young and adult animals following irradiation.

Irradiation-induced injury was only detectable using the IntelliCage system

Although the effects of irradiation on proliferation and differentiation are clear both from ours studies and other studies, it has proven more difficult to detect behavioral effects of this injury to the hippocampus. The effect of irradiation on learning and memory is therefore not entirely clear so far. Water maze tests (like Morris Water Maze) have traditionally been used to investigate effects on hippocampal-dependent learning and memory in research animals. In the radiation research field, there have been contradictory results from these behavior tests. While some have demonstrated impairments (Raber et al., 2004a; Rola et al., 2004; Snyder et al., 2005; Wojtowicz et al., 2008) others have not (Clark et al., 2008; Hernandez-Rabaza et al., 2009; Meshi et al., 2006; Saxe et al., 2006). Contextual fear conditioning is one test where irradiated animals have been shown to be impaired (Hernandez-Rabaza et al., 2009; Raber et al., 2009; Saxe et al., 2006; Winocur et al., 2006; Wojtowicz et al., 2008). While Morris water maze is recognized as a behavior test that is specific for hippocampus, contextual fear conditioning however also involves in addition to the hippocampus, the amygdala, frontal cortex and cingulate cortex (Buccafusco, 2009; Hernandez-Rabaza et al., 2008; Phillips and LeDoux, 1992). The amygdala has been implied in regulation of stress and memory (Roozendaal et al., 2009), and as fear conditioning reasonably involves higher levels of stress, it is more difficult to draw conclusions about the hippocampus from this test. One solution can be to use a cued fear conditioning in conjunction with the contextual fear conditioning, as this is thought to rely on the amygdala (Buccafusco, 2009). In the cued fear conditioning, a cue (usually a tone)

is used during the aversive stimulus. In contrast, during the contextual fear conditioning, only the aversive stimulus is present. In our study, we chose to use trace fear conditioning, where the aversive stimulus is presented several seconds after the cue (a tone). This test relies on the hippocampus to learn the separation of the cue and the stimulus (Bangasser et al., 2006; Buccafusco, 2009). We were unable to demonstrate any effect of irradiation on trace fear conditioning nine weeks after irradiation. Both the irradiated and control animals showed a strong response to the context and increased freezing even further after the tone. Six weeks after the first test, the animals still had a strong response to the context, but neither group responded to the tone this time. One explanation for not finding a difference between the groups might be that the association between the stimulus and both the context and tone was not strong enough. More training sessions could yield different results. This is supported by a lack of impairment in contextual fear conditioning after irradiation when using a similar training protocols to ours (Clark et al., 2008).

In paper I, we wanted to investigate if behavioral changes following irradiation could be detected using the IntelliCage system. We demonstrated that irradiated animals had a lower ratio of attempts to drink in the correct corner (nose pokes) and saw a trend that irradiated animals also made fewer attempts to drink once they were in the correct corner, indicating a change in behavior following irradiation. In paper III, we further set out to characterize the effects of irradiation on the animals using IntelliCage. We followed the animals for a total of 15 days. The 15 days were divided into three different corner periods, each lasting for five days, with each animal being randomized to a different corner each period.

After the animals had been assigned a corner, the number of visits and attempts to drink (nose pokes) would increase. During the first corner period, control animals decreased their number of nose pokes each day slightly more so than the irradiated

animals (10% vs. 5%) and by the last day, this resulted in an estimated 30% difference in the total number of nose pokes. This difference was not seen during the other corner periods. This could reflect an initially equal level of purpose-oriented exploratory behavior that gradually leads to a lower level of activity in the control animals, but not in the irradiated animals to the same degree, as they adapt to the new conditions. This is supported by the slightly more rapid decrease in total number of visits for the control animals during corner period one (7% vs. 3%), with irradiated animals showing slightly higher activity. No difference in the animals' latency to visit all the corners in the IntelliCage was seen. To further investigate levels of activity, the mice were subjected to an open field test, but we were unable to demonstrate any differences between the animals using this test. Research from our group has previously shown pronounced changes in activity following irradiation (Naylor et al., 2008). An explanation for this difference could be the slightly older animals that we have used (P14 vs. P9 in Naylor et al). At P9, the rodent brain grows more compared to at P14 (Dobbing and Sands, 1979) and is therefore more sensitive to irradiation compared to P14. This is also true in humans, where cranial radiotherapy in very young children is avoided (Mulhern et al., 2004) due to the more severe negative consequences in the younger brain.

To further investigate learning, we looked at several different parameters in the IntelliCage. We found that the irradiated mice made attempts to drink in the incorrect corners (nose pokes) to a higher degree compared to the control animals, indicating that they had problems learning and remembering which corner was the correct one. When the irradiated animals were in the incorrect corner, they also persisted in trying to drink there by performing more nose pokes compared to control animals. This is further demonstrated by the longer time spent in the incorrect corners. This difference seemed to increase as the task grew more difficult (i.e. when the animals had to relearn the task when a new corner was assigned). Animals with hippocampal lesions have previously been shown to have

impairments in reversal learning using water test, with the animals continuing to visit the old site (Whishaw and Tomie, 1997). To further investigate the animals' behavior as the task grew more difficult, we looked at the number of attempts to drink (nose pokes) in the corner that had previously been correct. Irradiated animals made significantly more attempts to drink in the previously correct corner compared to control animals and after the first corner change (period two), while the control animals made fewer attempts each day and seemed to relearn the task (15%), irradiated animals improved only slightly (2%). This resulted in a 94% difference during the last day. At the last corner change, there was a 29% difference between the two groups. The learning problems of the irradiated animals is further shown by the higher ratio of attempts to drink in the previously correct corner during corner period two that resulted in a 141% difference in odds by the last day. Although we found in paper II using isoflurane greater differences in the ratio of incorrect visits, this was not so apparent in the irradiation experiment. Based on this it could mean that visit parameters are more useful if a negative stimulus that promotes correct visits is used. We found however that when using air puffs as negative stimulus in the irradiation experiment, there was an unacceptably high number of both control and irradiated animals (60%) that had to be excluded as the mice avoided the corners completely. As this test appeared more stressful to these animals, it could mean that the amygdala was involved to a higher degree and in for instance the Morris water maze, stress has a negative impact on performance (Hölscher, 1999).

Taken together, it appears that the irradiated animals have learning difficulties associated with the declarative memory as they seem to learn the correct corner to a lesser extent compared to controls. Furthermore, as the animals were required to relearn a task, irradiated animals performed even worse, indicating a problem with replacing one declarative memory with another one. But as the animals improved over time, this would imply a maintained procedural memory. The declarative

memory is dependent on the hippocampus (Manns and Eichenbaum, 2006), but extrahippocampal structures may have a role in place learning (Whishaw and Tomie, 1997). Not all studies have demonstrated impaired spatial learning following irradiation. While many studies have demonstrated impairments in contextual fear conditioning, we were unable to demonstrate such impairments using our protocol.

IntelliCage versus other behavior tests

We have used the IntelliCage system to investigate learning and memory impairments following isoflurane anesthesia or irradiation to the young brain. IntelliCage was recently developed and so far, few published studies have utilized this new behavior analysis method. Other behavior tests that have been used to study memory and learning include for instance Morris water maze, radial arm maze and fear conditioning tests such as contextual fear conditioning and trace fear conditioning (for more information, see Buccafusco, 2009). The IntelliCage has the advantage that animals can be tested in social groups in a home cage-like environment. It also requires minimum interaction with the animals while an experiment is running. In contrast, other behavior tests, such as Morris water maze and radial arm maze often require extensive handling over several days during the training. The handling itself can be a stress factor for the animals and therefore handling needs to be highly consistent throughout an experiment, but the test itself can also be stressful. In the case of Morris water maze, it has been shown that mice perform worse on water tests compared to rats (Whishaw, 1995; Whishaw and Tomie, 1996). One reasonable explanation for this is likely the natural behavior of these animals. While rats are natural swimmers, mice on the other hand are primarily terrestrial animals and normally do not swim (Wotjak, 2004). One other confounding factor when using mice in spatial learning and memory tests is that they have poor vision and instead rely on auditory and olfactory cues to orient themselves. IntelliCage therefore seems suitable as it is a dry land test where the

animals are able to create their own olfactory cues and rely less on visual cues while any possible bias from the experimenters are avoided. Further research is however needed to validate this method and correlate it to other commonly used behavior tests.

General conclusion

This thesis concludes that IntelliCage is a suitable method to detect learning and memory impairments. Furthermore, using the IntelliCage, it is concluded that both isoflurane exposure and irradiation affect brain development and result in learning and memory deficits.

Specific conclusions

I. Using the IntelliCage system, behavioral effects following a moderate dose of irradiation were detected that could not be detected using other established methods (open field and trace fear conditioning). Irradiated animals showed impaired learning and as the task grew more difficult, they performed worse and had difficulties unlearning the old task and learn the new one. In addition, it was confirmed that irradiation impaired cell proliferation in the dentate gyrus.

II. Isoflurane exposure during the development of the brain, but not in the adult, led to fewer neural stem cells and less proliferation, eventually leading to fewer neurons in the hippocampus. This occurred in the absence of apparent cell death. Animals that were exposed to isoflurane at a young age later developed impairments in learning and memory.

Clinical perspective and future directions

Anesthesia is used for a wide number of procedures, both in children and in adults. Although previously considered safe, there is a growing awareness that anesthesia may have detrimental effects on the brain. Recently, two studies were published where isoflurane was linked to learning disabilities (Kalkman et al., 2009; Wilder et al., 2009). Although these studies should be considered indicative and in no way present any evidence, further research in this field is needed to elucidate the effects of anesthesia on the brain. Although the use of isoflurane in clinical settings have declined, many patients have been anesthetized using this anesthetic agent and other anesthetic agents related to isoflurane are still in use. In addition, many other commonly used anesthetics have been shown to have similar effects. This study provides further evidence of side effects that can occur following anesthesia. In this thesis it was shown that the common anesthetic agent, isoflurane, caused a reduction in neural stem cells in the hippocampus and eventually led to impairments in memory and learning. No cell death was detected following isoflurane anesthesia and future studies will have to investigate the mechanisms behind the decline in neural stem cells and proliferation to learn more about possible side effects. A greater understanding of the side effects of anesthesia and the mechanism behind it could lead to more efficient use of anesthesia and possibly to development of better anesthetics.

Irradiation is a vital part of treatment for cancer. Since the start of this type of treatment, radiation therapy has become more and more refined. This in turn has led to many more children surviving their cancer and reaching adulthood. As the surviving children age, the side effects on healthy tissue after this type of treatment become noticeable. These side effects include for instance lower intelligence and memory problems. Several studies have characterized what happens following irradiation in the brain in animal models and also what the behavioral effects are.

For obvious reasons, these are things that are difficult to investigate in humans. Proper animal models as well as functional tests are required to perform any type of intervention studies before studies can be made in humans. In this thesis, the behavioral effects of irradiation were investigated using the IntelliCage system. Irradiated animals were found to have impaired learning and memory that was accompanied with a reduction in proliferation in the hippocampus. IntelliCage has the advantage of being a home-cage test with the potential to analyze many animals in social groups at the same time without a major workload, compared to other traditional tests. The IntelliCage system therefore has great potential in future studies where the focus is finding ways to spare cognition after irradiation as well as in other studies where there is a need to investigate cognition in the animal models. Future studies are also needed to further validate this behavioral test by comparing it with other commonly used behavior tests.

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