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APOPTOSIS AND INFLAMMATION REGULATION AFTER INJURY TO THE DEVELOPING BRAIN

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Apoptosis and Inflammation Regulation after Injury to the Developing Brain

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Even if the open windows of science at first make us shiver after the cosy indoor warmth of traditional humanizing myths, in the end the fresh air brings vigour, and the great spaces have a splendour of their own.

Bertrand Russell

To my readers

ABSTRACT

The brain shows greater plasticity in early life than in maturity, which paradoxically renders the organ more vulnerable to hypoxia-ischemia (HI)- and cranial irradiation (IR)-induced damage. Apoptosis after a HI insult is more pronounced in the immature vs. mature brain and develops over time; hence, blockade of the apoptotic cascade provides a target for delayed neuroprotective interventions aimed at reducing HI-provoked brain damage. Moreover, inflammation subsequent to the initial insult exacerbates HI-induced brain damage, and is also a target for interventions. Meanwhile, the profound progressive decrease in neurogenesis after IR is associated with the deleterious effects of chronic inflammation. The overall goal of this thesis was to investigate potential neuroprotective strategies to decrease brain injury through the regulation of apoptosis and inflammation. First, the impact of a cell-penetrating, Bax-inhibiting peptide (BIP) was assessed in a neonatal mouse model of HI. BIP administration moderated injury to the gray and white matter, and ameliorated sensorimotor and cognitive deficits. These actions were attributed to diminished Bax activation and decreased mitochondrial release of the pro-apoptotic proteins, cytochrome c and apoptosis-inducing factor (AIF). Next, the influence of delayed and extended systemic administration of a caspase inhibitor, Q-VD-OPh, was examined in neonatal HI. Consequently, Q-VD-OPh decreased the expression of pro-inflammatory chemokines, reduced signs of brain injury, and transiently overturned HI-induced sensorimotor deficits and hyperactivity. The present findings also revealed a novel mechanism amenable for therapeutic strategies after neural progenitor cell (NPC) transplantation into the brain, and showed that active cell death of NPCs plays a key role in the release of heat-stable, neuroprotective proteins. Specifically, conditioned medium (CM) originating from dying NPCs safeguarded hippocampal neurons against glutamate toxicity and trophic factor withdrawal *in vitro*, and exerted protective actions against ischemic brain damage *in vivo.* Finally, the current data demonstrated that peripheral macrophages do not contribute to the inflammatory response in the hippocampus after IR. Moreover, the microglial response was more pronounced and protracted in the juvenile vs. adult brain, and the inflammatory response appeared to be chronic, lasting at least 1 month after IR. Taken together, the observations presented herein provide insight into the control of apoptosis and inflammation after ischemic or IR injury to the developing brain.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following manuscripts, which are referred to in this document by their corresponding Roman numerals.

I. Xiaoyang Wang[*], **Wei Han[*],** Xiaonan Du, Changlian Zhu, Ylva Carlsson, Carina Mallard, Etienne Jacotot, and Henrik Hagberg (2010). Neuroprotective effect of Bax-inhibiting peptide on neonatal brain injury. Stroke 41, 2050–2055.

II. **Wei Han,** Yanyan Sun, Xiaoyang Wang, Changlian Zhu[#], and Klas Blomgren[#] (2014). Delayed, long-term administration of the caspase inhibitor Q-VD-OPh reduced brain injury induced by neonatal hypoxia-ischemia. Dev Neurosci 36, 64–72.

III. Eva-Maria Meißner[*], **Wei Han[*],** Stefanie Neunteibl, Jörg Kahnt, Amalia Dolga, Cuicui Xie, Changlian Zhu, Klas Blomgren[#], and Carsten Culmsee[#]. Phoenix rising: Neural progenitor cell death confers neuroprotection. *Manuscript.*

IV. **Wei Han,** Takashi Umekawa, Kai Zhou, Changlian Zhu[#], and Klas Blomgren[#]. Blood-derived macrophages do not contribute to the inflammatory response after cranial irradiation in either the juvenile or the adult brain. *Submitted*.

Publications not included in this thesis:

Han W., Song J., Liu A., Huo K., Xu F., Cui S., Wang X. and Zhu C. (2011). "Trends in live births in the past 20 years in Zhengzhou, China." Acta Obstet Gynecol Scand **90**(4): 332-337.

Hellgren G.,*** Han W.,*** Wang X., Lofqvist C., Hagberg H., Mallard C. and Hellstrom A. (2011). "Safety aspects of longitudinal administration of IGF-I/IGFBP-3 complex in neonatal mice." Growth Horm IGF Res **21**(4): 205-211.

Wang X., Leverin A. L., **Han W.,** Zhu C., Johansson B. R., Jacotot E., Ten V. S., Sims N. R. and Hagberg H. (2011). "Isolation of brain mitochondria from neonatal mice." J Neurochem **119**(6): 1253-1261.

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Shared senior authorship.

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LIST OF ABBREVIATIONS

AIF	Apoptosis-inducing factor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Apaf-1	Apoptotic protease activating factor 1
Bax	Bcl-2-associated X protein
Bak	Bcl-2 homologous antagonist killer
BBB	Blood brain barrier
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
Bid	BH3 interacting-domain death agonist
BIP	Bax inhibiting peptide
CA	Cornu ammonis
Caspase	Cysteine-aspartic proteases
CCL2	Chemokine (C-C motif) ligand 2
CCL3	Chemokine (C-C motif) ligand 3
CCR2	C-C chemokine receptor type 2
CM	Conditioned medium
CNS	Central nervous system
CS	Conditional stimulus
CX3CR1	CX3C chemokine receptor 1
Cytc	Cytochrome c
DAB	3,3' Diaminobenzidine
dATP	Deoxyadenosine triphosphate
DISC	Death-inducing signaling complex
DG	Dentate gyrus
GABA	γ-Aminobutyric acid
GCL	Granular cell layer
GFP	Green fluorescent protein
HIE	Hypoxic-ischemic encephalopathy
IL-1β	Interleukin 1β
IL-18	Interleukin 18
IR	Irradiation
KA	Kainic acid
MCP-1	Monocyte chemotactic protein 1

MEF	Mouse embryonic fibroblast
MOMP	Mitochondrial outer membrane permeabilization
MPT	Mitochondrial permeability transition
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NPC	Neural progenitor cell
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PND	Postnatal day
Q-VD-OPh	Quinoline-Val-Asp(OMe)-CH2-PH
RFP	Red fluorescent protein
RNOS	Reactive nitrogen oxide species
ROS	Reactive oxygen species
RT	Room temperature
SGZ	Subgranular zone
SVZ	Subventricle zone
TBS	Tris-buffered saline
TNF-α	Tumor necrosis factor α
US	Unconditional stimulus

1 Introduction

1.1 Clinical background

Infants in the neonatal period, or the first 28 days of life, have the highest childhood mortality rate, accounting for 44 % of all deaths in children under 5 years of age (UNICEF 2014). Perinatal asphyxia, a severe condition characterized by impaired respiratory gas exchange, is a leading cause of neonatal death (23 %) (Liu, Johnson et al. 2012). However, thanks to advances in perinatal care over the past two decades (including improvements in safe childbirth procedures and more effective care of newborns), the neonatal death rate has significantly declined (Lawn, Kinney et al. 2012). Perinatal asphyxia is often followed by dysfunction of one or more organs or organ systems, typically the central nervous system (CNS) because of its high demand for oxygen and blood flow (Martin-Ancel, Garcia-Alix et al. 1995). Perinatal hypoxic-ischemic encephalopathy (HIE) is a common form of brain damage triggered by asphyxia, manifests itself by difficulty in initiating and maintaining respiration, and also by depression of tone and reflexes, subnormal consciousness, and frequently, by seizures. HIE is a serious disorder with critical neuropsychological sequelae among survivors. Available estimates suggest that all survivors of severe HIE show neurological deficits at one time or another, whereas in moderate HIE, the number is ~30–40 % (Robertson, Finer et al. 1989; Dixon, Badawi et al. 2002).

Although only ~7 % of reported brain and CNS tumors occur in children aged 0–19 years (Dolecek, Propp et al. 2012), brain tumors are the second most common type of cancer (after leukemia) in children, and the most common type of solid tumor. In fact, brain tumors are responsible for an estimated 17 % of all childhood cancers (Howlader, Noone et al. 2015). The 5-year survival rate for children with brain tumors has dramatically increased due to treatment advances (Armstrong, Stovall et al. 2010; Hellings, Peeters et al. 2010). Treatment modalities vary according to the affected cell type, tumor grade, and location in the brain, but mainly consist of surgery, radiation therapy, and chemotherapy. Unfortunately, these effective treatments are associated with late-onset adverse events (e.g., endocrine disorders, cognitive deficits, and developmental impairments occurring months or years after treatment termination) (Danoff, Cowchock et al. 1982; Ellenberg, Liu et al. 2009; Gunn, Lahdesmaki et al. 2015). Children diagnosed with brain tumors at a younger age and subjected to radiation therapy, in addition to chemotherapy and surgery, are more likely to develop severe late-onset effects (Robinson, Fraley et al. 2013). As the survivor population grows, new measures to improve patient quality of life become increasingly important.

1.2 HIE

Clinical and experimental findings underscore the evolving nature of HIE, which develops during the onset of the insult and encompasses the recovery period after resuscitation (Azzopardi, Wyatt et al. 1989; Roth, Edwards et al. 1992; Takeoka, Soman et al. 2002). The failure in cellular bioenergetics after ischemic insult to the immature brain displays a biphasic pattern (Wyatt, Edwards et al. 1989; Lorek, Takei et al. 1994; Blumberg, Cady et al. 1997), with an initial energy failure followed by a secondary prolonged and prominent energy failure over the first 48 h after reperfusion. This creates a valuable treatment window for the application of neuroprotective strategies to rescue injured but still viable brain cells.

Mitochondria are implicated in cell damage during hypoxia-ischemia (HI) and the subsequent reperfusion (Kristian 2004; Hagberg, Mallard et al. 2009; Rousset, Baburamani et al. 2012). Mitochondrial ATP production via oxidative phosphorylation is impaired during ischemia. Upon reperfusion, mitochondrial function is transiently restored due to the resupply of cerebral oxygen, but the functional restoration is followed by delayed post-ischemic mitochondrial respiratory failure. Several mechanisms are linked to delayed mitochondrial damage, particularly the induction of mitochondrial permeabilization (Griffiths and Halestrap 1995; Wang, Carlsson et al. 2009). Mitochondrial dysfunction then triggers a number of pathophysiological responses, including an accumulation of glutamate and the overactivation of its receptors, an increase in intracellular calcium levels, the generation of reactive oxygen species (ROS) and nitric oxide (NO), and the release of pro-apoptotic proteins (Rousset, Baburamani et al. 2012).

HI-induced excitotoxicity and oxidative damage cascades can instigate microvascular injury and blood-brain barrier (BBB) dysfunction, eliciting robust post-ischemic inflammation in the penumbra surrounding the infarct core, as evidenced by microglial activation (Giulian and Vaca 1993; McRae, Gilland et al. 1995) and cytokine release (Bona, Andersson et al. 1999). Together with apoptosis, inflammation critically participates in delayed neuronal cell death, and the magnitude of apoptosis and inflammation eventually reflects the severity of HIE.

1.2.1 Apoptosis

Cumulative evidence shows that brain cells die by either necrosis or apoptosis, or a combination of the two, based on biochemical and morphological criteria (Northington, Ferriero et al. 2001). Necrosis, manifested by swelling of the cytoplasm and organelles and

the loss of membrane integrity, is the predominant cell death mechanism initiated during the acute injury phase and in more severe cases of ischemic damage (Bonfoco, Krainc et al. 1995; Towfighi, Zec et al. 1995; Carloni, Carnevali et al. 2007). By contrast, apoptosis, a process whereby cells require appropriate levels of intracellular ATP to commit suicide (Eguchi, Shimizu et al. 1997; Nicotera, Leist et al. 1998), occurs several days after the insult in milder cases of ischemic injury, especially within the penumbra area (Bonfoco, Krainc et al. 1995; Northington, Ferriero et al. 2001). Brain maturity also has an impact on the preferential utilization of cell death mechanisms (Zhu, Wang et al. 2005). Pro-apoptotic proteins are highly expressed in the developing brain and decline during neuronal maturation (Hu, Liu et al. 2000). Therefore, it is not surprising that the activation of apoptotic machinery predominates during HI-induced pathogenesis in the immature brain. The neuronal cell death mechanisms then shift to predominantly necrosis during brain development (Liu, Siesjo et al. 2004).

Two categorized pathways have been identified to lead to apoptosis, an intrinsic and an extrinsic pathway. There is now considerable evidence that cerebral ischemia stimulates expression of Fas ligand, which is implicated in the extrinsic pathway of caspase activation (Rosenbaum, Gupta et al. 2000). The onset of ischemia also initiates the accumulation of intracellular calcium and oxidative stress, which subsequently triggers the intrinsic apoptotic pathway (Dirnagl, Iadecola et al. 1999). Although the triggers vary, they are all converged to a mitochondrion-centered control mechanism (Kroemer, Galluzzi et al. 2007).

1.2.1.1 The BCL2 family

The BCL2 (B-cell lymphoma 2) gene was originally identified by virtue of its deregulation via a chromosomal translocation commonly occurring between chromosome 14 and 18 in follicular B-cell lymphomas. This translocation decreases the propensity of B-cells to undergo apoptosis (Vaux, Cory et al. 1988). Approximately 25 BCL2 family proteins with a regulatory function in apoptosis have been described to date. These proteins contain at least one conserved BCL2 homology (BH) domain (BH1–BH4), allowing them to direct apoptosis through intermolecular interactions with other BCL2 family members.

The pro-survival members of the BCL2 protein family (BCL2 and BCL-XL) contain all four BH domains, and are essential for cell survival and function. Contrarily, some pro-apoptotic members of the BCL2 family (e.g., Bax and Bak) contain three BH domains, and are required for increasing mitochondrial permeability and releasing apoptotic regulators via channel or pore formation in the outer mitochondrial membrane. Other pro-apoptotic

BCL2 proteins (e.g., Bid) contain only the critical BH3 death domain. These one-BH domain proteins bind to and inhibit pro-survival BCL2 family members, thereby facilitating the activation of pro-apoptotic Bax and Bak proteins (Willis, Fletcher et al. 2007).

Under normal conditions, Bax resides primarily in the cytoplasm in its monomeric form. In response to apoptotic stimuli, Bax translocates to the mitochondria in association with other Bax and/or Bak monomers to form pores in the outer mitochondrial membrane (Yethon, Epand et al. 2003; Dewson and Kluck 2009; George, Targy et al. 2010). Mitochondrial outer membrane permeabilization due to a disturbance in the balance of pro- and anti-apoptotic members of the BCL2 family, stimulates the initiation of caspase-dependent and -independent cell death through the release of pro-apoptotic proteins. For example, cytochrome c translocates to the cytosol (Northington, Ferriero et al. 2001), where it together with apoptotic protease-activating factor 1 and dATP drive assembly of the apoptosome (Gill, Soriano et al. 2002), which goes on to trigger the proteolytic caspase-9/-3 cleavage cascade (Li, Nijhawan et al. 1997; Benjelloun, Joly et al. 2003). Meanwhile, AIF relocates to the nucleus and mediates large-scale DNA fragmentation through a caspase-independent pathway (Zhu, Qiu et al. 2003; Zhu, Wang et al. 2007).

1.2.1.2 Caspases

Caspases make up a family of intracellular cysteine proteases with unique substrate specificity, requiring an aspartate residue at the cleavage site. These proteases are essential for the execution of apoptosis (Stennicke and Salvesen 2000; Ferri and Kroemer 2001). The caspase proteins are synthesized as inactive zymogens containing three domains, a variable length amino-terminal prodomain, a large subunit domain (~20 kDa), and a small subunit domain (~10 kDa). Fourteen caspases have been identified so far, comprising the initiator caspases, the effector caspases, and a group of caspases involved in inflammatory cytokine processing. Initiator caspases (e.g., caspase-2, -8, and -9) directly catalyze the proteolytic maturation of effector caspases (caspase-3 and -7). In this manner, the effector caspases stimulate the cleavage of numerous important proteins, including structural proteins within the cell, eventually culminating in cellular apoptosis. Activation of inflammatory caspases (e.g., caspase-1, -4, -5, and -11) results in the production of pro-inflammatory cytokines and promotion of innate immune responses to various stimuli.

By now, three caspase-related signaling pathways are recognized that can lead to apoptosis. The intrinsic apoptotic pathway involves the activation of caspase-9 via the assembled apoptosome, followed by caspase-3 activation (Zou, Henzel et al. 1997). Extrinsic

apoptosis is elicited by the activation of death receptors, inducing the formation of the death-inducible signaling complex (DISC). DISC in turn mediates the activation of caspase-8 and the downstream caspases, caspase-3 and -7 (Salvesen and Dixit 1997). Caspase-8 also prompts the proteolytic cleavage of Bid, followed by the generation of truncated Bid and the insertion of Bax or Bak into the mitochondrial membrane (Li, Zhu et al. 1998; Luo, Budihardjo et al. 1998). The third pathway is associated with caspase-2 activation, also increasing mitochondrial permeabilization and the release of pro-apoptotic proteins (Enoksson, Robertson et al. 2004).

Caspase activation is a prominent feature of the developing brain after HI (Hu, Liu et al. 2000; Liu, Siesjo et al. 2004). Caspase-3 is the most abundant effector caspase present and predominates in apoptotic cell death in the immature brain, displaying increased activity for at least 6 days after HI (Wang, Karlsson et al. 2001). Notably, caspase activation appears to be more important for injury progression in the immature vs. adult brain (Hu, Liu et al. 2000; Blomgren, Zhu et al. 2001). These observations support the findings that caspase inhibitors utilized in treatment modalities ameliorate HI-provoked neonatal brain damage.

1.2.2 Inflammatory response

The immune system is generally divided into the innate immune system and the adaptive immune system. In contrast to the nonspecific defenses afforded by the innate immune system, the adaptive immune system confers an antigen-specific immunity driven by lymphocytes and the development of immunological memory. Researchers have long regarded the brain (except for the meningeal compartment) as relatively isolated from immunosurveillance under normal conditions due to the existence of the BBB and the formation of an immunological barrier, as well as a limited adaptive response (Reese and Karnovsky 1967; Schwartz, Moalem et al. 1999). However, a recent study described the existence of a functional network of lymphatic vessels lying parallel to the dural sinuses, prompting new consideration of immune responses in the CNS (Louveau, Smirnov et al. 2015).

Inflammation occurs in response to various forms of CNS injury and disease, and is characterized by activation of microglia, astrocytes, and endothelial cells, in addition to enhanced expression of inflammatory mediators. The peripheral immune cells can also cross a permeable BBB and contribute to such a process. The relative contribution of systemic and local inflammation, and the link between the two, varies depending on the injury setting. For instance, stroke in the adult brain promotes the infiltration of peripheral

myeloid cells into the brain parenchyma (Dirnagl, Iadecola et al. 1999), probably by increasing BBB permeability and stimulating a persistent inflammatory reaction (Gidday, Gasche et al. 2005).

The inflammatory process begins in the intravascular compartment at the onset of ischemia and progresses toward the parenchymal processes leading to brain damage over time (Iadecola and Anrather 2011). In the context of hypoxic-ischemic encephalopathy, inflammation is critical for the delayed cell death and contributes importantly to the progression of brain injury (Inder and Volpe 2000; Kaushal and Schlichter 2008; Liu and McCullough 2013). Inflammatory responses in the CNS are initially characterized by the activation of resident microglia. Activated microglia release reactive oxygen species (ROS), proinflammatory cytokines and matrix metalloproteinase 9 that cause neurotoxicity. Next, peripheral macrophages, monocytes, and neutrophils infiltrate the brain (Zheng and Yenari 2004; Liu and McCullough 2013; Umekawa, Osman et al. 2015), amplifying the inflammatory response.

1.2.2.1 Microglia

Microglia are distributed throughout the brain parenchyma and form the first and main line of defense against insults (Stoll and Jander 1999; Nimmerjahn, Kirchhoff et al. 2005; Tambuyzer, Ponsaerts et al. 2009). Microglia arise from hematopoietic stem cells in the yolk sac that migrate to the brain during early embryonic development. Thus, microglia are endowed with a self-renewing capacity to sustain the resident population (Ginhoux, Greter et al. 2010; Schulz, Gomez Perdiguero et al. 2012; Gomez Perdiguero, Schulz et al. 2013). Under normal conditions, microglia search for alterations in homeostasis by constantly scanning the environment with dynamic cytoplasmic extensions (Nimmerjahn, Kirchhoff et al. 2005; Hanisch and Kettenmann 2007). By doing so, they contribute to the maintenance of normal CNS functions; they also play an essential role in hippocampal neurogenesis by phagocytosing apoptotic newborn cells (Sierra, Encinas et al. 2010).

Under pathological conditions (e.g., HI), microglia rapidly react to activation stimuli and adjust their functions accordingly. In the immature rodent brain, microglial activation appears within 2 h after HI, reaches a peak after 2–3 days, and remains elevated until ~14 days after the insult (McRae, Gilland et al. 1995). Additionally, a retrospective clinical study on postmortem brains from human neonates with proven HIE showed a dense infiltrate of microglial cells in the polymorphous layer of the dentate gyrus (DG) (Del Bigio and Becker 1994).

Excessive microglial activation in the developing brain exerts toxic effects by generating an overabundance of pro-inflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-18, and others), excitotoxic neurotransmitters, NO, and ROS (Bhalala, Koehler et al. 2014). The net result is disruption of the immature BBB and worsening of inflammation and brain damage (Bona, Andersson et al. 1999; Yenari, Xu et al. 2006; Kichev, Rousset et al. 2014). Systemic administration of the antibiotic minocycline affords neuroprotection by inhibiting microglial activation, reducing BBB disruption and the production of inflammatory mediators (Arvin, Han et al. 2002; Yenari, Xu et al. 2006). Caspases, in addition to regulating apoptosis, also participate in microglial activation, providing an enzymatic target for microglial inhibition (Burguillos, Deierborg et al. 2011). In the subacute injury phase, however, activated microglia facilitate phagocytic clearance of cell debris and stimulate the reorganization of neuronal circuits. Moreover, pharmacological depletion of microglia before neonatal stroke amplifies local inflammation and aggravates damage (Faustino, Wang et al. 2011), suggesting that a subpopulation of microglial cells are beneficial after injury and contribute to endogenous brain defenses.

1.2.2.2 Cytokines and Chemokines

Cytokines and chemokines produced during inflammatory responses in the CNS mediate the mobilization/infiltration of inflammatory cells and the initial recognition of the infection or injury site. For example, cytokines and chemokines orchestrate leukocyte recruitment, adhesion, extravasation, and localization at the site of inflammation (Saliba and Henrot 2001). Cytokines also induce the expression of adhesion molecules on the vascular endothelium (Meager 1999). The tight adherence of leukocytes to the activated endothelium and their directed extravasation into inflamed tissues is promoted by chemokines (Rot and von Andrian 2004).

Cytokines and chemokines can be either pro- or anti-inflammatory, exacerbating or diminishing the immune reaction, respectively. The levels of the pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, are elevated in human newborns with HIE relative to healthy controls. IL-1β upregulation correlates positively with HIE severity (Aly, Khashaba et al. 2006; Liu and Feng 2010), while interference with IL-1β expression ameliorates HI-induced brain damage and neurological deficits (Martin, Chinookoswong et al. 1994; Hagberg, Gilland et al. 1996). The increased expression of cytokines following HIE is also accompanied by upregulation of chemokines, such as macrophage inflammatory protein (MIP)-1α and monocyte chemotactic protein-1 (MCP-1, also called chemokine ligand 2 (CCL2)) (Ivacko, Szaflarski et al. 1997; Bona, Andersson et al. 1999). In the neonatal brain,

HI stimulates the expression of CCL2 in the parenchyma at 4 h after insult, and CCL2 upregulation continues for ~48 h (Ivacko, Szaflarski et al. 1997). CCL2 exerts powerful activating and recruiting effects on mononuclear phagocytes, T-cells, and B-cells, and contributes to the pathogenesis of acute neonatal brain injury (Chen, Hallenbeck et al. 2003; Hagberg, Mallard et al. 2015).

1.2.3 Excitotoxicity

Glutamate is the major excitatory neurotransmitter in the brain. The excitatory response is generated following an interaction of glutamate with ionotropic glutamate receptors termed ligand-gated ion channels. Excessive activation of ligand-gated ion channels causes cell damage and death. This process, termed excitotoxicity, is an important mechanism of neuronal cell death after neonatal HI (Choi and Rothman 1990). The excitatory effects of glutamate are exercised via activation of three major receptor types located at post-synaptic membranes: the N-methyl-D-aspartic acid (NMDA) receptor, the α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor, and the kainic acid (KA) receptor.

In both preclinical animal models and human infants, cellular energy depletion during HI promotes neural and glial membrane depolarization and glutamate release into the extracellular space (Hagberg, Andersson et al. 1987; Hagberg, Thornberg et al. 1993). In addition, energy-dependent glutamate transporters that terminate the excitatory signal via glutamate reuptake become impaired (Swanson, Farrell et al. 1995). This results in an accumulation of glutamate to excitotoxic levels and the ensuing overactivation of NMDA receptors, which eventually increases the intracellular influx of calcium to initiate apoptotic cell death.

The immature brain is especially vulnerable to NMDA receptor activation. As an important trophic factor for the immature brain, glutamate promotes neuronal precursor proliferation and migration through NMDA receptors, as well as synaptic development and plasticity (McDonald and Johnston 1990; Komuro and Rakic 1993). In keeping with these functions, the activity of NMDA receptors changes during CNS development, resulting in a period of enhanced sensitivity to excitotoxic insults. In the immature brain, NMDA receptors are hyperactive and show enhanced density, excitability, and the capacity to provoke toxicity (Ben-Ari, Cherubini et al. 1988; Hamon and Heinemann 1988). In agreement with these findings, NMDA receptor antagonists are effective neuroprotective agents in preclinical animal models (McDonald, Silverstein et al. 1987). However, clinical trials studying the direct antagonism of NMDA receptors as a therapeutic treatment have proven

disappointing, perhaps because of interference with physiological NMDA receptor function and/or a narrow therapeutic window (Ikonomidou and Turski 2002).

1.2.4 Oxidative stress

Free radicals are byproducts of normal cellular metabolism. These molecules are extremely reactive and can inflict damage on cells (Dawson and Dawson 1996). There are many types of free radicals, but those of most concern in biological systems, the ROS, are derived from oxygen. Under normal conditions, our biological system is able to counteract or detoxify ROS via antioxidant-mediated neutralization. Nonetheless, in extreme situations such as HI, ROS production is accelerated upon reperfusion, when oxygen is reintroduced to the ischemic tissue. Hence, the effectiveness of the antioxidant defense system is decreased, and the ROS/antioxidant balance is disturbed (Chan 2001). A major consequence of the resultant oxidative stress is damage to cellular macromolecules, causing fragmentation of, or structural changes in, lipids, proteins, and nucleic acids. The neonatal brain is characterized by high concentrations of unsaturated fatty acids and oxygen consumption rates, low concentrations of antioxidants, and the availability of redox-active iron for the catalytic formation of free radicals, rendering the organ particularly vulnerable to oxidative damage (Ferriero 2004).

Mitochondria are a major source of ROS production in both healthy and diseased tissues, including those affected by HI-induced injury. Approximately 2 % to 5 % of oxygen utilization by resting mitochondria is lost to ROS generation, and ROS production is enhanced by increased electron-transport activity (Boveris and Chance 1973). In keeping with its high energy demands, mitochondrial respiration is higher in the brain than in most other organs, thus increasing the risk of elevated free radical production (Schonfeld and Reiser 2013). Data on mitochondria isolated from ischemic brain and from *in vivo* metabolic imaging studies suggest that ischemia-reperfusion engenders both short-and long-term alterations in mitochondrial function (Bainbridge, Tachtsidis et al. 2014). Oxidative damage to mitochondria and resulting metabolic impairment may have several consequences, including downregulated ATP synthesis, decreased mitochondrial membrane potential, and enhanced release of pro-apoptotic factors (Rousset, Baburamani et al. 2012).

1.2.5 Neuroprotective strategies

Numerous research efforts are directed toward the investigation of experimental therapies targeting specific pathways in the pathophysiology of HI-induced brain injury. Nevertheless, only a few of these strategies have presently met with clinical success. Hypothermia, when initiated within 6 h of birth, can successfully decrease mortality rates

and improve neurologic outcomes after moderate HIE in full-term infants, and is becoming a standard therapy for HIE management (Clark and Snedeker 2006; Jacobs, Morley et al. 2011).

The ideal aim of HIE treatment is to reduce mortality and restore long-term motor and cognitive deficits. A better understanding of the biochemical and cellular mechanisms behind HI-provoked brain damage would assist in uncovering effective interventions to terminate or mitigate deleterious injury cascades. In addition, the optimal therapeutic window for various neuroprotective strategies must be investigated to achieve maximum treatment efficiency, and to eventually improve the care of perinatal HI patients. In this regard, pharmacological interventions as well as regenerative therapies for HI have been proposed and evaluated (Badr Zahr and Purdy 2006). For instance, allopurinol is an antioxidant that inhibits the formation of free radicals. This agent can decrease HI-induced brain damage in experimental animal models and in the clinical environment (Palmer, Towfighi et al. 1993; Torrance, Benders et al. 2009). Other possible candidates for HI management include caspase inhibitors and anti-inflammatory drugs, which show neuroprotective effects even when delivered after the onset of HI-facilitated injury (Cheng, Deshmukh et al. 1998; Khan, Sekhon et al. 2004).

Endogenous neurogenesis seems to increase after HI (Felling, Snyder et al. 2006; Yang, Covey et al. 2007). However, the attempt at neuronal repopulation appears futile, because the supply of endogenous stem cells is either insufficient or the number of surviving stem cells is too low to replace lost neurons, let alone to reconstruct the three-dimensional architecture of the brain (Martino and Pluchino 2006). Transplantation of stem cells into injured or degenerating brain regions can be beneficial in cases of acute brain damage and neurodegenerative disease (Lindvall, Kokaia et al. 2004; Lindvall and Kokaia 2011). Regardless, very few exogenous stem cells survive the transplantation process, and those that do survive rarely integrate into the injured brain tissue (Chen, Li et al. 2002; Menasche 2005), thus challenging the concept of functional tissue replacement by stem cells. As such, the precise mechanisms underlying the therapeutic effects observed after stem/progenitor cell transplantation into the brain remain unclear.

1.3 IR-induced brain injury

IR-induced brain injury refers to functional deficits that arise predominantly from DNA damage, as well as to anatomical deficits. Both may occur either directly or indirectly from exposure of tissues to IR (Hall 2000). IR directly damages tissue by interacting with and lesioning DNA. Indirect damage is attributed to the actions of ROS and reactive nitrogen oxide species (RNOS), which are formed from radiation/water molecule interactions. Overt white matter necrosis is among the most debilitating reactions to IR (Ruben, Dally et al. 2006). However, with the improvement in radiotherapy protocols, such as divide the total dose of radiation into a number of small doses, these problems have been significantly reduced. Nevertheless, many patients exhibit progressive learning and memory deficits after IR with no overt injuries, especially when the radiation field involves the temporal lobe, where the hippocampus resides (Packer, Sutton et al. 1989; Mulhern, Merchant et al. 2004).

The hippocampal formation is essential for cognitive function, because it processes short-term declarative memory and spatial information prior to long-term memory storage in the neocortex (Eichenbaum 2000; Eichenbaum 2001; Clark, Broadbent et al. 2007). The mechanisms underlying IR-provoked cognitive deficits are only partly understood, but probably include reduced hippocampal neurogenesis (Raber, Rola et al. 2004; Rola, Raber et al. 2004). Like cancer cells, neural progenitor cells are vulnerable to the effect of IR than normal cells given to their high proliferative capacity. At least two mechanisms causing reduced neurogenesis after radiotherapy are possible: 1) direct depletion of NPCs via apoptosis, stemming from DNA double-strand breaks, and 2) IR-induced neuroinflammation, which alters the microenvironment and shifts the proliferative response of NPCs from neurogenesis to gliogenesis (Monje, Mizumatsu et al. 2002).

1.3.1 Hippocampal neurogenesis

Neurogenesis, or the process whereby new neurons are generated from neural stem/progenitor cells, is most pronounced during the embryonic/prenatal period and continues throughout life (Seki and Arai 1995; Kuhn, Dickinson-Anson et al. 1996; Eriksson, Perfilieva et al. 1998). Neurogenesis transpires in the subventricular zone located in the walls of the lateral ventricles, and also in the subgranular zone (SGZ) of the hippocampal DG (Altman and Das 1965; Altman and Das 1967; Caviness 1973; Gueneau, Privat et al. 1982; Kuhn, Dickinson-Anson et al. 1996; Eriksson, Perfilieva et al. 1998). This thesis focuses on neurogenesis and IR effects in the SGZ.

Neural stem cells in the SGZ comprise a narrow layer of cells between the hilus and the granule cell layer (GCL), and give rise to DG granule neurons through a multistep process upon extrinsic and intrinsic stimulation (Goldman and Chen 2011). Newborn neurons integrate into, and form synapses within, the existing hippocampal circuitry, receiving functional inputs by extending dendrites toward the molecular layer and projecting axons through the hilus toward the CA3 region of the hippocampus (Hastings and Gould 1999; Overstreet-Wadiche and Westbrook 2006; Zhao, Teng et al. 2006). The integration process is stereotypic in nature and initially activated by ambient gamma-aminobutyric acid (GABA), followed by dendritic GABAergic and glutamatergic synaptic inputs, and finally, by perisomatic GABAergic inputs (Esposito, Piatti et al. 2005; Overstreet Wadiche, Bromberg et al. 2005; Tozuka, Fukuda et al. 2005). Although newborn neurons exhibit hyper-electrical and enhanced synaptic properties during immature stages, they are already functional (Schmidt-Hieber, Jonas et al. 2004; Ge, Sailor et al. 2008). After structural maturation of the newborn neurons, their electrophysiological properties become indistinguishable from those of adult granule cells (Ge, Yang et al. 2007; Laplagne, Kamienkowski et al. 2007).

As noted above, the hippocampus is crucial for the temporal formation of memories, including declarative memory and spatial memory (Burgess, Maguire et al. 2002). Given the continuous integration of new neurons into the existing hippocampal circuitry, the effect of neurogenesis on hippocampus-related behavior becomes an important question. Several studies have shown strong positive correlations between the rate of hippocampal neurogenesis and performance in learning and memory tasks; furthermore, manipulation of hippocampal neurogenesis causes corresponding changes in cognitive performance (Epp and Galea 2009; Deng, Aimone et al. 2010). For example, voluntary exercise and environmental enrichment both increase neurogenesis and improve the performance of aged mice in spatial learning tasks (Kempermann, Gast et al. 2002; van Praag, Shubert et al. 2005). However, specific ablation of adult-born hippocampal neurons via inducible gene expression in transgenic mice impairs hippocampal-dependent memory performance, as does administration of methylazoxymethanol acetate or glucocorticoid (Shors, Miesegaes et al. 2001; Dupret, Revest et al. 2008; Atsak, Hauer et al. 2012). Therefore, disruption of hippocampal neurogenesis can have severe clinical outcomes.

Endogenous neural stem and progenitor cells seem to be particularly vulnerable to IR-induced damage, because exposure to even a low dose of X-rays during stem cell proliferation can trigger DNA damage and apoptosis (Mizumatsu, Monje et al. 2003).

Moreover, stem cell proliferation remains depressed for prolonged periods of time, and newborn neurons are not capable of repopulating or regenerating the SGZ (Tada, Parent et al. 2000). Fukuda *et al* indicated that a single dose of IR (8 Gy) almost completely blocked the growth of the hippocampal DG, as assessed by quantification of DG size. The growth inhibition was accompanied by deficits in hippocampal-dependent behaviors (Fukuda, Fukuda et al. 2005; Karlsson, Kalm et al. 2011). Together with ablation of the precursor pool, IR further reduced the ability of the remaining stem cells to adopt a neuronal fate, probably by triggering intrinsic cellular damage and also by altering a neurogenesis-promoting environment. In support of this idea, gliogenesis was relatively preserved following exposure of the brain to radiation (Monje, Mizumatsu et al. 2002). The persistent and progressive impairment of neurogenesis after IR has been linked to the deleterious effects of chronic inflammation (Monje, Mizumatsu et al. 2002).

1.3.2 IR-induced neuroinflammation

Appropriate regulation of neuroinflammation facilitates CNS recovery by containing and eliminating noxious stimuli while initiating tissue repair. Despite these efforts, prolonged neuroinflammation can induce secondary injury and harm the developing brain. Compelling evidence suggests that IR provokes a chronic state of inflammation and affects the fate of the precursor cell pool by altering the surrounding microenvironment. Importantly, irradiated precursor cells can still differentiate into neurons *in vitro* when given the requisite proliferative signals (Monje, Mizumatsu et al. 2002). Therefore, anti-inflammatory treatment can restore neurogenesis, at least in part, and mitigate IR-induced cognitive deficits (Monje, Toda et al. 2003; Jenrow, Brown et al. 2013).

Macrophages are an important type of leukocyte involved in directing immune responses. These cells were first discovered in 1884 by Élie Metchnikoff, a Russian bacteriologist, and derive their name from the Greek term for “big eaters”. As scavengers, macrophages primarily function to engulf and digest invading pathogens, cell debris, and apoptotic cells in a process called phagocytosis. In addition, macrophages are capable of responding rapidly to injury conditions, express a plethora of chemical substances and mediators to regulate inflammatory processes, and help to establish adaptive immunity by recruiting other immune cells (Fujiwara and Kobayashi 2005; Dheen, Kaur et al. 2007). Macrophages are found in essentially all tissues. In the CNS, microglia represent the resident macrophages.

Macrophages can either increase inflammation and stimulate the immune system, or decrease inflammation and dampen immune reactions through the release of specific

cytokines (Dalton, Pitts-Meek et al. 1993; Gordon 2003; Butterfield, Best et al. 2006). For decades, CNS macrophages were regarded as a functionally homogeneous population due to the existence of the BBB, and invulnerable to peripheral leukocyte entry. (Wilson, Weninger et al. 2010). However, an abundance of data now demonstrates that the macrophage population in the injured brain consists of both resident microglia and infiltrating monocyte-derived macrophages (Shechter, London et al. 2009; Jin, Yang et al. 2010). Furthermore, the latter perform indispensable roles that cannot be provided by their resident counterparts (Shechter, London et al. 2009; Shechter, Raposo et al. 2011). Nonetheless, whether peripheral macrophages contribute to the persistent negative effects of IR remains unknown and requires elucidation.

Monocytes expressing the chemokine receptor CCR2 are selectively recruited to injured tissue in response to CCL2, where they become macrophages (Auffray, Fogg et al. 2007). The finding that CCL2 deficiency is sufficient to restore hippocampal neurogenesis after IR suggests that pro-inflammatory monocytes may contribute to chronic IR-induced neuroinflammation (Lee, Haditsch et al. 2013). However, given the lack of specific markers, it is difficult to distinguish resident microglia from infiltrating macrophages derived from blood-borne, peripheral monocytes via conventional techniques. Moreover, little is known about the relative contributions of resident microglia and infiltrating macrophages to the inflammatory response in the CNS at different maturation stages. A better understanding of these two evolutionarily distinct groups of antigen-presenting cells will be important in the search for efficient clinical interventions for IR-induced brain damage.

2 Aims of the thesis

The overall goal of this thesis was to investigate the contributions of apoptosis and inflammation to the development of HI- and IR-induced brain injury, and to explore potential neuroprotective therapies for each condition.

Four specific aims were addressed:

I. To determine the neuroprotective effect of a cell-penetrating Bax-inhibiting peptide (BIP) after HI in neonatal mice.

II. To investigate the effects of delayed, long-term administration of the caspase inhibitor, Q-VD-OPh, on apoptosis and inflammation after HI in neonatal mice.

III. To evaluate the neuroprotective potential of the conditioned medium (CM) obtained from apoptotic NPCs both *in vitro* and *in vivo*.

IV. To characterize the relative contributions of resident microglia and infiltrating macrophages in the inflammatory response after IR in both juvenile and adult brains.

3 Methods

This section briefly describes the most important methods used in this thesis. For detailed information regarding specific experimental procedures, please refer to the Materials and Method section of each individual manuscript.

3.1 Animals (I-IV)

Manuscripts I, II, and III describe the use of C57BL/6 mice to produce a neonatal HI model to compare the efficacy of different therapeutic interventions. Strain differences in the susceptibility to brain damage were previously documented in a study of neonatal mouse HI models. In particular, CD1 and 129Sv mice were quite susceptible or enormously resistant to HI insult, respectively, whereas C57BL/6 mice were intermediately sensitive and showed an increasing degree of brain damage with the increasing duration of hypoxia (Sheldon, Sedik et al. 1998). For this reason, the C57BL/6 mouse was chosen for the present HI studies.

Manuscript IV describes the use of genetically modified $CX3CR1^{GFP/+}CCR2^{RFP/+}$ mice with a C57BL/6 background for the IR study. Resident microglia ($CX3CR1^{+}$) were labeled in these animals with green fluorescent protein (GFP), and monocytes ($Ly6C^{hi}CCR2^{+}CX3CR1^{-}$ cells) were labeled with red fluorescent protein (RFP) (Saederup, Cardona et al. 2010). The advantage of this reporter system is that resident microglia are readily distinguished from infiltrating monocyte-derived macrophages, allowing determination of the relative contribution of each population to the inflammatory response after IR.

Given the difference in brain anatomy between mice and humans, it is difficult to extrapolate the maturational age of the murine CNS to that of the human. Recent work compared five key events (neuroanatomy, cell proliferation, synaptogenesis, myelination, and inflammation) that accompany brain development in rodents and humans, and concluded that rodent at postnatal day (PND) 7–10 and 60 are equivalent to term infants and adult humans, respectively (Semple, Blomgren et al. 2013).

3.2 HI model (I-III)

The most commonly used neonatal HI model was modified in the early 1980s from the Levine technique (Levine 1960) for use with PND 7 rats (Rice, Vannucci et al. 1981; Vannucci and Vannucci 1997), and was later adapted again for use with PND 9 mice (Sheldon, Sedik et al. 1998). This model consists of unilateral common carotid artery ligation followed by a period of systemic hypoxic exposure. Ligation alone is insufficient

to induce brain injury in rodents, because these animals have extensive collaterals. However, when combined with hypoxia, the animals undergo a dramatic decrease in systemic blood pressure and cerebral blood flow, as well as a compensatory vasodilation of the vessels, instigating a reduction of regional blood flow to the ipsilateral hemisphere and the development of global cerebral ischemia. Cerebral blood flow is restored to control levels immediately upon return to a normoxic environment (Vannucci and Hagberg 2004).

The brain damage incurred in the above model is restricted to the ipsilateral hemisphere, leaving the contralateral hemisphere unaffected. Therefore, the contralateral hemisphere serves as an internal control (Grafe 1994; Towfighi, Housman et al. 1994). Animals that survive the insult generally live as long as normal animals, permitting assessment of the impact of various therapeutic interventions both histologically and functionally. The drawbacks of this model are the lack of multi-organ dysfunction and high variability (Grafe 1994; Hagberg, Bona et al. 1997). The latter limitation is circumvented by including a large number of animals in each experimental group.

3.3 IR model (IV)

PND 10 (juvenile) or PND 90 (adult) mice of both genders were used for the IR procedure. Animals were placed onto a carved Styrofoam bed adjusted for body size with a source-to-sample distance of 50 cm in an X-RAD 320 Biological Irradiator (Precision X-Ray, North Branford, CT, USA) and anesthetized with isoflurane (5 % for induction and 2 % for maintenance). The entire brain was covered by a radiation field of 2 cm × 2 cm (dose rate = 0.72 Gy/min at 320 kV and 12.5 mA). A single dose of 8 Gy was delivered, yielding a time for the IR procedure of 11 min and 3 sec. The total procedure, including induction of anesthesia, required ~13 min for each animal. Sham control animals were anesthetized but not subjected to IR. The dose applied to the experimental animals was equivalent to 18 Gy delivered in 2 Gy fractions, a treatment protocol described for prophylactic IR in selected cases of acute childhood lymphatic leukemia (Fowler 1989).

3.4 Cell culture and preparation of conditioned medium (CM, III)

Mouse NPCs were provided by Professor Fred H. Gage (Salk Institute, La Jolla, CA, USA) (Ray and Gage 2006). Cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with nutrient mixture F12 in a standard humidified tissue culture incubator at 37°C and 5 % CO_2 (Sato, Shinjyo et al. 2013). SNL 76/7 feeder cells were obtained from Sigma-Aldrich (St. Louis, MO, USA). HT22 hippocampal neuronal cells were originally generated by David Schubert (Salk Institute, San Diego, USA) and obtained from Gerald

Thiel (Homburg-Saar) Primary mouse embryonic fibroblasts (MEFs) were obtained from embryonic day 17–18 C57BL/6 mice. MEFs, SNL cells, and HT22 cells were cultured according to established protocols.

CM was procured from NPCs, MEFs, SNL cells, and HT22 cells. Cells were grown in tissue culture flasks until they reached ~70 % confluency. Culture media were removed, and cells were washed once with PBS. Next, the cells were treated with Earle's Balanced Salt Solution with or without phenol red (Sigma-Aldrich) to induce starvation. CM samples were collected, centrifuged at 1,000 rpm for 10 min, and filtered through a 0.22-µm membrane filter to remove dead cells and cellular debris. The filtered samples were heated at 60°C for at least 10 min for heat activation. To test the ability of secreted cellular components to safeguard hippocampal neurons against glutamate toxicity *in vitro*, HT22 cells were pretreated with the various heat-inactivated CM samples for 6 h, followed by glutamate treatment in the continued presence of the individual CM fractions.

3.5 Behavioral evaluation (I and II)

HI-induced brain damage is observed in the cerebral cortex, striatum/thalamus, hippocampus, and subcortical/periventricular white matter (Rice, Vannucci et al. 1981; Vannucci and Hagberg 2004). Surviving neural tissue surrounding the lesion cannot fulfill the extremely high regenerative demands of the brain, exemplified by deficits in both motor and cognitive functions. In this thesis work, behavioral assessments after HI were conducted by applying the cylinder-rearing test, the open-field test, and trace-fear conditioning test.

The cylinder-rearing test was first designed for evaluating locomotor asymmetry in Parkinson's disease model rats, and was later adapted to other animal models for assessing sensorimotor asymmetry (Iancu, Mohapel et al. 2005; Brooks and Dunnett 2009). The animals are placed in an open-top glass cylinder, and filmed with video equipment from the side. Forelimb activity while rearing against the wall is recorded, and forelimb use is defined by the contact of the entire palm against the wall during rearing or lateral exploration, indicating the use of the palm for body bearing. Paw usage is calculated as a percentage by measuring the number of impaired forelimb wall contacts subtracted from the number of contacts for the contralateral paw, divided by the total number of contacts.

The open-field test is a simple test of locomotor activity involving the observation of an animal's movements within an open arena (Nilsson, Markinhuhta et al. 2006). When placed

in an unfamiliar field, a mouse will typically explore the whole arena while keeping close to the walls. Once the animal becomes habituated to the environment, it increasingly moves from one wall to another by crossing the central portion of the arena. Here, the number of stops and time spent in the middle of the arena were recorded and later analyzed with Ethovision 3.1 video-tracking software (Noldus Information Technology Inc., Leesburg, VA, USA). The analysis produces a behavioral track record, allowing determination of the total distance traveled during the observation period, as well as evaluation of attention-deficit hyperactivity disorder after HI.

Trace-fear conditioning is an associative learning task employed for the assessment of hippocampus- and amygdala-dependent learning deficits in rodents (Clark and Squire 1998; Goosens and Maren 2001). In this test, animals are subjected to a neutral conditioned stimulus (CS, such as a tone) that is paired with an aversive unconditioned stimulus (US, such as a foot shock). The CS and US are presented at discrete time points, and are separated by a stimulus-free trace interval. Once the animals learn to associate the CS with subsequent presentation of the US, the CS alone will elicit specific behavioral responses (e.g., freezing behavior, defined as the absence of movement except for respiration).

3.6 Immunohistochemistry (I-IV)

Paraffin sections (manuscripts I–III) were deparaffinized in xylene and rehydrated in a series of graded ethanol solutions. Antigen retrieval was performed by heating the slides in 10 mM sodium citrate buffer, pH 6.0, for 10 min. After blocking, sections were incubated for 60 min with primary antibodies diluted in PBS, and then incubated for 60 min with biotinylated secondary antibodies diluted in PBS. After blocking endogenous peroxidase activity with 3 % hydrogen peroxide, antigen staining was visualized by using an ABC Kit (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine. In manuscript IV, free-floating sections were rinsed with Tris-buffered saline (TBS; 50 mM Tris-HCl in 150 mM NaCl, pH 7.5) and incubated with 10 mM sodium citrate at 80°C for 30 min for antigen retrieval. The sections were then incubated in blocking solution (3 % donkey serum in TBS containing 0.1 % Triton X-100) for 1 h at room temperature (RT), followed by incubation with primary antibodies overnight at 4°C. On the second day, the sections were rinsed three times with TBS, incubated with the appropriate fluorophore-conjugated secondary antibodies for 1 h at RT, and mounted in Prolong® Antifade Mountant with DAPI (Life Technologies, Carlsbad, CA, USA).

4 Results and discussion

4.1 Bax inhibiting peptide (BIP) reduces neonatal HI brain injury (I)

To evaluate whether HI stimulates Bax translocation and activation, neonatal mouse brain sections were stained with an anti-Bax antibody (6A7) that primarily detects the pro-apoptotic Bax conformation (Yethon, Epand et al. 2003). An early progressive increase in 6A7 Bax immunostaining was observed after HI. This finding, together with data showing that a) Bax gene deficiency confers neuroprotection and b) Bax functions in mitochondrial permeabilization *in vivo* and *in vitro* (Gibson, Han et al. 2001; Wang, Carlsson et al. 2009), strongly indicates that Bax is a potential target for pharmacological neuroprotection of the immature brain.

Ku70 is a critical component of the Ku70/80 complex required for nuclear repair of DNA double-strand breaks. The cytosolic levels of Ku70 reportedly decrease in response to apoptotic stimuli, prompting the dissociation of Ku70 from Bax (Gama, Yoshida et al. 2006). This dissociation in turn elicits Bax-dependent mitochondrial permeabilization, and initiates caspase-dependent and -independent cell death through the release of pro-apoptotic proteins (Li, Nijhawan et al. 1997; Northington, Ferriero et al. 2001; Benjelloun, Joly et al. 2003; Zhu, Qiu et al. 2003). BIP is derived from the Bax-binding domain of Ku70 and exists in the cytosol, where it binds and inhibits Bax (Nothwehr and Martinou 2003; Gomez, Gama et al. 2007). We therefore investigated the effects of BIP on apoptosis after HI insult.

Injection of BIP into the brain ventricles via intracerebrovascular (ICV) administration just prior to the onset of hypoxia reduced Bax activation/Bax 6A7 staining at 1.5 and 3 h after HI. The decrease in Bax 6A7 staining was accompanied by reduced AIF translocation at 3 h after HI, diminished cytosolic cytochrome c immunoreactivity at 6 h, and downregulated caspase-3 activity at 24 h. These findings suggest that Bax activation is required for mitochondrial permeabilization and the subsequent release of pro-apoptotic proteins and downstream activation of executioner caspases in neonatal HI. Histological analysis demonstrated that BIP reduced HI-induced brain injury in terms of infarction volume and tissue loss in the entire hemisphere and specifically in the white matter at 5 days after the insult. Furthermore, evaluation of injury severity via neuropathological scores showed that BIP-facilitated neuroprotection was at its highest in the cortex and striatum. The neuroprotective actions of BIP were sustained over time, as evidenced by a continued reduction in tissue loss at 7 weeks after HI.

HI-induced brain damage can engender substantial long-term neurobehavioral deficits in sensorimotor as well as cognitive function (Jansen and Low 1996; Bona, Johansson et al. 1997; Ikeda, Mishima et al. 2001; Ten, Bradley-Moore et al. 2003). Neuroprotective strategies that exert morphological protection do not necessarily ameliorate the decline in functional activity (Farrell, Evans et al. 2001). Therefore, it is important to assess neurological function along with morphological outcomes. Here, the cylinder-rearing and trace-fear conditioning tests were used evaluate locomotor asymmetry and associative learning, respectively, at 7 weeks after HI. Consequently, BIP delivered before HI significantly improved functional performance in both tests relative to the vehicle control.

Altogether, the data in aim I/manuscript I demonstrate that BIP administered immediately before HI reduced gray and white matter injury and enhanced sensorimotor and cognitive function. The neuroprotection afforded by the peptide was associated with the inhibition of Bax-dependent mitochondrial permeabilization and the release of pro-apoptotic proteins.

However, there are potential limitations to this approach that hinder its translation into clinical applications. First, BIP was given prior to the onset of HI, yet in the clinical setting, HIE diagnosis is based on physical and neurological exams, as well as on the results of laboratory tests that can only be performed after birth (Azzopardi, Strohm et al. 2009). Interventions are applied even later. Therefore, further clinical application-related investigation (i.e., post-HI BIP administration) is required to validate the histological and functional effects of the peptide. Second, BIP was delivered directly into the lateral ventricles, because of the poor bioavailability of neuropeptides to the brain, high clearance by the liver, and the presence of the BBB (Egleton and Davis 2005). Nevertheless, ICV administration is an invasive approach, and should be avoided for possible clinical development of peptide drugs, especially in light of alternative strategies designed to improve bioavailability and BBB transport of such compounds (Pardridge, Buciak et al. 1991; Egleton, Mitchell et al. 2000). Future research is thus required elucidate BIP efficacy when delivered by less invasive routes (i.e., intravenous or intraperitoneal).

4.2 Role of caspases in apoptosis and inflammation after neonatal HI-induced brain injury (II)

Caspase-3 is a key executor of apoptosis in the developing brain after HI (Blomgren, Zhu et al. 2001; Wang, Karlsson et al. 2001; Zhu, Wang et al. 2005). Application of caspase inhibitors before or at the onset of the insult is an effective treatment for HI-induced brain injury (Renolleau, Fau et al. 2007; Zhu, Wang et al. 2007). Considering that apoptotic neuronal death is a relatively slow, multistep process, HI pathology might provide a prolonged window for therapeutic interventions (Beilharz, Williams et al. 1995; Northington, Ferriero et al. 2001). Moreover, caspase signaling purportedly regulates microglia activation (Burguillos, Deierborg et al. 2011), which may then aggravate brain damage (Bona, Andersson et al. 1999; Kichev, Rousset et al. 2014). Therefore, we next investigated the impact of delayed, long-term administration of the caspase inhibitor, Q-VD-OPh, on apoptosis and inflammation after neonatal HI-provoked brain injury.

Delayed administration of Q-VD-OPh initiated at 12 h after HI decreased HI-induced caspase-3 activity by 23 % and total brain tissue loss by 31 %. However, the decrease in caspase activity was lower than that observed in our previous work (57 %), where Q-VD-OPh was applied at the onset of and immediately after HI (Zhu, Wang et al. 2007). Additionally, administration of a less potent pan-caspase inhibitor, boc-aspartate fluoromethylketone (BAF), at 3 h after HI successfully decreased caspase-3/caspase-3-like activity by 50 % (Cheng, Deshmukh et al. 1998). These results suggest that much of the caspase activation after HI already occurs at 12 h after the insult, and that the attenuated enzyme inhibition observed herein (23 %) was probably due to the delayed onset of treatment.

This supposition is supported by the 48 % reduction in tissue loss reported in our earlier work after only two injections of Q-VD-OPh, while in the current study, a 31 % reduction in tissue loss was noted at 4 months after HI and 14 Q-VD-OPh injections. Nevertheless, it must be emphasized that the interval between HI and the evaluation of brain injury was very different in the two studies (3 days vs. 4 months). Taking into account the progressive nature of HIE, an accurate comparison will require evaluation of tissue loss at 4 months after HI and with two injections of Q-VD-OPh.

In addition to inhibiting caspase activation, Q-VD-OPh may overturn certain aspects of HI-induced brain injury by reducing inflammation. Cytokines and chemokines produced by resident immune cells, mainly microglia, trigger reactive changes in the damaged brain.

Some of these inflammatory mediators, such as CCL2 and CCL3, are pro-inflammatory and guide monocytes/macrophages toward the ischemic area, intensifying cerebral injury (Kim, Gautam et al. 1995). Others, like IL-4 and IL-10, are anti-inflammatory (Vila, Castillo et al. 2003) and exert protective or regenerative actions after ischemic brain injury (Spera, Ellison et al. 1998; Xiong, Barreto et al. 2011). Accordingly, we assayed the expression levels of CCl2, CCL3, IL-4, and IL-10 at 48 h after HI and quantified the number of microglia 4 months later. Two injections of Q-VD-OPh decreased CCL2 and CCL3 expression levels by 29.3 and 29.1 %, respectively, but did not alter IL-4 or IL-10 content. Long-term Q-VD-OPh treatment also failed to alter microglia density or morphology, and all discerned microglia displayed a ramified, surveillance morphology.

The role of Q-VD-OPh in post-ischemic inflammation is unclear. In light of recent findings demonstrating that microglial activation requires caspase activation (Burguillos, Deierborg et al. 2011), we speculate that the agent might reduce microglia-mediated inflammation, particularly because the anti-inflammatory cytokines, IL-4 and IL-10, were unaffected by its administration. Whether Q-VD-OPh-mediated caspase inhibition in microglia specifically blocks formation of the pro-inflammatory M1 phenotype as opposed to the regenerative M2 phenotype is unknown. Alternatively, the lower levels of pro-inflammatory chemokines observed following Q-VD-OPh treatment may simply be secondary to the prevention of tissue loss. Investigation of the anti-inflammatory mechanism of Q-VD-OPh will require detailed time courses of cytokine expression levels and caspase-3 activity in the presence or absence of caspase inhibition.

We utilized the cylinder-rearing and open-field tests to evaluate functional performance after caspase inhibition. Q-VD-OPh treatment ameliorated HI-induced loss of sensorimotor function and reduced HI-induced hyperactivity at 3 and 7 weeks, respectively, after the insult. The current study differs from previous reports of Q-VD-OPh treatment in ischemia-induced brain injury, in that long-lasting neurobehavioral outcomes were assessed as well as short-term consequences. After PND 60, when brain development in the rodent is complete (Semple, Blomgren et al. 2013), the two functional tests were repeated. Disappointingly, the functional improvements conferred by Q-VD-OPh at 3 and 7 weeks were no longer observed. The mechanisms underlying the discrepancy between the long-term reduction in tissue loss and the transient functional protection remain to be determined.

Several magnetic resonance imaging studies in human infants showed that neurodegeneration in multiple integrated brain regions evolves over time after the initial HI

insult (McKinstry, Miller et al. 2002; Stone, Zhang et al. 2008). Moreover, transplantation of mesenchymal stem cells in a mouse model of HI markedly ameliorated sensorimotor deficits and decreased brain tissue loss, but analogous to our findings, long-term functional impairment still persisted (van Velthoven, Kavelaars et al. 2010). Thus, even though Q-VD-OPh reduced long-term tissue loss in the current study, neurobehavioral deficits after HI were probably merely postponed.

4.3 Neural stem cell-based interventions for neonatal HI-induced brain injury (III)

Stem cell-based therapies reportedly promote recovery in animal models of acute brain damage or neurodegenerative disease (Lindvall, Kokaia et al. 2004; Lindvall and Kokaia 2011). However, the number of stem cells that survive the transplantation process is quite limited, and even fewer integrate into the injured brain tissue (Menasche 2005; Silva, Litovsky et al. 2005). These observations calling into question the notion that transplanted stem cells replace functional tissue.

Here, we provide evidence that apoptotic NPCs release critical components that promote neuroprotection. We first confirmed that grafted NPCs undergo apoptosis as early as 6 h after HI. Viable NPCs are no longer detectable at 7 and 14 d after the insult, suggesting that the transplanted cells do not integrate into the circuitry of the injured brain. Next, growth factors were withdrawn from NPCs *in vitro* in an attempt to mimic the inhospitable environmental conditions after NPC transplantation *in vivo*. Such growth factor withdrawal induced cell death in the cultured NPCs within 24 h.

Treating cultured neurons with conditioned medium (CM) generated by NPCs undergoing caspase-dependent cell death preserved mitochondrial integrity and function. Moreover, CM-treated neurons were protected from both glutamate-induced toxicity and trophic factor withdrawal. In contrast, medium that was obtained from NPCs that were growth factor–deprived and rescued using the caspase inhibitor Q-VD-OPh failed to confer neuroprotection, indicating that caspase-dependent NPC death—and their release of protective components—are required for mediating the observed protective effects of the resulting CM. Importantly, protection was observed in CM harvested specifically from apoptotic cells that were neuronal in origin, including NPCs and immortalized hippocampal neurons; in contrast, medium obtained from other cell types, including Mesc, MEFs, SNL feeder cells did not provide protection. This finding engenders a new concept of neuroprotection through the death of progenitor cells, in contrast to previous views linking the defensive effects of progenitor or stem cell therapies to tissue replacement and/or functional integration of transplanted cells into injured host neural networks. This concept is further supported by our results obtained using an *in vivo* model of cerebral hypoxia/ischemia, in which an intraventricular injection of NPC-derived CM confers robust neuroprotection, avoiding the need for cell transplantation.

We next identified protein constituents as the main mediators of neuroprotection in NPC-derived CM, and attributed the protective properties of the medium to the combination of peroxiredoxin-1 (prdx-1) and galectin-1. Because protein synthesis inhibitors did not affect the neuroprotective capacity of CM rescued from dying NPCs, the bioactive proteins were presumably already expressed in NPCs under standard culture conditions and released upon apoptotic stress. The proposed ability of apoptotic cells to provide defensive signaling cues to surrounding cells has been previously suggested in other settings, such as β-cell mass regeneration (Bonner, Bacon et al. 2010), wound healing with inhibition of inflammation (Li, Huang et al. 2010), and planarian regeneration (Pellettieri, Fitzgerald et al. 2010). The actions of apoptotic cells to rescue surrounding cells in invertebrates as well as vertebrates indicates a fundamental and evolutionarily conserved regeneration mechanism underlying the protective potential of stem/progenitor cells.

Most intriguingly, incubation at 60–80°C significantly increased the neuroprotective capacity of NPC-derived CM, suggesting that the bioactive protein constituents are stable at temperatures up to 80°C. This finding agrees with the identification of galectin-1 and prdx-1 in the CM, which retain their activity at 60°C. Nonetheless, the increase in neuroprotective potential after incubation at high temperatures could be also explained by heat denaturation of proteins and enzymes that otherwise mediate inhibitory or even toxic effects. The identification of both protective and inhibitory components in NPC-derived CM requires further investigation.

Our MALDI-TOF (matrix-assisted laser desorption ionization time of flight) mass spectrometry analyses detected prdx proteins in all investigated neuroprotective CM samples. Prdx proteins comprise a family of small (22–27 kDa) non-seleno peroxidases with six mammalian isoforms. Their role as antioxidants stems from their capacity to reduce and detoxify hydrogen peroxide, peroxynitrite, and a wide range of organic hydroperoxides (Wood, Schroder et al. 2003). The physiological importance of the prdx proteins is illustrated by their abundance, as well as by loss-of-function studies in knockout mice (Neumann, Krause et al. 2003; Yang, Rabinovich et al. 2008; Yang, Song et al. 2011). In the CNS, prdx proteins are thought to act as free radical scavengers and are capable of protecting a variety of cell types as reported in *in vitro* models of ischemic neuronal death, overexpression models and neurodegenerative diseases (Boulos, Meloni et al. 2007; Fang, Nakamura et al. 2007; Hattori and Oikawa 2007; Botia, Seyer et al. 2008; Smith-Pearson, Kooshki et al. 2008).

Our experiments using recombinant prdx-1 support the notion that prdx-1 has neuroprotective properties. However, an anti-prdx-1 antibody only partially overturned the protective effects of the medium, confirming that other components contribute to the protective effects of CM. For example, galectin-1 was also identified in NPC-derived CM. Galectins function in the cytoplasm, where they regulate signal transduction pathways via protein-protein interactions, independent of their lectin activity. Galectins can also be released, where their extracellular functions are mainly dependent on their sugar-binding lectin capacity (Yang, Rabinovich et al. 2008). Galectin-1, for its part, can assume many roles, including in immune responses, tumor formation, and CNS, peripheral nervous system, and muscle tissue development. In neural cells, galectin-1 promotes proliferation of neural stem cells (Sakaguchi, Imaizumi et al. 2010), drives neurite outgrowth (Puche and Key 1995), enhances axonal regeneration (Horie, Inagaki et al. 1999) and inhibits glutamate toxicity (Lekishvili, Hesketh et al. 2006) and neuronal death in a mouse model of amyotrophic lateral sclerosis (Chang-Hong, Wada et al. 2005).

In summary, our findings offer an alternative explanation for the beneficial effects observed after NPC transplantation in brain injury paradigms, where NPC death is an essential step in the release of protective proteins mediating neuroprotection. From a clinical perspective, we suggest that conditioned medium derived from NPCs has high therapeutic potential, avoiding the complications associated with the transplantation of stem/progenitor cells.

4.4 Involvement of monocyte-derived macrophages in the inflammatory response after IR (IV)

The persistent and even progressive (Bostrom, Kalm et al. 2013) impairment in neurogenesis after IR has been linked, at least in part, to the deleterious impact of chronic inflammation (Monje, Mizumatsu et al. 2002; Monje, Toda et al. 2003). The second part of this thesis shows that IR can induce a state of chronic inflammation in the immature and the adult brain, which is probably mediated by astrocytes and microglia, but not by peripheral macrophages.

CCL2 regulates BBB permeability and recruits neutrophils and macrophages to injury sites under multiple pathological conditions (Sheehan, Zhou et al. 2007). In this manner, the chemokine plays an important role in directing the inflammatory response, and increased CCL2 expression is associated with decreased neurogenesis (Villeda, Luo et al. 2011). In aim IV/manuscript IV, we demonstrated that CCL2 expression peaked at 6 h after IR and decreased thereafter. In contrast to previous studies showing a return of CCL2 content to baseline levels within 24 h after injury (Kalm, Fukuda et al. 2009; Lee, Haditsch et al. 2013), our results showed upregulation of the chemokine for as long as 1 month after IR, indicative of a chronic, radatiation-induced inflammatory process in both juvenile and adult brains.

CCL2 is expressed by many types of cells, including microglia, astrocytes, and endothelial cells (Luo, Laning et al. 1994; Hayashi, Luo et al. 1995; Thibeault, Laflamme et al. 2001). We previously found that CCL2 expression induced by IR in the juvenile rat brain was mainly restricted to astrocytes, with some expression in microglia (Kalm, Fukuda et al. 2009). Considering the transient nature of microglial activation after injury in juvenile as well as adult brains, we speculate that the initial burst in CCL2 expression was due to reactive microglia, whereas chronic CCL2 expression stemmed from other cells (e.g., astrocytes).

We found no evidence for the recruitment of peripheral monocytes into either the juvenile or adult hippocampus for at least 1 month after IR, possibly due to maintenance of BBB integrity. In support of this hypothesis, an earlier study employing high-dose radiation (20 Gy in a single dose) reported increased BBB permeability and the presence of adherent leukocytes in pial vessels, but not after subjection of animals to relatively low-dose radiation (5 Gy) (Yuan, Gaber et al. 2003). On the other hand, our results and those of others demonstrated BBB disruption and massive peripheral monocyte-derived macrophage

invasion in models of more pronounced tissue injury, such as after severe ischemia (Tanaka, Komine-Kobayashi et al. 2003; Umekawa, Osman et al. 2015).

Upon IR, the adult vs. juvenile brain underwent a milder and relatively transient microglial activation in the GCL, as assessed by comparatively lower CCL2 expression levels and reduced microglial density, proliferation, morphology, and expression of CD68. The diminished microglial response probably reflected the lower level of neurogenesis in the mature brain and consequently, fewer dying neural stem and progenitor cells after exposure to radiation. Microglia rapidly proliferate during injury-induced activation (Dihne, Block et al. 2001; Kato, Takahashi et al. 2003), and accordingly, we quantified proliferating microglia by assessing double-positive cells for Ki-67 and $CX3CR1^{GFP/+}$. We found that the number of proliferating microglia contributed to 50 % of the microglial increase in the GCL of the immature brain at 6 h after IR. A further increase in microglial numbers at 1 day after IR was most likely due to microglial migration from the molecular layer (ML) to the GCL, because scarcely any proliferating microglia were observed at this time point. Moreover, microglial numbers in the ML decreased without any signs of microglial cell death.

Neurogenesis was virtually eliminated by IR in the adult brain, and very few Ki-67-positive cells were observed at either 6 h or 1 day after IR. None of the Ki-67-positive cells co-localized with $CX3CR1^{GFP}$-positive cells, signifying an absence of microglial proliferation. An observed increase in microglial density at 6 h after IR in the adult brain was probably not due to microglial migration from neighboring areas parts of the DG, because no decrease in microglial number was observed in the hilus or the ML. Still, the possibility of migration from other parts of the hippocampus or brain cannot be excluded.

Little is known about the mechanism(s) behind the decrease in microglial density during the late injury phase following IR. Possible mechanisms include microglial migration away from injury sites (Giulian, Chen et al. 1989; Angelov, Gunkel et al. 1995) and cell death (Gehrmann and Banati 1995; Dihne, Block et al. 2001; Kalm, Lannering et al. 2009). In this work, however, the decreased microglial density at later time points apparently did not result from IR-induced apoptosis, because no active caspase-3- or TUNEL-positive microglia were found in the DG at 6 h or 1 day after IR. Additional research on microglial migration and other modes of microglial death is required to explain the observed changes in microglial numbers.

5 Clinical implications and future perspectives

The overall goal of this thesis work was to provide insights into efficient clinical therapies for HIE and IR-induced brain injury. To this end, pharmacological interventions aimed at the control of apoptotic and inflammatory responses after HI were studied. A previously unknown mechanism for the therapeutic effects of neural stem cell-based therapies was revealed, and the application of NPC-derived CM to promote neuroprotection after brain injury was proposed. In addition, we found that peripheral monocyte-derived macrophages do not participate in the inflammatory response in the irradiated brain, contributing to a better understanding of neurogenesis impairment after exposure to radiation.

Apoptosis plays a prominent role in the immature vs. adult brain in response to HI. Therapeutic strategies targeting programmed cell death have been extensively studied, achieving promising outcomes. Here, BIP administered before HI prevented delayed cell death by inhibiting the activation of Bax, thus providing an additional intervention target. However, it is still too early to conclude that BIP can be utilized in clinical treatment modalities. Unlike in animal models, it is not always possible to define the onset of ischemia and reperfusion in humans; therefore, additional research regarding the effects of delayed BIP administration in rodent models might help to understand its therapeutic impact when given during later stages of HIE. On the other hand, we did study the effect of delayed Q-VD-OPh administration in mice with HI-induced brain damage. The caspase inhibitor decreased long-term tissue loss, but only transiently enhanced behavioral performance. The question remains if complete restoration could be achieved with caspase inhibitors. Considering that neuronal damage after HI involves multiple signaling pathways, combinatorial therapy for HIE is probably required to achieve the best outcome, as long as the cascade of damaging events and the temporal window of efficacy for the tested drugs are fully defined.

Macrophages elicit inflammatory responses under neuropathological conditions through the production of various cytokines, growth factors, and chemokines. Functional macrophage heterogeneity has long been recognized in the peripheral nervous system, but until recently, was largely ignored in the CNS. In the injured brain, the macrophage population consists of both resident microglia and infiltrating monocyte-derived macrophages that perform distinct functions. The post-ischemic inflammatory response, comprising activation of resident microglia and infiltration of peripheral macrophages, contributes to ischemic brain injury. Anti-inflammatory and immunomodulatory strategies are neuroprotective in the treatment of brain damage, and both diminish microglial activation. Interestingly, caspase-8

and caspase-3/-7 are reportedly mediators of microglial activation. Here, Q-VD-OPh diminished post-ischemic inflammation and reduced tissue loss after HI, but its direct effect on microglial activation was unclear. Additional exploration of the impact of caspase inhibitors on the modulation of the microglial phenotype and the infiltration of peripheral macrophages would assist in addressing this question.

Regarding IR-induced brain injury, neuroinflammation after radiation exposure has been suggested to alter the microenvironment of the neurogenic niche, thereby preventing neuronal differentiation of precursor cells. Unlike the case of spinal cord injury, where monocytes are recruited to the lesion site and participate in repair, peripheral monocyte-derived macrophages do not appear to contribute to the inflammatory response in the irradiated hippocampus. Instead, our findings indicate that the acute inflammatory state is largely mediated by resident reactive microglia, whereas chronic inflammation is mediated by other cells, particularly activated astrocytes. Of note, β-1,4-galactosyltransferase-6 is upregulated in activated astrocytes in multiple sclerosis lesions, which drives the transcription of neuroinflammatory-associated genes and contributes to disease pathogenesis. Further studies are needed to similarly unravel the functions of activated astrocytes and microglia in IR-provoked brain lesions.

This thesis shows that caspase-dependent NPC death is essential for the release of components that mediate the resilience of neural cells. This concept differs from previous concepts linking the protective effects of stem cell therapy to repair mechanisms via cell replacement. We propose that the therapeutic application of stable, well-defined NPC-derived CM fractions obtained under standardized conditions *in vitro* could achieve better results than cell-based therapies. The latter are hindered by various inherent obstacles, including variability among different cell transplant preparations, uncontrolled cell migration, nonfunctional cell integration into host tissue and circuitry, and tumorigenesis. By contrast, NPCs are relatively easy to isolate and expand, and large amounts of CM can be produced fairly quickly. Because the CM is highly stable at 37°C for several days, a continuous infusion of fresh CM or active CM constituents (e.g., prdx-1, galectin-1) is possible, thereby likely increasing therapeutic benefits.

Finally, this thesis highlights apoptosis and inflammation as two crucial components of neuropathogenesis in HI- and IR-induced brain injuries. The determination of an optimal therapeutic window is of utmost concern for the development of efficacious clinical interventions for such conditions. If a therapeutic window cannot be identified, delayed

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