Doctoral thesis for the Degree of Doctor of Philosophy, Faculty of Medicine

Inflammatory mechanisms in experimental neonatal brain injury and in a clinical study of preterm birth;

Involvement of galectin-3 and free radical formation

Christina Doverhag

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Perinatal Center
Department of Physiology
Institute of Neuroscience and Physiology
The Sahlgrenska academy
University of Gothenburg
Sweden

UNIVERSITY OF GOTHENBURG

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Dedication

Some people believe in you from the first time you open your eyes, some people know what you will become long before you even learn to speak.

My grandfather believed in me from the first day I opened my eyes; he never stopped believing that I would be something great.

My grandmother mounted the expression; "She's so distracted and confused, I bet you she's going to be a professor when she grows up."

In loving memory

Rolf Doverhag	*11/4 1924 †25/8 1994
Irene Svantesson	*7/9 1936 †1/4 2008

Abstract

Inflammation in experimental neonatal brain injury and in a clinical study of preterm birth; Involvement of galectin-3 and free radical formation

Christina Doverhag

Perinatal Center, Department of Physiology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Box 432, 405 30 Göteborg, Sweden

Introduction; Intrauterine infection/inflammation is associated with preterm delivery (PTD) and severe neonatal morbidity. Inflammation is also important in the secondary neurotoxic cascade leading to perinatal brain injury. The aims were to investigate (a) the novel inflammatory marker galectin-3 that affects accumulation, apoptosis and activation of inflammatory cells and (b) free radical formation in particular by the enzyme NADPH-oxidase responsible for free radical formation in inflammatory cells, in threatening PTD (III) and in the development of experimental perinatal brain injury (I,II).

Materials and methods; Amniotic fluid (AF) and placentas were obtained from 83 women with threatening PTD presenting with preterm labour (PTL) or premature prelabour rupture of membranes (pPROM) and from 15 term controls. AF was analysed for galectin-3, ascorbyl radicals and antioxidative capacity (AOC) and results were correlated to signs of intrauterine infection/inflammation, PTD and severe neonatal morbidity (III). In the experimental studies, models of term hypoxia-ischemia (HI) (I,II) and of excitotoxic white matter injury (I) were used in knock-out (KO) mice for galectin-3 (II) and $gp91^{phox}$ (a subunit in NADPH-oxidase) (I) and together with two inhibitors of NADPH oxidase (I). Lesion size, inflammation (microglia accumulation, inflammatory mediators), free radical formation, apoptosis and trophic factors were studied. In vitro studies were used to investigate free radical formation in microglial cells (I) and the effect of AF on pre-activated neutrophils (III).

Results; Galectin-3, ascorbyl radicals and AOC were detected in all AF samples. Women with threatening PTD and infection/inflammation had higher levels of galectin-3 and slow AOC but lower levels of ascorbyl radicals than term controls but without correlation to PTD. Mothers of girls had higher levels galectin-3 than mothers of boys. AF also quenched free radical formation in primed neutrophils in vitro. Women with PTL did not differ from women with pPROM. Only AOC was increased in mothers to infants with severe morbidity (III). After experimental HI, mRNA expression of galectin-3 and NADPH-oxidase subunits (I,II) as well as galectin-3 protein expression were increased (II). Galectin-3 KO mice had reduced injury but increased microglial density and reduced expression of inflammatory factors (MMP-9) and markers for oxidative stress (nitrotyrosine). The protection was more pronounced in males. Apoptosis was altered in un-operated KO mice but not after HI. No difference was seen for trophic factors (II). Pharmacological inhibition of NADPH-oxidase reduced free radical formation in vitro and markers of oxidative stress in vivo, but resulted in an increase in apoptotic markers and lesion size after excitotoxic injury. Genetic inhibition did not reduce injury but resulted in an increase of the inflammatory markers galectin-3 and IL-1β (I).

Conclusion; Galectin-3 is increased in women with threatening PTD and associated infection/inflammation,but our findings suggest a strong antioxidative capacity and not an increased oxidative stress as previously suggested (III). Galectin-3 contributes to neonatal brain injury by modulating the inflammatory response rather than affecting apoptosis or trophic factors and the mechanisms may be sex dependent (II). Contrary to findings in the adult brain, perinatal brain injury was unaltered or aggravated after genetic and pharmacological NADPH-oxidase inhibition (I).

Key words: preterm labour, premature prelabour rupture of membranes, chorioamnionitis, hypoxia-ischemia, neonatal brain injury, NADPH oxidase, galectin-3, MMP-9, ascorbyl radicals, antioxidative capacity.

List of original papers

1. **Doverhag C**, Keller M, Karlsson A, Hedtjarn M, Nilsson U, Kapeller E, Sarkozy G, Klimaschewski L, Humpel C, Hagberg H, Simbruner G, Gressens P, Savman K. **Pharmacological and genetic inhibition of NADPH oxidase does not reduce brain damage in different models of perinatal brain injury in newborn mice**. *Neurobiol Dis.* 2008 Jul;31(1):133-44. Epub 2008 Apr 25.
2. **Doverhag C**, Poirier F, Hedtjarn M, Hagberg H, Karlsson A, Savman K. **Galectin-3 contributes to neonatal hypoxic-ischemic brain injury.** *(in press)*
3. **Doverhag C**, Jacobsson B, Holst RM, Wennerholm UB, Nilsson UA, Hagberg H, Karlsson A, Savman K. **Galectin-3 and oxidative stress in association with chorioamnionitis and preterm birth** *(manuscript)*

Paper not included in this thesis:

Holst RM, Laurini R, Jacobsson B, Samuelsson E, Savman K, **Doverhag C**, Wennerholm UB, Hagberg H. **Expression of cytokines and chemokines in cervical and amniotic fluid: relationship to histological chorioamnionitis.** *J Matern Fetal Neonatal Med.* 2007 Dec;20(12):885-93.

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Abbreviation

ABTS	2,2´azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
AF	Amniotic fluid
AIF	Apoptosis inducing factor
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AOC	Antioxidative capacity
ATP	Adenosine triphosphat
BSA	Bovine serum albumin
CA	Chorioamnionitis
CL	Chemiluminescence
CLD	Chronic lung disease
CP	Cerebral palsy
CRP	C-reactive protein
CS	Caesarean section
DAB	3,3'-diaminobenzidine
DCF	2',7'-dichlorofluorescin
ELISA	Enzyme-linked immunosorbent assay
ESR	Electron spin resonance
FIRS	Foetal inflammatory response syndrome
GA	Gestational age
Gal3	Galectin-3
GFAP	Glial fibrillary acidic protein
GH	Growth hormone
HCA	Histological chorioamnionitis
HI	Hypoxia ischemia
HIV	Human immunodeficiency virus
HNE	4-hydroxynonenal
IAI	Intra amniotic inflammation
Iba-1	Ionizing calcium-binding adaptor molecule -1
i.c.	Intra cerebral
IF	Immunofluorescens
IGF-1	Insulin like growth factor-1
IHC	Immunohistochemistry
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
i.p.	Intra peritoneal
IVH	Intra ventricular haemorrhage
KO	Knock out
LPS	Lipopolysaccharide
MAP-2	Microtubule associated protein – 2
MBP	Myelin basic protein
mGluR	Metabotropic glutamate receptor
MIAC	Microbial invasion of the amniotic cavity
MMP	Matrix metalloproteinase
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
NEC	Necrotizing enterocolitis
NeuN	Neuronal nuclei
NF	Neuronal filament
NMDA	N-metyl-D-aspartat
NO	Nitric oxide
NOS	Nitric oxide synthase
i/e/nNOS	Inducible/endothelial/neuronal NOS
P5	Postnatal day 5
pAkt	Phosphorylated Akt
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCR	Polychain reaction
PMN	Polymorphonuclear cells
pPROM	Preterm pre-labour rupture of membranes
PTB	Preterm birth
PTD	Preterm delivery
PTL	Preterm labour
PVL	Periventricular leukomalacia
RMA	Robust multiarray average
ROS	Reactive oxygen species
SCFPMN	Subchorionic fibrin polymorphonuclear cells
S.E.M	Standard error of mean
TIMP	Specific tissue inhibitor of matrix metalloproteinases
WB	Western blot
w GA	Weeks of gestational age
WMD	White matter damage
WT	Wildtype

Introduction

Intrauterine infection/inflammation is an important cause of preterm delivery and severe neonatal morbidity, including perinatal brain injury (Gomez et al., 1998; Martinez et al., 1998; Romero et al., 2007; Wu and Colford, 2000; Yoon et al., 1997). Gestational age (GA) is important for the type of brain injury the infant sustains. Preterm infants subjected to chorioamnionitis (CA) are at risk of developing white matter injury (Gomez et al., 1998; Wu et al., 2003), while term infants exposed to birth asphyxia develop hypoxic-ischemic (HI) injury with a secondary inflammatory response (Volpe, 2008). Furthermore, infection/inflammation can be an antecedent of asphyxia and increase the risk of neurological sequelae (Peebles and Wyatt, 2002). Thus, inflammation is a major contributor to both types of injury; a better understanding of the complex inflammatory system in the perinatal setting is of great importance.

Clinical background

Preterm pre-labour rupture of membranes and preterm labour in association with preterm delivery

Preterm delivery (PTD) accounts for almost 1/3 of all global neonatal mortality; the risk of death is highest during the first day of life. Together with PTD, asphyxia and severe infections are the most common causes of neonatal deaths (Lawn et al., 2005). The incidence of PTD remains stable in Sweden (Morken et al., 2008), although the survival rate has increased due to better obstetric and neonatal care (Goldenberg et al., 2000). In Sweden, 30% of babies born at 22-24 weeks of GA (w GA) die within the first week, while the corresponding mortality in babies born between 25 and 26 w GA is only 11% (Bennis et al., 2009). As more babies survive at lower GA, neonatal morbidity is increased; the most severe illness is seen in the very preterm babies (Fellman et al., 2009).

PTD is caused by multiple gestation, intrauterine death and physician-induced labour related to maternal pre-eclampsia or intrauterine growth restriction. The largest aetiological group is, however, spontaneous preterm delivery, accounting for nearly 60% of all PTDs (Goldenberg et al., 2000; Morken et al., 2005). Spontaneous PTD presents as

either preterm pre-labour rupture of membranes (pPROM) or preterm labour (PTL). pPROM is defined as rupture of foetal membranes prior to 37 w GA, and accounts for approximately half of spontaneous PTDs, and PTL is defined as labour prior to 37 w GA (Hagberg and Wennerholm, 2000; Romero et al., 2006).

Although the mechanisms behind pPROM/PTL are partly unknown, intrauterine infection/inflammation is an important risk factor (Hagberg and Wennerholm, 2000; Romero et al., 2006). Infection or inflammation can induce local inflammatory mediators such as interleukin (IL)-1 (Romero et al., 1989) and IL-6 (Greci et al., 1998; Holst et al., 2007) that eventually cause spontaneous uterine contractions, leading to PTL or pPROM. Although PTL and pPROM share many mechanisms, it has been suggested that pPROM is associated with an excessive release of free radicals and/or limited antioxidant defences (Wall et al., 2002; Woods, 2001) as well as increased release of inflammatory mediators such as metalloproteinases (MMPs) (Menon and Fortunato, 2004). Spontaneous PTD, especially at <30 w GA, is associated with a high risk of severe neonatal morbidity and mortality (Goldenberg et al., 2000; Gomez et al., 1998; Romero et al., 2007; Yoon et al., 1997).

Inflammation/infection in the chorioamnion

CA is defined as intrauterine inflammation and is considered to be caused by infection, although bacteria are only detected in the amniotic fluid (AF) in less than 1/3 of the women that deliver preterm after PTL or pPROM and intraamniotic inflammation (IAI) is detected in approximately 50% of women with threatening PTD in Sweden (Jacobsson et al., 2003a; Jacobsson et al., 2003b) and abroad (Romero et al., 2006; Yoon et al., 1997).

The infection normally ascends through the vagina and enters the chorio-decidual space, passing to the chorioamnion or directly into the AF, and is often asymptomatic (see Figure 1). This makes identification of women with intrauterine infection very difficult. However, as the infection intensifies it becomes symptomatic, resulting in PTL or pPROM (Goldenberg et al., 2000; Romero et al., 2006) with or without clinical signs of CA (maternal fever, abdominal pain, foul discharge, maternal and foetal tachycardia).

Microbial invasion of the amniotic cavity (MIAC) is defined as bacteria detected in the AF. The microbes most commonly associated with spontaneous PTD are low-virulence bacteria such as Ureaplasma urealyticum, Mycoplasma hominis and Gardnerella vaginalis (Goldenberg et al., 2000; Romero et al., 2006).

IAI is defined as elevation of inflammatory mediators such as IL-6 and IL-8 in the AF and is found in more than 50% of cases with pPROM and PTL (Jacobsson et al., 2003a; Jacobsson et al., 2003b). It is associated with the same clinical outcome as that in women with MIAC (Shim et al., 2004).

Histological chorioamnionitis (HCA) is defined as infiltration of inflammatory cells in the placental membranes, detected at histological evaluation after delivery, and is found in most cases of pPROM and PTL (Romero et al., 2006). HCA is associated with higher levels of IL-6 and IL-8 in AF, compared with controls (Holst et al., 2007).

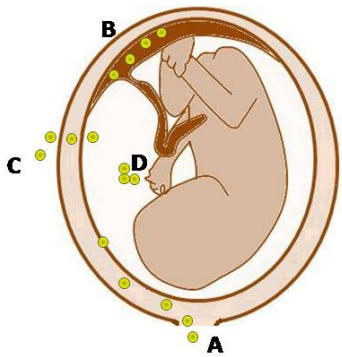

Figure 1. Potential pathways by which the foetus can be exposed to infection/inflammation

Infection/inflammation can reach the foetus through several pathways. It can ascend from the vagina and pass through the foetal membranes (A) or come from the maternal blood through the placenta (B). It can also pass from the peritoneal cavity through the membranes (C) or directly into the amniotic fluid by accident during invasive procedures (D). Figure modified from Goldenberg et al 2000.

Chorioamnionitis-related severe neonatal morbidity

Intrauterine infection and CA are well-known risk factors for severe neonatal morbidity such as early-onset sepsis, chronic lung disease (CLD), necrotizing enterocolitis (NEC), preterm brain injury with periventricular leukomalacia (PVL) and severe intraventricular haemorrhage (IVH) and subsequent cerebral palsy (CP) (Baud et al., 1999; Gomez et al., 1998; Greenough, 2008; Jobe, 2005; Martinez et al., 1998; Romero et al., 2007; Wu and Colford, 2000; Yoon et al., 1997).

Neonatal Sepsis

Neonatal sepsis is an important cause of neonatal morbidity and mortality. As infants are more susceptible to infection and less capable of responding to infection in combination with a variety of clinical manifestations, even the slightest sign of infection such as an increase in C-reactive protein (CRP) is used to diagnose suspected sepsis. Not all infants with clinical infections have positive blood cultures. Other markers of infection are neutropenia and elevated inflammatory markers (IL-6, IL-8) (Kliegman et al., 2007). Early-onset infection (during the first week after delivery), of which intrauterine infection transmitted to the foetus is a major cause, is commonly acquired before or during delivery (Kliegman et al., 2007; Klinger et al., 2009).

Chronic Lung Disease

CLD is a result of lung injury and is mostly seen in preterm infants with low birth weight (Jobe, 2005; Kliegman et al., 2007). The pathogenesis is multifactorial and the prognosis includes the risk of long-term pulmonary complications (Greenough, 2008; Kliegman et al., 2007). CA is a risk factor for CLD, but mechanical ventilation, glucocorticoid therapy and oxygen can also affect outcome (Jobe, 2005; Kliegman et al., 2007).

Necrotizing enterocolitis

NEC is a life-threatening condition entailing various degrees of mucosal or transmural necrosis of the intestines. The aetiology is largely unknown (Kliegman et al., 2007) but CA is a risk factor for NEC and preterm infants are at higher risk of developing the condition (Gomez et al., 1998; Kliegman et al., 2007). The prognosis is poor, including a risk of major intestinal complications and sepsis and, later on, suboptimal growth and adverse neurodevelopmental outcome (Kliegman et al., 2007; Patole, 2007).

Perinatal brain injury

Periventricular leukomalacia and intraventricular haemorrhage

PVL and severe IVH are the major neuropathological lesions in preterm infants with very low birth weight (Khwaja and Volpe, 2008; Whitelaw, 2001) and CA is an important independent risk factor for both conditions (Wu et al., 2003; Yoon et al., 1997). In the

gelatinous subependymal germinal matrix, blood vessels are numerous but the tissue provides poor vascular support, predisposing for haemorrhage. IVH is graded from I to IV; grade I is isolated bleeding in the subependymal area and grade IV is a haemorrhage extending into the parenchyma, creating a haemorrhagic infarction. Extreme prematurity and HI injury are both risk factors for severe IVH (grade III-IV). PVL consists of focal necrotic lesions in periventricular white matter and/or diffuse white matter damage (WMD). Another factor that makes the white matter in neonates so vulnerable is the maturation of the oligodendrocyte precursor cells which is easily affected by free radicals in the surroundings and insufficient antioxidant defence (Back, 2006; Volpe, 2008). Infants with both severe IVH and PVL are at high risk of death or CP. There are no currently available treatments for either of these conditions, although some of the complications to IVH can be treated (Volpe, 2008).

Hypoxic-ischemic brain injury

HI brain injury is an important cause of neonatal mortality as well as of CP and mental retardation. Foetal hypoxia due to maternal factors (hypoventilation, heart or respiratory failure, low blood pressure), premature separation of the placenta, compression or knotting of the umbilical cord and placental insufficiency are causes of HI. It can also arise after birth due to severe circulatory or respiratory failure. While preterm infants subjected to HI predominantly contract injuries in the white matter, term infants sustain focal or wide-spread neuronal injuries resulting in GA-specific neuropathology in both white and grey matter (Volpe, 2008).

Mechanisms of perinatal brain injury

When the brain is exposed to HI, the injury evolves in two phases (McLean and Ferriero, 2004; Perlman, 2006; Volpe, 2008). The first is the primary, local, injury that occurs during the HI; it affects the exposed part of the brain and mainly consists of necrosis (Northington et al., 2001; Volpe, 2008). The secondary injury is characterised by delayed energy depletion (Azzopardi and Edwards, 1995; Lorek et al., 1994) and takes place as the blood flow is restored during the reperfusion phase. This phase consists mainly of a process including inflammatory activation and predominantly apoptotic cell death (Azzopardi and Edwards, 1995; Lorek et al., 1994; Northington et al., 2001). It can continue

for several days, up to weeks, after the initial injury and can affect other areas of the brain, not primarily exposed to the insult (Nakajima et al., 2000).

Primary injury

During the primary insult, cells suffer from acute energy depletion. This process can lead to immediate necrotic cell death, to an extent determined by the severity of the insult (Blomgren et al., 2003; Skulachev, 2006; Volpe, 2008). The adenosine triphosphate (ATP) level is one factor that may determine whether the cell will undergo apoptosis or necrosis when it dies. More extensive or more prolonged energy depletion causes the cell to undergo necrosis while transient, more minor ATP depletion results in apoptosis (Skulachev, 2006; Volpe, 2008).

As the HI progresses, the excitotoxic amino acid glutamate accumulates extracellularly (Hagberg et al., 1987) due to impaired uptake in the astrocytes and presynaptic nerve endings and to decreased ATP levels in the neurons. This leads to depolarisation of the neurons and release of glutamate into the synapses (Volpe, 2008). Glutamate utilises three types of ionotropic receptors, i.e. the NMDA, AMPA and Kainate receptors, but it also binds to a metobotropic glutamate receptor (mGluR) (Degos et al., 2008; Johnston et al., 2002; Volpe, 2008). Activation of these receptors during HI leads to excessive calcium influx that can induce activation of enzymes responsible for reactive oxygen species (ROS) formation (nitric oxide (NO) synthase (NOS), xanthine oxidase, phospholipase A) and immediate cell degradation (lipases, endonucleases and proteases) (Blomgren and Hagberg, 2006; Ferriero, 2004).

Secondary injury and secondary energy failure

During the secondary injury phase, blood flow and energy supply are restored. This is, however, not equivalent to restored mitochondrial function and injured cells may suffer from delayed injury, characterised by predominantly apoptotic cell death, after reperfusion (Blomgren et al., 2003; Skulachev, 2006; Volpe, 2008). After HI, apoptotic mechanisms, including apoptosis-inducing factor (AIF) and caspases, are much more prominent in the neonatal than in the adult brain (Hu et al., 2000; Zhu et al., 2005).

When blood flow is restored, a substantial inflammatory process will be induced. This leads to oedema formation and infiltration of inflammatory cells such as macrophages and neutrophils into the brain, but also to relocation and proliferation of resident microglia at the injured site (Alvarez-Diaz et al., 2007; Bona et al., 1999; Degos et al., 2008; Hudome et al., 1997; McRae et al., 1995). The inflammatory cells can produce several pro-inflammatory mediators that increase the inflammation further, as well as neurotrophic substances that promote neuronal survival (Alvarez-Diaz et al., 2007; Bona et al., 1999; Degos et al., 2008; Hedtjarn et al., 2004).

Apoptosis

As mentioned above, neuronal cells start to undergo apoptosis to a greater extent during the reperfusion phase (Northington et al., 2001). The apoptotic cascade can be induced through two major pathways, the caspase-dependent and the caspase-independent pathway.

The caspase-dependent pathway has two major activation mechanisms both leading to an increase in caspase-3, which may eventually lead to apoptosis. One of these mechanisms is the binding of external activating factors to the FAS ligand. This results in a cascade leading to increased caspase-8, which in turn activates caspase-3. HI also leads to the release of Cytochrome C from the mitochondria, resulting in apoptosome assembly and subsequent caspase-9 and -3 activation. The caspase-independent pathway results in AIF translocation from mitochondria to the nucleus, leading to chromatinolysis (Blomgren et al., 2003; Hagberg, 2004; Kroemer and Martin, 2005).

During the last decade, apoptotic mechanisms after neonatal HI have been shown to be gender-dependent. While female animals tend to undergo caspase-3-dependent apoptosis, apoptosis in males depends more on AIF and the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) (Zhu et al., 2006).

Inflammation

The inflammatory process

Inflammation was first described by Celsus (30 BC – AD 38) as rubor (redness), tumor (swelling), calor (warmth) and dolor (pain), a definition that was later amended to also include functio laesa (impaired function) by Galenos (A.D. 129-200). Inflammatory symptoms are a result of increased blood flow in the area (rubor, calor) and increased permeability in the blood vessels (tumor). The release of several nerve-triggering substances and swelling contribute to the pain (Mölne J and Wold, 2007).

Inflammation is a complex process that can be triggered by both injured cells and infection. The process involves invasion of inflammatory cells, such as neutrophils, monocytes/macrophages/microglia, and the production of inflammatory mediators such as cytokines, chemokines and MMPs, as well as ROS, anti-inflammatory mediators and neurotrophic factors (Mölne J and Wold, 2007).

Inflammation has traditionally been considered to be detrimental both in experimental brain injury (Arvin et al., 2002; Dommergues et al., 2003) and in the clinical setting, e.g. intrauterine infection as an independent risk factor for white matter injury and subsequent CP in the preterm infant (Back, 2006; Gomez et al., 1998; Wu et al., 2003). Inflammatory mediators such as IL-1β (Hagberg et al., 1996; Martin et al., 1994), IL-18 (Hedtjarn et al., 2002) and MMP-9 (Svedin et al., 2007) also contribute to the development of HI brain injury in rats and mice. Results concerning inflammatory inhibition after experimental HI are, however, contradictory (Arvin et al., 2002; Tsuji et al., 2004) and recent evidence supports the possibility that microglial cells may even be protective in experimental adult stroke (Imai et al., 2007; Kitamura et al., 2004).

Inflammatory cells

Neutrophils

Neutrophilic granulocytes are a part of the innate immune system and are considered to be a first line of defence (Mölne J and Wold, 2007). They appear in the neonatal brain within 24h after experimental brain injury (Benjelloun et al., 1999; Bona et al., 1999). Several

reports suggest that inhibition of these cells is protective in both adult and immature brain injury models (Hudome et al., 1997; Jiang et al., 1995; Matsuo et al., 1994; Nijboer et al., 2008). Since neutrophil infiltration seems to be limited in the immature brain after HI (Bona et al., 1999), it has been suggested that the protective effects of neutrophil depletion in both the adult and immature brain are due to effects on brain microcirculation (Palmer et al., 2004; Ritter et al., 2000).

Microglia/monocytes/macrophages

Microglial cells are resident macrophages in the brain and represent 5-20% of the neuroglial population (Kim and de Vellis, 2005). They are more abundant in grey matter than in white matter and can change their phenotype according to the degree of activation (Kim and de Vellis, 2005). Microglial cells are activated after neonatal HI injury (Bona et al., 1999; McRae et al., 1995) and when activated they participate in several processes such as phagocytosis of cell debris, regulation of homeostasis, nerve growth and modulation of inflammation and immune response (Kim and de Vellis, 2005). They produce several substances known to be involved in the development of neonatal brain injury, such as excitatory amino acids (Piani et al., 1992), ROS (Bagenholm et al., 1997), pro-inflammatory cytokines such as IL-1 (Hagberg et al., 1996; Martin et al., 1994) and IL-18 (Hedtjarn et al., 2002), and proteases, including MMP-9 (Svedin et al., 2007). The issue of whether microglia and invading macrophages have the same phenotype and function is under debate (Lalancette-Hebert et al., 2007). The role of microglia is being discussed, as several studies suggest that implanting exogenous microglial cells is effective for post-injury treatment in an adult stroke model in rats (Imai et al., 2007; Kitamura et al., 2004), and microglial inhibition can either attenuate (Arvin et al., 2002) or aggravate experimental neonatal HI (Tsuji et al., 2004). This demonstrates the complexity of the inflammatory system and the role of inflammatory cells.

NADPH oxidase

Neutrophils and microglia/monocytes/macrophages cells contain an enzyme, NADPH oxidase or Nox2, that produces ROS to help in the process of degrading phagocytosed particles such as microbes (Babior, 1978a; Babior, 1978b). This enzyme consists of several subunits that assemble upon activation and the active enzyme can then produce

superoxide. Gp91^{phox} is one of the crucial subunits for the function of the enzyme (see Figure 2) (Babior, 1999; Bedard and Krause, 2007; Infanger et al., 2006; Leusen et al., 1996). This subunit is membrane-bound and can mutate in humans to cause chronic granulomatous disease (Bedard and Krause, 2007; Leusen et al., 1996) which involves increased inflammatory response (Warris et al., 2003). Nox2 is part of a larger family of Noxes, consisting of at least five known members, and Nox2 has been found in both neurons and resident microglia in the brain. There is also evidence that Nox1, Nox4 and Nox5 are present in the brain, although Nox2 is the most dominant type in neonates (Bedard and Krause, 2007; Infanger et al., 2006). Microglial cells express NADPH oxidase but they seem to produce less superoxide than neutrophils (Sankarapandi et al., 1998).

NADPH oxidase in neuronal injury models

NADPH oxidase is important for injury development in the adult stroke model; mice lacking functional NADPH oxidase sustain almost 40% less injury (Walder et al., 1997). Neutrophil depletion decreases ROS formation in adult mice (Matsuo et al., 1995) and decreases injury in both adult and neonatal mice (Hudome et al., 1997; Matsuo et al., 1994).

Pharmacological inhibition of NADPH oxidase

Two different methods to pharmacologically inhibit NADPH oxidase have been used in this thesis.

Gp91ds-tat

Gp91ds-tat is a pharmacological substance that inhibits functional NADPH oxidase and its production of superoxide. It enters the cells aided by the human immune deficiency virus (HIV) viral coat "tat", binding to the subunit gp91^{phox} on the binding site for p47^{phox}, thereby making assembly of the enzyme impossible (Jacobson et al., 2003). Gp91ds-tat effectively blocks ROS formation with a resulting decrease in inflammation, both in vitro and in vivo (Jacobson et al., 2003; Schiffrin and Touyz, 2003).

Apocynin

Apocynin (4′-hydroxy-3′methoxyacetophenone) is an inhibitor of NADPH oxidase that interacts with the assembly of its cytosolic components (Stolk et al., 1994). It has been

tested both in vivo and in vitro in several models of inflammation-induced/-associated disease (t Hart et al., 1990) as well as in several neurological conditions (Simons et al., 1990). It has low toxicity and does not interfere with the killing capacity of the neutrophils (Stolk et al., 1994)

Free radicals

Reactive oxygen species

ROS are oxygen-derived, highly reactive compounds that could either be free radicals or rapidly form free radicals when reacting with other molecules. ROS are produced at several locations and in several processes in the cell; they are mainly produced in the mitochondria as a result of electron leakage from the electron transport chain. ROS formation is also

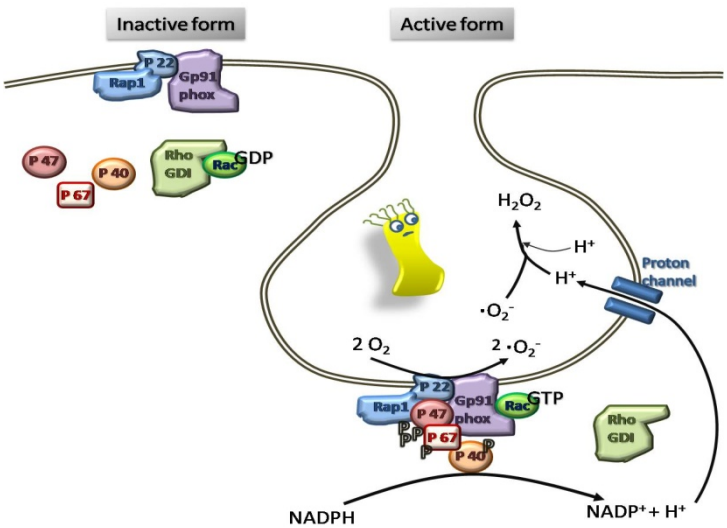

Figure 2. The assembly of NADPH oxidase and its catalytic effect

When inactive, the NADPH oxidase consists of the membrane bound subunits $p22^{phox}$ and $gp91^{phox}$ that together form the cytochrome b_{558}, as well as the cytosolic component consisting of $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$. Another component necessary for activation of the enzyme is the Rac2 protein. When inactive, it is bound to the Rho-GDI and Rap1A dimere. Upon activation, Rac2 binds GTP and migrate towards the core complex. When assembled, the enzyme can now catalyse the reaction: $2\ O_2 + NADPH \rightarrow 2\ O_2^- + NADP^+ + H^+$. The O_2^- can in turn react with hydrogen ions to form hydrogen peroxide: $2\ O_2^- + 2\ H^+ \rightarrow H_2\ O_2$

induced by the conversion of hypoxanthine to xanthine in the presence of oxygen, in prostaglandin synthesis and through activation of NADPH oxidase that results in superoxide formation (Blomgren and Hagberg, 2006; Buonocore et al., 2001). ROS formation is increased after perinatal HI (Bagenholm et al., 1997; Welin et al., 2005) and contributes to injury (Ferriero, 2004; Shadid et al., 1998). As previously mentioned, excessive ROS formation has also been suggested as a mechanism underlying pPROM (Woods, 2001).

Nitrotyrosine

NOS converts L-arginine to citrulline and NO. There are three types of NOS: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). NO can react with superoxide to form peroxynitrite (Blomgren and Hagberg, 2006; Buonocore et al., 2001), that can in turn lead to protein nitrosylation, causing protein deformation. The formation of nitrotyrosine-positive proteins is increased after HI in the immature brain (Peeters-Scholte et al., 2002; Zhu et al., 2004) and occurs mainly in areas with loss of neuronal markers (Zhu et al., 2004). Inhibitors of iNOS (Ishida et al., 2001) and nNOS gene deficiency (Ferriero et al., 1996) have been shown to protect the immature brain.

Antioxidative capacity

Antioxidants counteract the effects of ROS. Antioxidative capacity (AOC) is defined as the ability of the tissue or body fluid to buffer ROS. The AOC therefore consists of several molecules, such as vitamins and plasma proteins, the role of which is to protect the tissue from ROS-mediated injury. AOC has previously been demonstrated in AF from women in both the second and third trimesters, although ROS was only present in half of the samples, suggesting the importance of protection against ROS formation (Burlingame et al., 2003). In vitro studies of the foetal membranes suggest that antioxidants such as vitamin C and E can prevent ROS-mediated injury and thereby prevent pPROM (Plessinger et al., 2000).

Inflammatory mediators

Inflammatory cells can produce several inflammatory mediators, such as ILs, MMPs and a newly detected galactoside, galectin-3.

Interleukin-1β

IL-1β is a pro-inflammatory mediator that contributes to neonatal brain injury. Studies show that both IL-1 receptor antagonist (IL-1ra) (Hagberg et al., 1996; Martin et al., 1994) and caspase-1 (IL-1 converting enzyme) gene deletion are protective after moderate neonatal HI injury (Liu et al., 1999). IL-1 also seems to have different roles in adult and neonatal brains, as mice lacking IL-1α and IL-1β are protected against adult stroke (Boutin et al., 2001) but not against neonatal HI injury (Hedtjarn et al., 2005b). Nonetheless, IL-1β is often used as a marker of inflammation after HI, as it increases within 12h after injury in both adult (Hill et al., 1999) and neonatal brains (Hagberg et al., 1996).

Interleukin-6

The cytokine IL-6 is produced during the inflammatory process and is involved in redirecting the inflammatory response into a healing process, as well as in activation of the specific immune system and in the production of antibodies (Mölne J and Wold, 2007). Elevated IL-6 levels are present in IAI but are also associated with the foetal inflammatory response syndrome (FIRS) that is an independent risk factor for severe neonatal morbidity and brain injury (Romero et al., 2006). Increased IL-6 is seen both in the brain after experimental neonatal HI (Bona et al., 1999) and in cerebrospinal fluid from asphyxiated infants (Savman et al., 1998).

Interleukin-8

IL-8 is a chemokine produced by macrophages and endothelium; it functions as a chemoattractant for neutrophils, leading to accumulation and extravasation (Mölne J and Wold, 2007). Like IL-6, IL-8 is also increased in AF from women with HCA in a dose-dependent manner; a higher degree of inflammation is associated with higher levels of IL-8 (Holst et al., 2007). The mouse equivalent of IL-8 is elevated after experimental HI (Bona et al., 1999) and IL-8 is elevated in cerebrospinal fluid from asphyxiated infants with abnormal outcome (Savman et al., 1998).

Interleukin-18

IL-18 is a cytokine produced by astrocytes and microglial cells (Hedtjarn et al., 2005a). It is up-regulated at both the mRNA and protein levels after neonatal HI (Hedtjarn et al.,

2002). IL-18-deficient mice are protected against brain injury, including white matter injury, after neonatal HI (Hedtjarn et al., 2002; Hedtjarn et al., 2005a).

Matrix Metalloproteinase-9

MMP-9 is one of several extracellular membrane-bound proteases. It consists of a pro-enzyme that can be activated by other proteases, forming active MMP-9, which is subsequently inactivated by binding to inhibitory proteins like specific tissue inhibitors of matrix metalloproteinases (TIMP) (Malla et al., 2008). MMP-9 is produced by microglial cells in the immature brain, and contributes to HI brain injury by disruption of the blood brain barrier, loss of white matter markers and increased microglial activation (Svedin et al., 2007). Together with MMP-2, it degrades active galectin-3 (Ochieng et al., 1994) and primed neutrophil cells expose the galectin-3 receptor on the cell surface during degranulation of MMP-9 containing granules (Almkvist et al., 2001). However, the interactions between these two inflammatory mediators have not been fully elucidated. MMP-9 has also been implicated in the induction of labour (Athayde et al., 1999) and an increase in MMP-9 can be associated with intrauterine infection and pPROM (Menon and Fortunato, 2004).

Galectin-3

Galectin-3 is a member of the growing family of β-galactosides. Galectin-3 has mainly been studied in association with cell migration and proliferation (Takenaka et al., 2004) but it is also important in multiple, both intra- and extracellular, processes such as the apoptotic cascade (mostly in tumour cells) and in inflammatory processes (Liu, 2005; Liu et al., 2002) (see Figure 3). Galectin-3 is present in several different cell types in humans, such as trophoblasts and decidual cells in the placenta, under normal conditions (Jeschke et al., 2007), but also during inflammatory processes (Ohshima et al., 2003) and in various tumours of brain origin (Park et al., 2008).

Inflammatory role

Galectin-3 is produced and secreted by macrophages (Liu et al., 1995) and microglial cells both in vitro (Pesheva et al., 1998) and in vivo (Walther et al., 2000; Yan et al., 2009). It has chemotactic properties, both for macrophages/monocytes (Hsu et al., 2000; Sano et al.,

2000) and neutrophils (Colnot et al., 1998b), when released into the extracellular space and contributes to increased inflammatory response in experimental peritonitis (Colnot et al., 1998b; Hsu et al., 2000). A possible mechanism behind the increased inflammatory response is the inhibition of apoptosis and activation of NF-kB in macrophages (Hsu et al., 2000). Galectin-3 also promotes the release of IL-1 in human monocytes in vitro (Jeng et al., 1994). It contributes to phagocytosis in both neutrophils and macrophages (Fernandez et al., 2005; Karlsson et al., 2009; Sano et al., 2003) and can also activate NADPH oxidase to produce a respiratory burst in monocytes (Liu et al., 1995) and in primed human neutrophils (Karlsson et al., 1998; Yamaoka et al., 1995).

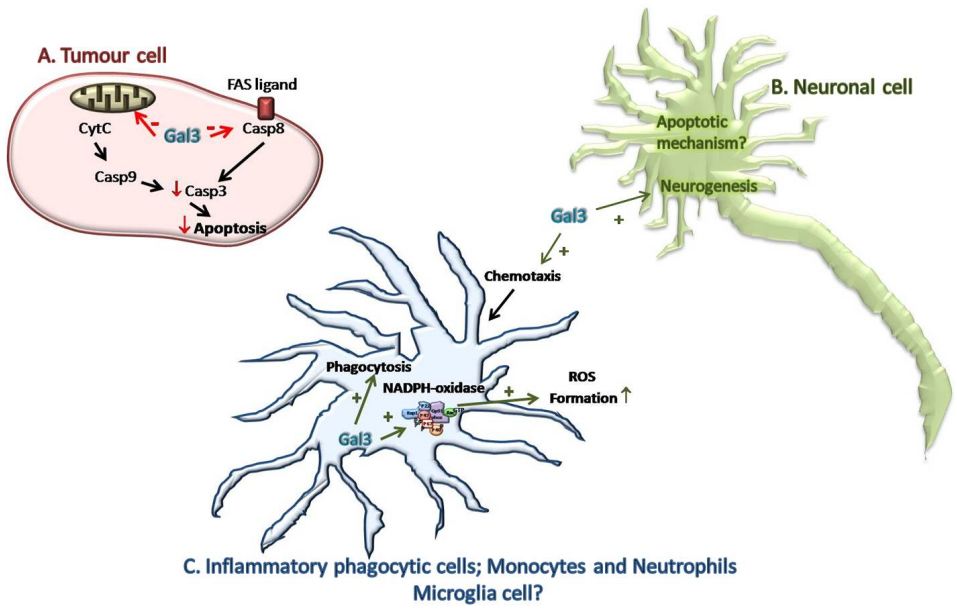

Figure 3. Overview of some of the intracellular and extracellular effects of galectin-3

Galectin-3 has anti-apoptotic properties and in tumour cells, galectin-3 can affect both the caspase-dependent intrinsic pathway, through regulation of mitochondrial stability, as well as the extrinsic pathway through inhibiting the caspase-8/CD95-ligand (A). It has also been suggested that galectin-3 promotes neurogenesis in the adult brain while it is unknown if it can affect neuronal apoptosis (B). Galectin-3 can modulate the activity of phagocytic inflammatory cells due to enhanced chemotaxis and through the ability to activate NADPH oxidase, although it is not known if this is also true for microglial cells (C)

Intracellular role

Studies on the intracellular role of galectin-3 show that it is involved in different anti-apoptotic pathways (Liu et al., 2002). It has been established that its structure resembles that of Bcl-2 and that it therefore might regulate mitochondrial permeability. In human prostate cancer cells, an increase of galectin-3 can inhibit apoptosis by affecting caspase-8 activation (Fukumori et al., 2004) or by reducing cytochrome C release, leading to a reduction in caspase-9 activation (Fukumori et al., 2006). In inflammatory cell lines, galectin-3 can also modulate apoptosis (Liu et al., 2002), resulting in a prolonged inflammatory response (Hsu et al., 2000). In the brain, it has been found in special kinds of neurons in primary cell cultures from mice (Pesheva et al., 2000) as well as in several kinds of human brain tumours (Park et al., 2008). However, it is not known whether galectin-3 influences neuronal apoptosis after HI injury and, if so, how.

Galectin-3 in the brain and in injury models

As mentioned above, galectin-3 has been identified in microglial cells (Pesheva et al., 1998; Walther et al., 2000; Yan et al., 2009), in a subgroup of neurons (Pesheva et al., 2000) and lately in astrocytes (Yan et al., 2009). Both galectin-3 protein and mRNA levels are up-regulated in different injury models, such as experimental bacterial meningitis (Bellac et al., 2007), and in prion-infected animals (Riemer et al., 2004). In the adult stroke model, galectin-3 protein expression is seen in microglial cells and it is increased after injury in rats (Lalancette-Hebert et al., 2007; Walther et al., 2000), although late inhibition of galectin-3 in the same model does not seem to reduce brain injury (Yan et al., 2009). It has also been suggested that galectin-3-positive microglial cells are a resident population with protective properties that produce insulin-like growth factor (IGF)-1 (Lalancette-Hebert et al., 2007). No studies have previously investigated galectin-3 in the perinatal setting.

Neurotrophic factors

Insulin-like growth factor-1

The mRNA of the peptide IGF-1 is increased during the late recovery phase (72 h) after neonatal experimental HI (Clawson et al., 1999). When given to neonatal rats, IGF-1 reduces brain injury after HI (Brywe et al., 2005). As previously mentioned, studies in adult

mice indicate that IGF-1 is produced in the same inflammatory cells as galectin-3 and that these cells are protective after adult stroke (Lalancette-Hebert et al., 2007).

Phosphorylated Akt

Akt is a downstream marker for the activation of several trophic factors, such as IGF-1 and growth hormone (GH) (Aberg et al., 2006). When phosphorylated, Akt becomes activated and forms pAkt (Dudek et al., 1997). Previous studies indicate that pAkt is decreased during reperfusion after injury (Noshita et al., 2001; Ouyang et al., 1999; Yoshimoto et al., 2001), including after neonatal HI (Brywe et al., 2005).

AIMs

The overall objective was to investigate inflammatory mechanisms and free radical formation in experimental immature excitotoxic and HI brain injury as well as in women with threatening PTD.

Specific objectives were

Paper I. To investigate if NADPH oxidase contributes to ROS formation and injury in two models of perinatal brain injury and whether genetic or pharmacological inhibition of NADPH oxidase reduces injury.

Paper II. To investigate the effect of galectin-3 on tissue loss, inflammatory activation, as well as the expression of trophic factors and apoptotic markers after neonatal HI brain injury.

Paper III. To investigate galectin-3, ROS formation and AOC in AF and whether these markers are altered in association with intrauterine infection/inflammation, PTD or severe neonatal morbidity in women with PTL or pPROM.

Materials and methods

Patient recruitment (III)

In Paper III, a prospective cohort study was performed on a study population consisting of women with singleton pregnancies in PTL (n=49) or with pPROM (n=34) at less than 34 w GA, presenting at the Sahlgrenska University Hospital, Gothenburg. The outcome variables used in this study are listed in Table 1.

Preterm labour

PTL was defined as regular uterine contractions (at least 2 uterine contractions/10 minutes for ≥ 30 minutes) in combination with one of the following findings on examination of the cervix (1 – 3: evaluated by digital examination): **(1)** ≤ 2 cm length + ≥ 1 cm dilatation or **(2)** ≤ 2 cm length + softening or **(3)** ≥ 1 cm dilatation + softening or **(4)** < 3 cm length on transvaginal sonography).

Preterm pre-labour rupture of membranes

pPROM was defined as amniorrhexis (visible amniotic fluid in the vagina) before the onset of spontaneous labour. Women with known uterine abnormalities, cervical cerclage, foetal malformations, significant vaginal bleeding, imminent delivery or foetal distress were not included. GA was determined in all cases by routine ultrasound in the second trimester (16^{th} to 19^{th} weeks of gestation). Tocolytic therapy was administered according to department protocol. Maternal background data was obtained from medical charts (Jacobsson et al., 2003a; Jacobsson et al., 2003b).

Table 1. Outcome variables used in Paper III

Outcome variables					
Women	PTL	pPROM	Threatening PTD (PTL+pPROM)		Term controls (no pPROM or PTL)
Inflammation	MIAC		IAI	HCA	CA (MIAC+IAI+HCA)
Onset of delivery from sampling	<7 days		>7 days		
GA at birth	<34weeks		>34weeks		
Gender	Boys		Girls		
Neonatal severe morbidity	CLD		PVL/IVH	NEC	Sepsis; suspected or verified

Galectin-3, ascorbyl radicals and rapid/slow AOC was measured in AF and data was then analysed for association with the outcome variables listed above.

Controls

Twenty-five women at term (≥ 37 w GA) were included as controls. They were all scheduled for elective caesarean section (CS) due to psychosocial indications, breech presentation or ≥ two previous CS.

Classification of chorioamnionitis (Paper III)

AF samples were obtained from all patients with PTL or pPROM by ultrasound-guided transabdominal amniocentesis, performed under antiseptic conditions within 12 h of admission. The samples were immediately stored in a refrigerator (+4°C) and centrifuged at +4°C, within 5 h of sampling, at 855 g for 10 minutes. The supernatant was stored at -80°C until analysis.

Comment: Patients with CA were divided into 3 groups as follows: MIAC, IAI and HCA. If any of these conditions were diagnosed, the women were considered to have CA. AF samples were obtained when the women presented with PTL or pPROM, while HCA was determined by histopathological examination after delivery. This means that MIAC and IAI represent inflammation in the acute state of PTL/pPROM while HCA represents the state at delivery, occurring from 0 days to several weeks/months after AF sampling.

Microbial invasion of the amniotic cavity

A sample of uncentrifuged AF was immediately sent for polymerase chain reaction (PCR) analysis of Ureaplasma urealyticum and Mycoplasma hominis as well as for aerobic and anaerobic culture. MIAC was defined as positive PCR and/or growth of any bacteria in the AF with the exception of coagulase-negative Staphylococci which was assessed as skin contamination unless the women also had IAI, as defined below.

Intra-amniotic inflammation

AF was analysed for IL-6 and IL-8 using an ELISA with commercially available paired antibodies from R&D Systems (Minneapolis, Minnesota, USA). The samples were run in duplicates, as previously described (Jacobsson et al., 2003a). The diagnostic levels for IAI were set at IL-6 ≥ 1.5 ng/ml and/or IL-8 ≥ 1.3 ng/ml, according to previous results in women

with PTL (Jacobsson et al., 2003b), or at IL-6 ≥ 0.80 ng/ml and/or IL-8 ≥ 0.42 ng/ml for women with pPROM (Jacobsson et al., 2003a). The detection level was 150pg/ml for IL-6 and IL-8.

Comment The levels of IL-6 and IL-8 in AF increased with increasing inflammation. The levels of IL-6 and IL-8 are also higher in AF from women in PTL than from women with pPROM; hence the two different limits for classification of IAI (Holst et al., 2007).

Histological chorioamnionitis

After delivery, the placenta was examined by a perinatal pathologist. Tissue samples were obtained from umbilical cord (proximal and distal samples), membrane "rolls", umbilical cord insertion and 2 full-thickness samples of placenta, yielding a minimum of six routine samples. Furthermore, a varying number of extra samples were obtained, depending on gross examination findings. HCA was defined as a combination of funiculitis, CA in extraplacental membranes, foetal vessel vasculitis and subchorionic fibrin polymorphonuclear (SCFPMN) infiltration. Detection of diffuse infiltration by polymorphonuclear cells (PMNs) in all the above locations was required for HCA diagnosis (Holst et al., 2007).

Comment: As discussed in Holst et al. (2007), there is no standardized way to define HCA and it is thus difficult to compare our results with those of others. In this study we used a rather conservative definition requiring PMN infiltration in all locations of the placental unit.

Severe morbidity (Paper III)

Neonatal medical charts were examined for severe morbidity known to be associated with intrauterine infection/inflammation. The following conditions were included: 1. PVL; 2. IVH (grade III-IV); 3. NEC; 4. CLD, defined as requirement for extra oxygen at 36 w GA; 5. Early-onset sepsis, verified if the infant had positive bacterial cultures and suspected if CRP was › 20 mg/L within 7 days of delivery.

The clinical study was approved by the Local Ethics Committee at Sahlgrenska University Hospital, the University of Gothenburg (nr 349-95, 271-98). All women gave informed consent before enrolment in the study.

Experimental studies of perinatal brain injury

As the methods used in Papers I and II are very similar and all are aimed at investigating the extent of injury, inflammatory response and apoptosis, they are summarised in Table 2.

Genetically modified mice (Papers I, II)

Gp91^{phox} -/- (knock-out=KO) C57/Bl6 mice lacking functional NADPH oxidase were originally obtained from Jackson Laboratories (Pollock et al., 1995) and were bred separately with wild type (WT) C57/Bl6 mice for the first series of experiments with non-littermates, including both female and male pups. Littermate KO and WT animals were then bred from heterozygote (+/-) females and KO (0/-) males, resulting in WT (0/+) and KO (0/-) male littermates.

Galectin-3 -/- SV129 mice were obtained from Dr Francoise Poirier (Colnot et al., 1998a). As the SV129 background is not suitable for this model of neonatal HI (Sheldon et al., 1998), the galectin-3 null mutant mice were back-crossed for 4 generations with C57/Bl6 mice, resulting in 93.75% C57/Bl6 / 6.25% SV129 mice. A similar breeding strategy has previously been used by our group for HI experiments (Hagberg et al., 2004). Galectin-3 heterozygote (+/-) mice were then bred to obtain littermate WT (+/+) and KO (-/-) mice.

Both animal strains were bred at Experimental Biomedicine, Sahlgrenska Academy, Gothenburg University, Sweden, with free access to food and water.

Comment: The gp-91^{phox} mutation is x-linked. This affects the breeding of littermates as males only carry one allele and mating with a heterozygote female can only yield male littermates (+/0 or -/0). The gp91^{phox} mutant mice appear to develop normally; however, they have increased susceptibility to some bacterial infections (Pollock et al., 1995). The galectin-3 KO mice are reported to undergo normal development but they can be susceptible to infections due to the altered inflammatory response (Colnot et al., 1998a).

Table 2. Overview of the methods used in Paper I, II

	Injury model	Method	Analysis	Antibodies/ primers	paper
Verification of genetical KO	Hypoxia ischemia model	Extraction of neutrophils	Chemilunimecens	fMLF	I
		PCR	Qualitatitive analysis	See table 2	I,II
		WB	Qualitatitive analysis	Galectin-3	II
Cell localisation	Hypoxia ischemia model	IF	Qualitatitive analysis	Galectin-3/Iba-1	II
				Galectin-3/lectin	
				Galectin-3/NeuN	
				Galectin-3/GFAP	
Verification of gender	Hypoxia ischemia model	Visual examination	Qualitatitive analysis		I,II
	Excitotoxic model	PCR	Qualitatitive analysis	SRY-gene	I
Injury evaluation	Hypoxia ischemia model	IHC	Area measurement, injury volume	MAP-2	I,II
			Neuropathological scoring	MAP-2	I,II
	Excitotoxic model	IHC	Injury volume	Cresyl violett	I
White matter evaluation	Hypoxia ischemia model	IHC	Area measurement	MBP	I,II
				NF	I
	Excitotoxic model	IHC	Injury volume	Cresyl violett	I
Inflammatory evaluation	Hypoxia ischemia model	IHC	Cell count/ area measurement	Galectin-3	I
				Iba-1	II
		ELISA	Quantification/protein	IL-1β	I
				Galectin-3	
				IL-1β	II
				IL-18	
				MMP-9	
Free radical formation in vitro	Microglial cells	Ibotenate challange	Fluorescens intensity quantification	H2DCFDA	I
Free radical formation and markers of oxidative stress in vivo	Hypoxia ischemia model	ESR	Quantification/protein	Ascrobylic radicals	I
		IHC	Cell counting	Nitrotyrosine	I
		WB	Quantification/protein	Nitrotyrosine	II
	Excitotoxic model	IHC	Cell counting	HNE	I
Free radical formation in inflammatory cells in vivo	Hypoxia ischemia model	IF	Cell counting	Nitrotyrosine/Iba-1	I
Apoptotic evaluation	Hypoxia ischemia model	IHC	Cell counting	AIF	II
		WB	Quantification/protein	AIF	II
		Caspase activity measurement	Quantification/protein	Casp-3, -8, -9	II
	Excitotoxic model	IHC	Cell counting	Cleaved casp-3	I
Apoptosis in inflammatory cells	Hypoxia ischemia model	IF	Cell counting	Iba-1/Casp-3	II
				Iba-1/AIF	
Evaluation of trophic factors	Hypoxia ischemia model	ELISA	Quantification/protein	IGF-1	II
		WB	Quantification/protein	pAkt	II

PCR= Polymerase chain reaction, WB= Western blot, IF= immunofluorescent staining, IHC= immunohistochemistry using DAB, ELISA= Enzyme-linked immunosorbent assay, ESR= Electron spin resonance, Quantification/protein= values are correlated with protein content, fMLF= formyl-methionyl-leucyl phenylalanine, Casp= caspase

Therefore, these two mice strains were handled carefully and separately. Both strains grew normally and no clinical signs of infection were observed during the study period.

Induction of neonatal brain injury (Papers I, II)

Induction of neonatal hypoxia-ischemia (Papers I, II)

Neonatal HI was induced using the Rice Vannucci model (Rice et al., 1981), modified for mice on postnatal day 9 as previously described (Hedtjarn et al., 2002; Rice et al., 1981). Pups were anaesthetised with isoflurane (Forene®: 3.5 % for induction, 1.5 % for maintenance) in a mixture of nitrous oxide and oxygen (1:1). The left common carotid artery was permanently ligated (prolene 6.0). The incision was closed (prolene 5.0) and infiltrated with local anaesthetic after the procedure. The duration of anaesthesia was <5 min. After a recovery period with the dam (≥ 1 h), the pups were exposed to a humidified gas mixture (10% oxygen in nitrogen) for 50 or 60 minutes at 36C°.

The animal experiments were approved by the local Ethics Committee of the University of Gothenburg (95/2003, 11/2004, 264-265/2007).

Comment: In Paper I, two sets of experiments were run, one with 60 min of hypoxia and one with 50 min of hypoxia. Sixty min of hypoxia induces severe brain injury with major brain tissue loss. We hypothesised (Liu et al., 1999; Svedin et al., 2007) that the inflammatory response might play a more important role in response to more moderate injury. Therefore, a second set of experiments was run with a shorter hypoxia duration that resulted in moderate brain injury.

Induction of neonatal excitotoxic injury (Paper I)

Induction of excitotoxic brain damage by intracerebral (i.c.) injection of the NMDA receptor agonist ibotenate was performed as previously described (Marret et al., 1995), using 5 day old CD1 pups weighing 3±0.3 grams. They were housed under standard conditions in a conventional animal facility with their dams. During the procedure the mice were anaesthetised with isoflurane and kept under a warming lamp. I.c. injections were performed with a 26-gauge needle attached to a 50 µl Hamilton syringe mounted on a

calibrated microdispenser. The needle was inserted stereotactically 2 mm under the external surface of the scalp in the fronto-parietal area of the right hemisphere, 2 mm from the midline in the lateral–medial plane and 3 mm from the junction between the sagittal and lambdoid sutures in the rostro-caudal plane. Two 1-µl boluses of ibotenate (5 µg/µl, from BioTrend) were injected 2 mm and 1 mm under the surface with a 20 s interval. The needle was left in place for 20 s after the second bolus to avoid leakage. After the injections, the animals were returned to their dams. Histopathology was used to confirm that the tip of the needle had reached the periventricular white matter.

All protocols complied with the European Community guidelines for the use of experimental animals.

Comment: This procedure results in a white matter lesion, mimicking the injury observed in preterm neonates. The development of the lesion in this model can be blocked by treatment with a NMDA receptor antagonist (Keller et al., 2006a; Marret et al., 1995).

Administration of pharmacological inhibitors of NADPH oxidase (Paper I)

The gp91ds-tat peptide and its inactive analogue scrambled tat were synthesized by the Protein Chemistry Facility of Tufts University (Boston, Mass, USA) and kindly provided by PJ Pagano, Detroit, USA. Apocynin was obtained from SIGMA, Germany.

Experimental groups in vivo

In the first experiment, the function and selectivity of gp91ds-tat and scrambled tat were tested. Mouse pups were randomised into two experimental groups: 1. ibotenate (10 µg) + scrambled tat (1 µM) i.c. and 2. ibotenate (10 µg) + gp91ds-tat (1 µM) i.c. The peptides were injected immediately following the injury. Both groups were analysed at 24 h and 120 h after injury.

Apocynin was tested by administering either apocynin (4 mg/kg body weight) or vehicle (PBS+0.01% Ethanol) by intraperitoneal (i.p.) injection. Pups were divided into 3 groups: 1. ibotenate i.c. + single injection of vehicle or apocynin, administered immediately after the

insult; 2. ibotenate i.c. + two injections of vehicle or apocynin administered one day before and immediately after the insult; 3. ibotenate i.c. + four repeated injections of vehicle or apocynin, starting immediately after the insult and daily until postnatal day nine. Animals were then sacrificed at 24h after injury (groups 1 and 2) and, in a second experiment, at five days after injury (all three groups). The absolute dose of apocynin was adapted daily to the current body weight of the pups. Finally, the dose of apocynin was increased to 150% of the initially tested dose. Following i.c. ibotenate injection, animals were randomised into two groups; one injected with apocynin (6.5 mg/kg) and a second group injected with vehicle. Both were administered immediately after the insult and animals were sacrificed at 4 and 24 h after the insult.

Comment: Maintaining sufficient plasma levels of apocynin is important in order to ensure inhibition of NADPH oxidase (Tsukagoshi et al., 1994) and several different regimens were thus tested.

Genotyping by PCR (Papers I, II)

The genotype of the gp91^{phox} KO and WT littermates and the galectin-3 KO and WT mice was determined by analysis of tail DNA, extracted from the tissue as previously described (Svedin et al., 2007). Approximately 2 mm of tissue was digested overnight at 60°C in buffer (50 mM Tris-HCL (pH 8.0) 100 mM EDTA, 100 mM NaCl, 1% SDS) containing 1 mg/ml proteinase K (Roche Diagnostics). In order to extract the DNA, half the original volume of potassium acetate, 5 M, was added and the mixture was centrifuged at 13 000 rpm for 20-30 min. The supernatant was saved and mixed with twice the original volume of 100% ethanol and incubated at -20°C for at least 30 minutes. To precipitate the DNA, the mixture was centrifuged at 13 000 rpm for 25-30 minutes at 4°C. Pellets were washed with 75% ethanol, and then left to dry after removal of the liquid. The DNA was dissolved in 100 µl sterile H_2O and DNA for gp91^{phox} was also diluted additionally 10 times before being added to the PCR mixture. 1-2 µl of the extracted DNA was added to the reaction mixture (1x PCR buffer (250 mM Tris-HCl (pH 8.3) 375 mM KCl, 15 mM $MgCl_2$, Sigma-Aldrich, Saint Louis, USA), 200 µM dNTP, 0.5 µM Primer 1-3 (Cybergene), 0.04 units/µl Taq polymerase (Sigma-Aldrich, Saint Louis, USA)), yielding a total volume of 25 µl. The

Table 3. The PCR reactions and the primers used (Paper I, II)

Gene	Primers	PCR reaction	Number of cycles	Band size
Gp91phox	Common; 5'-CGC ACT GGA ACC CCT GAG AAA GG-3' WT; 5'-AAG AGA AAC TCC TCT GCT GTG AA-3' KO; 5'-GTT CTA ATT CCA TCA GAA GCT TAT CG-3'	Denaturation: 94°C, 20 sec Annealing: 64°C, 30 sec Elongation: 72°C, 35 sec Denaturation: 94°C, 20 sec Annealing: 58°C, 30 sec Elongation: 72°C, 35 sec	12 25	KO; 240 bp WT; 195 bp
Galectin-3	Common; 5'-CAC GAA CGT CTT TTG CTC TCT GG- WT; 5'-TGA AAT ACT TAC CGA AAA GCT GTC TGC-3' KO; 5'-GCT TTT CTG GAT TCA TCG ACT GTG G-3'	Denaturation: 94°C, 45sec Annealing: 55°C, 30sec Elongation: 72°C 90sec	40	KO; 240 bp WT; 195 bp

PCR and primers used are listed in Table 3. The PCR products were then separated by electrophoresis on a 0.5 x TBE 1.5% agarose gel, labelled with ethidium bromide. The bands were visualised by a LAS 100 cooled CCD camera (Fujifilm, Tokyo, Japan).

Comment: There are two sets of PCR cycles for the $gp91^{phox}$ gene: one setting used for 12 cycles, followed by 25 cycles at another setting. This is the commercially used protocol for this mutation and the double settings are required because of the wide range in the amount of DNA retrieved.

Verification of absent functional NADPH oxidase (Paper I)

The absence of functional NADPH oxidase in KO animals was initially determined by isolation of activated neutrophils from the peritoneum, and subsequently by PCR. Mice were injected i.p. with 0.1 ml uric acid to induce inflammatory accumulation of neutrophils in the peritoneal cavity. After peritoneal lavage with 2x5ml cold PBS, cells were harvested and washed 4 h after injection. Red blood cells were eliminated by hypotonic lysis. After an additional wash, the cells were re-suspended in Krebs–Ringer phosphate buffer. The purity of the peritoneal neutrophils was 95% (determined by May-Grünwald-Giemsa staining) and the viability was >95 % (determined by trypan blue exclusion). The absence of ROS production was confirmed by chemiluminesence (CL), as previously described, after stimulation with the NADPH oxidase-activating peptide fMLF (Bylund et al., 2003).

Gender determination

A PCR for the Y- specific SRY gene was used retrospectively, as previously described (Keller et al., 2006b), to determine gender in the CD1 mice in the excitotoxic model. In the neonatal HI model, gender was determined in the C57/Bl6 mice by visual examination of the genitals by a trained investigator.

Genetic analysis of mRNA (Papers I, II)

The mRNA for gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox} (Paper I) and galectin-3 (Paper II) was analysed at different time-points after HI, as previously described (Hedtjarn et al., 2004). Animals were sacrificed by decapitation at 2, 8, 24 and 72h after HI. Un-operated controls, not subjected to HI, were sacrificed on postnatal day 9 and 12 (n=5 at each time-point). The brains were rapidly extracted and frozen on dry ice. RNA was isolated using the Rneasy (R) mini kit (Qiagen Inc) and quantified by spectrophotometry at 260 nm, and the OD was determined by a 260/280 ratio. The quality of the RNA was further checked by running the RNA samples on a 1.1% agarose/2.2 M formaldehyde gel to ascertain that there was no degradation. The mRNA was analysed by microarray (Affymetrix©) technique. Affymetrix CEL files were pre-processed using the robust multiarray average (RMA) algorithm, which performs three distinct calculations: global background normalisation, across-array normalisation, and log2 transformation of perfect match values.
(http://stat.www.berkeley.edu/users/bolstad/RMAExpress/RMAExpress.html)

Preparation of tissue (Papers I, II)

Preparation for immunohistochemistry and cell count (Papers I, II)

After neonatal HI, pups were sacrificed 3 h, 8 h, 24 h, 96 h and 7 days after the insult (p9-16) in different experimental settings for evaluation of tissue loss, inflammation and apoptosis. Animals were anaesthetised with thiopental (Pentothal® Sodium), perfusion-fixed with 5% paraformaldehyde (Histofix, Histolab, Göteborg, Sweden) and the brains were dehydrated and embedded in paraffin. Five-µm coronal sections were obtained evenly throughout the whole brain. Every 50th section was stained and analysed for Paper I, and every 70th section was stained and analysed for Paper II.

After excitotoxic injury, pups were euthanised by decapitation. Brains were immediately immersion-fixed in 4% formaldehyde for 72 h. Following dehydration and paraffin embedding, blocks were sliced into 10-µm coronal sections.

Preparation of tissue for electron spin resonance, ELISA and Western blot (Papers I, II)

P9 mice were sacrificed by decapitation at 0 min, 15 min, 30 min, 2 h and 24 h (Paper I) and at 3 h, 8 h and 24 h (Paper II) after HI. After sacrifice, both controls (P9 mice) and study mice underwent the following procedures. The brains were dissected on ice and the hemispheres immediately frozen in isopentane on dry ice. At the time of analysis, hemispheres were homogenised, in 500 µl ice-cold PBS (Paper I) or in 500 µl ice-cold homogenisation buffer containing 50 mM Tris base, 2mM EDTA (pH 7.3) and 0,5% protease-inhibiting cocktail (Paper II), and then aliquotted into smaller portions for each analysis. The aliquots were then stored at -80ºC until analysis (or after electron spin resonance (ESR) measurement, see ROS assessment, Paper I).

Immunohistochemistry and cell count (Papers I, II)

Immunohistochemistry after neonatal hypoxia-ischemia (Papers I, II)

For immunohistochemistry, sections were deparaffinised and boiled in citric acid buffer for 10 min, followed by incubation with 3% H_2O_2 in PBS to block endogenous peroxidase. The sections were incubated with blocking solution with 4% serum in 3% BSA for 1 h, followed by incubation with primary antibody (1 h or overnight incubation), 1 h incubation with the appropriate secondary antibody (1:250), and 1 h incubation with avidin-biotinylated enzyme complex (20µl/ml, Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, USA). For DAB staining, sections were incubated with a solution containing 3.3-diaminobenzidine (DAB 0.5 mg/ml), glucose oxidase (Sigma) and ammonium nickel sulphate for enhancement, followed by dehydration and mounting with DALAB mounting medium. All secondary antibodies were obtained from Vector Laboratories. The immunofluorescent stainings included an extra incubation with 2 M HCl for 1.5 h before incubation with blocking solution. Sections were immediately mounted with DACO

fluorescent mounting medium after incubation with the secondary antibody. All secondary antibodies for fluorescent stainings were obtained from Molecular Probes (Alexa fluor 488, 596 and 647) and Vector Laboratories (Texas red). All antibodies used are listed in Table 4.

Morphological analysis after neonatal hypoxia-ischemia (Papers I, II)

The different staining methods used are listed in Table 4. In order to visualise a timeline for galectin-3 protein expression, sections from both KO and WT animals at 3 h, 8 h, 24 h, 96 h and 7 days after injury were stained for galectin-3. This also ensured a lack of protein expression in the KO mice. Images were obtained with an Olympus microscope DP50. In order to determine the cellular location of galectin-3 protein, hippocampal and striatal sections from 3, 8, 24, 96 h and 7 days after injury were co-stained with galectin-3 and ionised calcium-binding adaptor molecule (Iba)-1, iso-lectin, Glial Fibrillary Astrocyte Protein (GFAP) or Neuronal Nuclei (NeuN), as well as triple-stained with Iba-1, MMP-9, and galectin-3. Images were then obtained with a Zeiss Meta Confocal microscope at the Centre of Cellular Imaging at the Sahlgrenska Academy.

To assess microglial cell accumulation at 96 h and 7 days after neonatal HI, one hippocampal and one striatal section were stained with galectin-3 (Paper I) or Iba-1 (Paper II) and the stained area was measured using Micro Image software. At earlier time-points, 8 and 24 h after neonatal HI, the number of cells was counted using Stereoinvestigator (MicroBright-Field System, Inc) with computer-generated counting frames within selected areas. Results are given as mean number of cells ± S.E.M per area unit in each group. Cell counts were undertaken in the hippocampus and striatum as well as at the hippocampal and striatal levels (frames 175x175 µm squares, approximately 25 squares per area unit). Results were then corrected for individual variation by subtracting the number of cells in the uninjured hemisphere from the number in the injured hemisphere before comparing KO and WT littermates. Neutrophils were counted in the same manner, using anti-neutrophil staining (Paper I).

For apoptosis, sections from 3 h, 8 h and 24 h after HI were stained for AIF (Paper II) and cells with nuclear translocated AIF were counted, as described above. To evaluate apoptosis in inflammatory cells, one section from the hippocampus and one from the

Table 4. Antibodies used for immunohistochemistry (Paper I, II)

Antibody	Company, product number	Dilution	Staining method	Additional treatment	Secondary antibody	Paper
Apoptosis Inducing Factor (AIF)	Santa cruz, Sc-9416	1:50	IHC, IF	1% BSA in TBS +0,1% triton	Horse anti goat, biotinylated (IHC) Streptavidin (IF)	II
Caspase-3 (biotinylated)	BD Pharmingen, 557038	1:50	IF		Streptavidin (IF)	II
Cleaved Caspase-3 (Asp175)	# 966, Cell Signaling	1:500	IHC		Goat anti rabbit, biotinylated (IHC)	I
Galectin-3	Gift from Anna Karlsson (anti-Mac-2, culture supernatant from the hybridoma M3/38, LGC Promochem/ATCC)	1:100	IHC, IF	3% BSA, 0,2% Triton-X	Goat anti rat, biotinylated (IHC) Followed by fluorescein (IF) Goat anti rat, alexa fluor 488 (IF) Goat anti rat, alexa fluor 546 (IF)	I, II
Glial Fibrillary Acidic Protein (GFAP)	Gift from Lars Rosengren	1:2000	IF	3% BSA	Goat anti rabbit, texas red (IF)	II
4-Hydroxynonenal (HNE)	#.393207, Calbiochem	1:1000	IHC	3% BSA	Goat anti rabbit, biotinylated	I
Iba-1	a. WAKO, 019-19741 b. Abcam, ab5076	1:500 1:100	IHC, IF IF	3% BSA	Goat anti rabbit, biotinylated (IHC) Followed by fluorescein, Alexa fluor 594 (IF) Donkey anti-goat, Alexa fluor 594 (IF)	II
Isolectin (FITC-labelled)	Sigma Aldrich, L2895	15µg/ml	IF		(FITC-labelled primary)	II
Microtubule Associated Protein (MAP)-2, clone HM-2	Sigma Aldrich, M4403	1:2000	IHC	3% BSA	Horse anti mouse, biotinylated (IHC)	I, II
Metalloproteinase (MMP)-9	Abcam, 16306	1:10 000	IF	3% BSA	Donkey anti rabbit, Alexa fluor 647 (IF)	II
Myelin Basic Protein (MBP)	BioSite, SMI-94R	1:10 000	IHC	3% BSA, 0,2% Triton-X	Horse anti mouse, biotinylated (IHC)	I, II
Neurofilament 68 (NF)	Sigma Aldrich, N5139	1:200	IHC	0,2% Triton-X	Horse anti mouse, biotinylated (IHC)	I
Neuronal Marker N (NeuN)	Chemicon International, MAB377	1:250	IF	3% BSA	Goat anti mouse, texas red (IF)	II
Nitrotyrosine	Molecular probes, A-21285	1:20	IHC, IF	0,2% Triton-X	Goat anti rabbit, biotinylated (IHC) Goat anti rabbit, texas red (IF)	I
Rat anti Mouse Neutrophils	AbD Serotec, MCA771B	1:100	IHC	Proteinase K 10µg/ml, 9 min 0,2% Triton-X	Goat anti rat, biotinylated (IHC)	I

Antibodies were diluted in PBS. IHC= immunohistochemistry using ABC kit followed by DAB, IF= immunofluorescent staining. For immunofluorescent stainings, sections were also incubated with 2M HCl for 1.5 h before incubation with block and antibodies to enhance the stainings. BSA= bovine serum albumine.

striatum, at 24 h after HI, were double-stained with anti-AIF and anti-Iba-1 as well as anti-cleaved caspase-3 and anti-Iba-1. To evaluate markers for oxidative stress, nitrotyrosine-positive cells were counted in sections from 3 h, 8 h and 24 h post-injury, using x100 objectives in hippocampal areas CA1, CA3 and dental gyrus (50x50 µm frames, approximately 25 squares per area unit). To evaluate nitrotyrosine in inflammatory cells in the hippocampus, one section from each brain was double- stained with galectin-3 and cells were then counted as described above.

Comment: ***GFAP*** is a protein in the cytoskeleton of the astrocyte (Hedtjarn et al., 2005a; Svedin et al., 2007). ***NeuN*** is a marker for neurons that visualises the cell body. ***Iso-lectin*** is a stain that binds to the endothelium of blood vessels as well as the surface of resting and activated microglial cells (Hedtjarn et al., 2002; Svedin et al., 2007). ***Iba-1*** is a marker for phagocytic cells and is used as a microglial staining (Ito et al., 1998; Kalm et al., 2009). There is no currently known staining method for separating resident microglial cells from invading macrophages. At 96 h and 7 days after neonatal HI, the inflammatory cells were too confluent and microglia accumulation was thus assessed by area measurement instead of cell count.

AIF is a marker of the caspase-independent apoptotic pathway and is translocated to the nucleus upon activation (Zhu et al., 2003).

Nitrotyrosine is a marker of oxidative stress. Its precursor peroxynitrate ($NOO^{\cdot}$) is produced as a result of free radical formation in the cell and binds to different proteins, forming nitrotyrosine. Microscopically, nitrotyrosine-positive cells were found in MAP-2-negative areas, as previously described (Zhu et al., 2004).

The 24 h time-point was used in staining for apoptosis in inflammatory cells as the cells were too confluent at 96 h and too scarce at 8 h. Both AIF and caspase-3 were used as apoptotic markers, since neonatal apoptotic mechanisms differ in male and female animals (Zhu et al., 2006).

Morphological analysis after excitotoxic injury (Paper I)

After de-paraffination, sections were incubated in 2% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity and then immersed in 10 mM citrate buffer (pH 6.0) for 15 minutes in a microwave oven. After blocking with 10% foetal calf

serum (cleaved caspase-3) or 5% normal goat serum (4-hydroxynonenal= HNE) in PBS for 90 minutes, sections were incubated with primary antibodies and appropriate secondary antibodies. After 45 min incubation with avidin-biotinylated enzyme complex (20µl/ml, Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, USA), sections were incubated with DAB, as described above.

After excitotoxic injury, the number of stained cells was counted in the neocortical layers and underlying white matter at the level of the ibotenate-induced lesions in the injured and un-injured hemispheres by two blinded observers. Four-six brains were randomly selected from each treatment group and 8–12 sections from each brain were examined. Data are given as the mean number of labelled cells in white and gray matter per brain, respectively, ±S.E.M.

Comment: In this model, cells stained for HNE, a marker for oxidative stress and lipid peroxidation, and cleaved caspase-3, a marker of apoptosis, were counted (Keller et al., 2006b). The time-point was chosen based on previous findings in which a significant microglial accumulation was described at 4h after injury in this model (Tahraoui et al., 2001).

Immunoblotting (Paper II)

After protein analysis, samples were mixed with 2% SDS and loading buffer in order to ensure 2.5 µg/µl protein per sample. Samples were then heated (70°C) for 10 min and loaded to a 4-12% Bis Tris gel (Novex, San Diego, CA) for Western blot analysis. Electrophoresis was run at 200V in MOPS buffer (nitrotyrosine, galectin-3) or MES buffer (pAkt) and proteins were transferred to Trans Blot, a supported nitrocellulose membrane (BioRad). Membranes collected for quantitative analysis were adjusted using either a collecting gel (nitrotyrosine) or four internal control samples (pAkt).

For staining, the membranes were first blocked in 30 mM Tris-HCl (pH 7.5) 100 mM NaCl, and 0.1% Tween 20 (TBS-T) containing 5% fat-free milk powder. After incubation with primary antibody (diluted in TBS-T containing 3% BSA and 0.2% NaN_3) overnight, and 1 h incubation with the corresponding secondary antibody (diluted in blocking solution),

Table 5. Antibodies used for Western blot (Paper I, II)

Antibody	Company product number	Concentration (primary)	Secondary antibody (peroxidase labelled)	Concentration (secondary)	paper
Nitrotyrosine	BioMol, SA-100, clone HM12	1:3000	anti mouse	1:3000	II
Galectin-3	Gift from Prof Anna Karlsson	1:50	anti rat	1:1000	II
pAkt	Cell signaling, #4060	1:1000	anti rabbit	1:3000	II
MMP-9	LS-C8814 LifeSpan Bioscience	1:1000	anti rabbit	1:5000	II
AIF	Santa Cruz Sc-9416	1:1000	anti goat	1:2000	II

immunoreactive bands were visualised using Super Signal Western Dura CL substrates (Pierce, Rockford, IL) and a cooled CCD camera (LAS1000; Fujifilm, Tokyo, Japan). Quantification was conducted using Image Gauge software (version 3.0; Fujifilm). Membranes were stripped for re-probing by incubation in 62.5 mM Tris-HCl (pH 6.7) 100 mM mercaptoethanol and 2% SDS at 50°C for 30 min.

The different antibodies used are listed in Table 5. Western blot for galectin-3 was used to ensure that galectin-3 KO mice did not produce any galectin-3 protein. Nitrotyrosine was used as a marker for oxidative stress and analysed in un-operated controls and at 8 h and 24 h after HI.

Comment: The selected time-points were chosen as a previous publication indicated that they were appropriate for assessing altered nitrotyrosine content (Zhu et al., 2004).

Injury evaluation and neuropathological scoring (Papers I, II)

After neonatal HI, 10-12 evenly spaced coronal sections were stained with MAP-2, labelling neurons and dendrites. Microscope images were captured by a CCD camera (Olympus DP50) and the area of MAP-2-positive staining in each hemisphere was measured (Olympus Micro Image, v4.0). The MAP-2-positive area in the injured hemisphere was subtracted from that in the uninjured hemisphere at each brain level, and the injury was expressed as percentage loss of MAP-2-positive area in the injured, compared with the uninjured, hemisphere.

The total tissue volume loss was calculated in MAP-2-stained sections at 96 h and 7 days after HI, using the Cavalieri Principle ($V=\Sigma A*P*T$, where V is the total volume, ΣA is the sum of all measured areas, P is the inverse of the section sampling fraction and T is the section thickness).

Semi-quantitative neuropathological scoring after neonatal HI was undertaken in MAP-2-stained sections by an observer blinded to the treatment of the animals. The cortical injury was graded from 0–4, where 0 was equivalent to no observable injury and 4 to confluent infarction involving most of the hemisphere. The damage in the hippocampus, striatum and thalamus was assessed both with respect to hypotrophy (shrinkage) (0–3) and observable cell injury/infarction (0–3), resulting in a neuropathological score for each brain region (0–6). The total score (0–22) was the sum of scores in all four regions (Hedtjarn et al., 2002). Since both determination of infarction area and neuropathological scoring were based on neuronal loss, white matter injury was determined separately.

After excitotoxic injury, every third section was stained with cresyl violet in order to determine lesion size, the total loss of viable brain tissue in periventricular white matter and cortical injury. Neocortical and white matter lesions were defined by the maximal length of three orthogonal axes: one horizontal and one radial axis in the coronal plane and one in the fronto-occipital axis (in a sagittal plane). Therefore, the entire brain was cut into coronal serial sections that permitted an accurate and reproducible determination of the maximal fronto-occipital diameter of the lesion (equal to the number of sections in which the lesion was present multiplied by 10 µm). The volume of the lesion was then assessed using an index of the linear measurement. Two observers, blinded to the treatment groups being analysed, independently determined the lesion size. Data are presented as mean length of the lesion in the rostro-caudal axis ±S.E.M.

Comment: Previous studies have shown a correlation between the maximal size of the lesion in the lateral–medial and fronto-occipital axes of the ibotenate-induced excitotoxic lesions (Gressens et al., 1997; Marret et al., 1995), as well as a correlation between the maximal fronto-occipital diameter of the lesion and the volume measured by stereology (Medja et al., 2006).

White matter injury evaluation (Papers I, II)

After neonatal HI, white matter injury was measured immunohistochemically by the loss of myelin basic protein (MBP)- (Papers I and II) and neurofilament (NF)- (Paper I) positive tissue in sections at the striatal and hippocampal levels, as previously described (Hedtjarn et al., 2005a), at 7 days after injury (Paper I) and at 24 h, 96 h and 7 days after injury (Paper II). Microscope images were captured (10× magnification) by a CCD camera (Olympus DP50) and the area of the subcortical MBP- and NF-positive staining in each hemisphere was measured (Olympus Micro Image, v.4.0). The injured hemisphere was compared with the uninjured hemisphere and the injury expressed as percentage MBP- or NF-positive tissue loss.

ELISA (Papers I, II, III)

Galectin-3 (Papers I, III), IL-1β (Papers I and II), IL-18 (Paper II) and MMP-9 (Papers II, III) protein content was measured in brain homogenates from uninjured and injured hemispheres at 24 h post-HI, in un-operated controls or in AF, with commercially available ELISA kits. Homogenates were centrifuged at 15,000×g at 4 °C for 2×15 min. The supernatants were collected and every sample was assayed in duplicate; analyses were performed according to manufacturer's instructions. The results were then corrected for protein content using a method for microplates, according to Whitaker and Granum (Whitaker and Granum, 1980) (paper I, II). For analysis of AF, samples were thawed and then analysed immediately without any correlation for protein content (Paper III). The ELISA kits used are presented in Table 6.

Comment: For the galectin-3 assay (Papers I and III), a human galectin-3 ELISA kit was used. Galectin-3 is a highly preserved protein, with little variation among species, and mammal lectins share considerable structural homology, which may explain the cross-reactivity (Cooper et al 1999). In Papers II and III, a mouse MMP-9 (total) ELISA kit was used. There are no ELISA kits currently available for analysis of the much smaller fraction of active MMP-9. All homogenate samples in Papers I and II were corrected for protein content to minimise the confounding effect of oedema.

Table 6. ELISA kits used in Paper I, II and III

Name	Company	nr	Remark	Paper
IL-1β/IL-1F2	R&D systems	MLB00B	Undiluted samples	**I,II**
IL-18	R&D systems	7625	Samples diluted 1:10	**II**
IGF-1	R&D systems	MG100	Samples diluted 1:5	**II**
MMP-9 (total)	R&D systems	MMPT90	Samples diluted 1:10 (II), undiluted (III)	**II, III**
Galectin-3	Bender MedSystems	BMS279/2	Human gal-3 kit, cross-reacts with mouse gal-3	**I,III**

ELISA for IGF-1 (Paper II)

IGF-1 was measured in homogenates from both uninjured and injured hemispheres at 24 h post-HI. Samples were first exposed to two freeze-and-thaw cycles to break cell membranes and thereafter diluted five times. Analysis was undertaken with a commercially available ELISA kit for mouse IGF-1. All procedures were performed according to manufacturer's instructions. All samples were assayed in duplicate and protein content was assessed as previously described and results were subsequently corrected for protein content.

Evaluation of free radical formation (Papers I, III)

In vitro evaluation of reactive oxygen species formation in microglia (Paper I)

Microglial cell cultures were obtained, as previously described (Salimi and Humpel, 2002) and 10 µM H_2DCFDA was used in each culture to visualise superoxide-anion formation. A metabolite of H_2DCFDA reacts with superoxide and forms a highly fluorescent agent 2',7'-dichlorofluorescein (DCF) on oxidation (Sohn et al., 2000). All reagents were obtained from Molecular Probes, Eugene, Oregon, USA. Fluorescence intensity correlating with ROS formation was measured in randomly selected areas in the cell culture, using a 488-nm excitation filter and a 590-nm pass filter for emission (Leica DM5000 Fluorescent microscope). Fluorescence was measured directly after adding ibotenate to the cell culture (baseline) as well as after 30 min. Data are given in percentage change to baseline fluorescence ±S.E.M. Microglial cell culture dishes were randomised to the following

groups: 1. scrambled tat + PBS; 2. scrambled tat + ibotenate (2 mM); 3. Gp91ds-tat (1 mM) + PBS; 4. Gp91ds-tat (1 mM) + ibotenate. Cells were pre-incubated with scrambled tat and gp91ds-tat for 30 min (including 10 µM of DCF) before administration of ibotenate.

Comment: H_2DCFDA is a non-fluorescent substrate that is added to the cell culture before the experiments, cleaved in the cells by endogenous esterases and oxidated to form DCF (Hempel et al., 1999).

In vitro evaluation of reactive oxygen species formation in primed neutrophils (Paper III)

AF samples from three control women, three women with no signs of CA (no MIAC, IAI or HCA) and three women with CA (MIAC and IAI) were added to the cell suspension with primed neutrophils in order to investigate the effect of AF on ROS formation in the neutrophils.

Neutrophils were isolated from buffy coats from healthy blood donors, using dextran sedimentation and Ficoll-Paque gradient centrifugation (Pharmacia, Uppsala, Sweden) (Boyum, 1968). The cells were washed and re-suspended (1x107/ml) in Krebs-Ringer phosphate buffer containing glucose (10 mM), Ca^{2+} (1 mM), and Mg^{2+} (1.5 mM) (KRG, pH 7.3). Neutrophils were incubated for one hour at room temperature to achieve mild priming, after which the cells were stored on ice until use. More substantial priming was achieved by incubation with 10 µg/ml lipopolysaccharide (LPS, *E. coli* serotype O111:B4; L-2630, Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C, as previously described (Almkvist et al., 2001).

The production of ROS by neutrophil NADPH oxidase was determined using a luminol-enhanced CL system (Dahlgren and Karlsson, 1999). Neutrophils were mixed with horseradish peroxidase (HRP; 4U, Boehringer Mannheim, Germany) and luminol (a CL substrate; 6x10-5 M, Sigma Chemical Co., St. Louis, MO). The CL activity was measured in a six-channel Biolumat LB 9505 (Berthold Co. Wildbad, Germany), using disposable 4 ml polypropylene tubes with a 0.46 ml reaction mixture containing $5x10^5$ neutrophils. The tubes were incubated in the Biolumat at 37°C, and the ROS production, detected as light

emission, was recorded continuously. After equilibration (when the cells had reached their maximal background level of ROS production), 40 µl of AF was added and the CL measurement was continued.

Evaluation of reactive oxygen species formation in vivo with electron spin resonance (Papers I, III)

To evaluate the ROS formation in vivo the ascorbyl radical intensity was measured in 0.2 ml brain homogenate (Paper I) or in 0.2 ml AF (Paper III), using the ESR signal, as previously described (Bagenholm et al., 1997). The radical signal intensity was measured with a Bruker ECS 106 EPR spectrometer. Spectrometer settings were as follows: field centre, 3478.5 G; modulation amplitude, 1.0 G; microwave power, 10mW; microwave frequency, 9.74 GHz; scan range, 10 G; scan rate, 60 G/min; time constant, 0.02 s. The sample used for ESR measurement was also used for protein measurement, as previously described, in brain homogenates. The ESR signal intensity was measured in arbitrary units and is expressed as signal intensity/mg protein, to correct for varying degrees of oedema in Paper I. In Paper III no correction for protein content was needed and the intensity is expressed in arbitrary units.

Comment: ROS and free radicals are normally short-lived molecules that quickly convert into more stable molecules/complexes. Ascorbic acid is present in the brain and biological fluids and can react with any radical produced, resulting in a semi-dehydroascorbate radical with low reactivity and a characteristic spectrum that can be measured by ESR. This method reflects the overall ROS formation but does not distinguish between extracellularly or intracellularly formed ascorbate (Bagenholm et al., 1997).

Evaluation of antioxidative capacity (Paper III)

Total AOC in AF was measured with the ABTS assay (Miller et al., 1993), using 2,2´ Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radicals produced by oxidation with 2,2´ Azo-bis (2-amidinopropane), resulting in a oxidised compound with a peak absorbance at 734 nm. When an antioxidant is added to the ABTS solution, the ABTS radicals will bleach in a dose-dependent reduction, producing a proportional decrease in

absorbance at 734 nm. AOC was calculated in our samples by relating the decrease in absorbance after addition of AF to a standard curve. The vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as a reference compound and the AOC in AF samples was expressed as mM of Trolox equivalents.

The absorbance appears in two phases; in order to separate them, an exponential regression was performed on the part of the curve beyond the 30-min value (between 30 and 60 min). This part of the curve represents the slow phase of the AOC. Exponential regression typically yields regression coefficients very close to 1 and the curve is extrapolated to time zero. This extrapolated curve was then used to correct the data in order to obtain the curve of the rapid phase. The corrected curve rapidly reaches an endpoint which is interpreted as the AOC due to low molecular-weight antioxidants (rapid AOC), whereas the "slope" of the regression curve may be used as a measure of the protein AOC in the sample (slow AOC) (Nilsson UA, personal communication regarding manuscript in progress).

Comment: All spectrophotometric assays for AOC are complicated by the fact that the absorbance change does not reach a true endpoint in complex systems such as blood plasma or AF, even after prolonged incubation. In these complex systems the time course of absorbance appears to have two phases, a rapid phase (within minutes) and a slower phase (accounts for the persisting absorbance change following incubation exceeding approximately 10 min). The slow phase is absent in simple solutions of low-molecular-weight antioxidants in which a true absorbance endpoint is reached within minutes. On the other hand, the rapid phase is largely absent in protein solutions; the slope of the remaining curve is proportional to the protein concentration in the sample.

Evaluation of apoptosis by caspase activity measurement (Paper II)

Homogenate from 24 h after HI and from un-operated controls were analysed for caspase-3, -8 and -9-like activity. Homogenate from 8 h after HI was analysed for caspase-3-like activity. Twenty µl homogenate was mixed on a microfibre plate (Microfluor, Dynatech, VA., USA) with 80 µl extraction buffer (50mM Tris (pH 7.3), 100mM NaCl, 5mM EDTA,

1mM EGTA, 1mM PMSF, 0,2%CHAPS, 1mM protease inhibitor cocktail (Sigma P8340) and incubated at room temperature for 15 min. 100µl assay buffer was added to each well. For caspase-3, 50mM Tris (pH 7.3), 100mM NaCl, 5mM EDTA, 1mM EGTA, 1mM PMSF, 10mM DTT and 10mM substrate for caspase-3 (Ac-Asp-Glu-Val-Asp-aminomethyl coumarine) was used. For caspase-8 and -9, 50mM Tris (pH 7.3), 100mM NaCl, 5mM EDTA, 1mM EGTA, 1mM PMSF, 10mM DTT and 20mM substrate for caspase-8 (Ac-Ile-Glu-Thr-Asp-AFC) or -9 (Ac-Leu-Glu-His-Asp-AFC) was used. The caspase activity was measured by Spectramax Gemini microplate fluorometer at 37°C, at a wavelength of 380-460 nm for caspase-3 and 400-505 nm for caspase-8 and caspase-9. Degradation of the substrate was observed at 2-min intervals for 2 h and V_{max} was then determined as the linear part of the curve. 10µl AMC (for caspase-3) or AFC (for caspase-8 and -9) was added to six selected wells on each plate to create a standard curve. All substrates, AMC and AFC were obtained from Enzyme Systems Products, Livermore, California, USA. Each sample was analysed in duplicate and protein concentrations were measured according to Whitaker and Granum (Whitaker and Granum, 1980), as previously described in this thesis. In the un-operated controls, values from the left and right hemispheres were pooled.

Comments: The caspase activity measurement is not entirely specific, as several caspases may cleave the same substrate. Caspase-3 activity measurement also detects caspase-7 and, to a lesser extent, other caspases, and the caspase-8 and -9 substrates are cleaved by both these caspases (Benkova et al., 2009; McStay et al., 2008; Pereira and Song, 2008). Caspase-3 is, however, the dominant caspase after neonatal HI (Blomgren and Hagberg, 2006).

Statistics

Data was tested for normal distribution using GraphPad Prism or Sigma Stat. The Student's t-test was used in cases of normal distribution when two experimental groups were compared and the paired t-test was used for comparisons between uninjured and injured hemispheres. To compare multiple groups, an ANOVA followed by Dunnet's correction for multiple comparisons was used. Data that was not normally distributed was analysed using non-parametric tests (Mann–Whitney U test, Wilcoxon sign rank test and Kruskall Wallis, followed by Dunn´s correction). Correlations were determined by the

Spearman rank test and the Fisher´s exact test was used for comparisons of proportions. All analyses were performed using GraphPad Prism or Sigma Stat for Windows Version 2.03. Results are given as mean ±S.E.M unless otherwise stated. Statistical differences were considered significant when $p<0.05$.

Summary of Results

Galectin-3, ascorbyl radicals and antioxidative capacity are altered in amniotic fluid from women with chorioamnionitis (Paper III)

Eighty-three women with threatening PTD were included and evaluated for CA. Women in PTL and with pPROM did not differ concerning any background data, except that more women with pPROM gave birth before 34 w GA. In all analyses, PTL and pPROM were pooled, because there were no differences between the PTL and pPROM group for any of the analysed markers.

Galectin-3 was present in all samples and increased in all CA types, compared with term controls, as well as in women with threatening PTD and MIAC, compared with women without MIAC. Galectin-3 levels did not predict delivery within 7 days or before 34 w GA. Galectin-3 in AF was dependent on foetal gender; mothers carrying female foetuses had higher levels than those with male foetuses. This difference was found both in mothers with signs of CA (MIAC, IAI or HCA) and in term controls.

MMP-9 was only detected in a minority of women. In this subgroup, the levels correlated positively with galectin-3 concentrations.

Ascorbyl radicals were detectable in all samples and threatening PTD was associated with decreased concentrations, regardless of whether CA was diagnosed. When AF was added to primed neutrophils, ROS production was completely extinguished by samples from women with threatening PTD with or without CA, as well as by samples from term controls, indicating that AF has strong antioxidative properties.

AOC was detectable in all samples and rapid AOC did not differ between any of the groups analysed. Slow AOC did, on the other hand, increase in women with signs of CA, compared with term controls. There were also significantly higher levels of slow AOC in women with IAI in the PTL/pPROM group, compared with women without IAI. Ascorbyl

radicals and AOC were not affected by short latency to delivery (≤ 7 days) or by preterm delivery before 34 w GA.

Nine children, all born before 34 w GA, were classified as suffering severe neonatal morbidity, with early- onset sepsis, PVL and/or CLD. Galectin-3 and ascorbyl radicals in AF were not altered in the mothers of these infants while slow AOC was increased supporting our earlier findings of strong antioxidant properties in AF also in pregnancies complicated by infection or adverse outcome.

For more detailed information, see Results section in Paper III.

Expression of NADPH oxidase subunits and galectin-3 after neonatal hypoxic-ischemic insult in mice (Papers I, II)

Increased mRNA for NADPH oxidase subunits and galectin-3 (Papers I, II)

The expression of mRNA for NADPH oxidase subunits was increased after HI. mRNA for $p22^{phox}$ was already increased at 8 h, but most subunits did not rise until 24 h ($p22^{phox}$, $p47^{phox}$, and $gp91^{phox}$) or 72 h ($p22^{phox}$, $p47^{phox}$, and $p67^{phox}$) after HI in the injured hemisphere.

The mRNA for galectin-3 was significantly increased at all time-points. At 8 h there was a 2.6-fold increase, at 24h a 5.0-fold increase and at 72 h a 6.9-fold increase after HI in the injured hemisphere (n=5 at each time-point).

Protein expression and location of galectin-3 (Papers I, II)

The protein expression of galectin-3 increased gradually after HI, reaching a peak at 96 h that persisted up to 7 days after HI (Paper II). High-magnification images showed that galectin-3-positive cells had a configuration of typically activated microglial cells (Papers I and II). No protein expression could be seen in the galectin-3-deficient mice (Paper II).

Double-labelling revealed that galectin-3 and two microglial markers, Iba-1 and iso-lectin, were co-localised and that virtually all galectin-3-positive cells were located adjacent to the injured site. Hardly any galectin-3-positive cells were found in the uninjured areas and none were found in iso-lectin-positive resting microglia or blood vessels. The galectin-3-positive cells in the uninjured areas were, however, co-localised with Iba-1 and iso-lectin. Galectin-3 did not co-localise with neuronal marker NeuN, but was occasionally seen in GFAP-positive astrocytes. Triple-labelling with MMP-9 and Iba-1 demonstrated only very few stained cells with both MMP-9 and galectin-3 staining, although both markers were co-localised with Iba-1 (Paper II).

Galectin-3-deficient mice are protected against neonatal hypoxic-ischemic brain injury but inflammatory response is altered (Paper II)

Galectin-3-deficient mice had significantly less MAP-2-positive volume loss than their WT littermates at 7 days post-HI ($p<0.02$). Sub-analysis of area loss at different brain levels revealed that the difference reached statistical significance at levels 4-7, corresponding to the striatum and part of the hippocampus (Figure 5). At 96 h after HI, no difference in tissue volume loss was found and only one hippocampal level showed significantly smaller

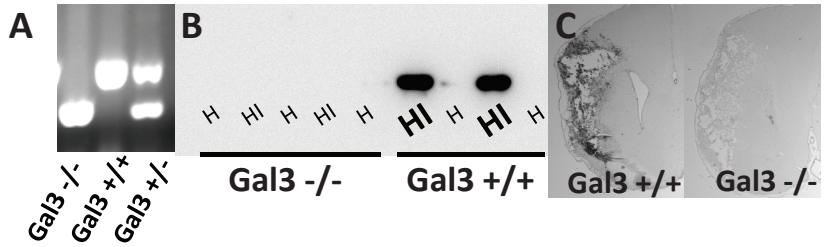

Figure 4. Expression of galectin-3 in +/+ and -/- mice at different time points after HI

All animals used in Paper II were carefully examined for expression of galectin-3 using PCR (A), western blot (B) and by immunohistochemistry (C). DNA for PCR was taken from all animals used in Paper II, both breeding and experimental animals. Western blot was performed on homogenates obtained at 24h after HI while immunohistochemistry was performed on sections from all time-points used in the study. The image in C demonstrates protein expression at 7 days after HI.

tissue area loss in the KO mice, compared with the WT mice. Neuropathological injury scores in the hippocampus ($p<0.05$) and striatum ($p<0.05$) at 7 days post-HI were significantly lower in the galectin-3 KO, mice compared to WT littermates. Expression of the white matter marker MBP was significantly reduced at 24 h after HI, but this reduction did not remain significant at later time-points.

The inflammatory response, demonstrated by both iba-1 staining and expression of MMP-9, was altered in the KO mice. The iba-1-positive area was significantly increased at the hippocampal level in the KO mice, compared with their WT littermates ($p=0.025$), while the same comparison did not reach statistical significance at the striatal level ($p=0.06$). At earlier time-points (24 h and 96 h) there were no significant differences in cell counts or area between the two groups.

Nitrotyrosine was significantly decreased in the KO mice, compared to the WT littermates, at both 8 h and 24 h after HI. Both groups also had increased levels of nitrotyrosine in the injured, compared with the uninjured, hemisphere at both 8 h and 24 h after HI.

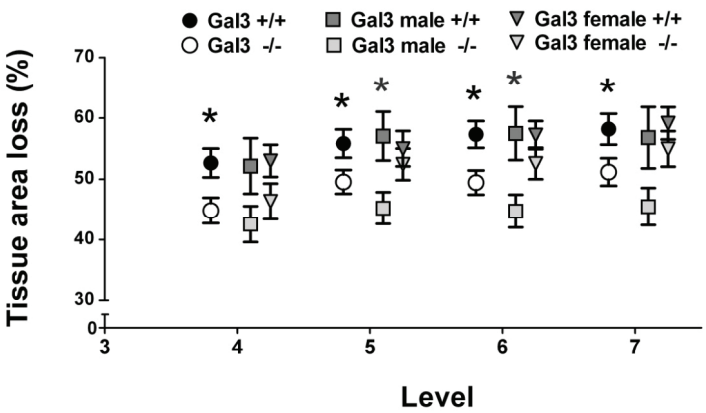

Figure 5. Subanalyses of tissue area loss in galectin-3 -/- and +/+ mice as well as for males and females

Tissue area loss (defined as percentage loss of Map-2 positive area in the injured compared with the uninjured hemisphere) was reduced in the -/- (KO) mice (n=30) compared to +/+ (WT) (n=22) littermates after neonatal HI (60 min hypoxia) at brain levels 4-7, corresponding to hippocampus and striatum. A similar pattern was seen in male (KO n=12 and WT n=9) but not female mice (KO n=18 and WT n=13). Analyses were performed with Student's t-test except for gender analyses where the Mann-Whitney U Test was used and results are given in mean (S.E.M), * $p<0.05$.

MMP-9 was increased in the injured, compared to the uninjured, hemisphere in both KO and WT mice. The KO mice also had significantly lower levels of MMP-9, compared to the WT mice ($p=0.02$), in the injured hemisphere at 24 h after HI (Figure 6). IL-1β increased at 24h after HI in the injured, compared to the uninjured, hemisphere in both KO mice and WT mice but there was no difference between the genotypes. There was no difference in IL-18 expression between the injured and uninjured hemisphere, or between KO and WT mice.

No difference was seen in the growth factor IGF-1 or the downstream marker pAkt between KO and WT mice. IGF-1 was, however, increased in the injured, compared with the uninjured, hemisphere in both groups at 24 h after HI. The levels of pAkt were significantly lower at 24 h after HI, compared with the un-operated controls, in both groups.

At 24 h after HI, caspase-3-like activity was higher in the left, injured, compared with the right, uninjured, hemisphere in both KO and WT mice. KO mice also had significantly lower levels of caspase-3-like activity than the WT mice ($p<0.05$). Caspase-8- and -9-like activity did not differ between KO and WT mice but only the WT mice had significantly higher activity of both caspases in the injured, compared with the uninjured, hemisphere. No differences were seen in caspase-3-, -8- or -9-like activity between KO and WT mice after sub-analysis for gender. Cell counts for AIF at this time-point only differed at the striatal level, at which KO mice had a lower number of cells than their WT littermates. At

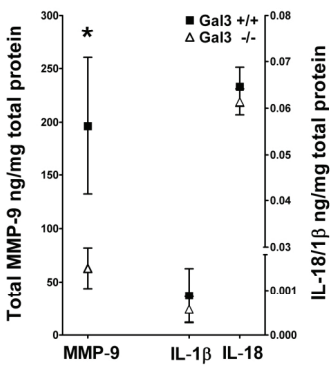

Figure 6. Protein levels of total MMP-9, IL-1β and IL-18 in the galectin-3 -/- compared with +/+ mice in the injured hemisphere at 24 h after HI

Total MMP-9 protein expression was increased at 24 h after HI (60 min hypoxia) in the galectin-3 -/- (KO) mice compared with +/+ (WT) littermates (KO n=12, WT=11). There was no difference in IL-1β (KO n=13, WT=10) or IL-18 (KO n=9, WT=9) protein expression between KO and WT mice (both groups visualised on the right y-axis). The protein levels were measured by ELISA and data was analysed with the Mann-Whitney U Test. Results are given as mean (S.E.M), * $p<0.05$.

8 h the same increase was found in caspase-3 activity in the injured and uninjured hemispheres in both KO and WT mice.

In the un-operated controls, caspase-3-like activity was lower in KO than in WT mice ($p<0.001$), while caspase-9- like activity was increased in KO, compared with WT, mice ($p<0.05$). No difference was seen between KO and WT mice when it came to caspase-8 like activity.

Staining with iba-1 and AIF at 24 h after HI revealed no double-labelled cells in KO or WT animals at the hippocampal or striatal levels and only very few cells double-labelled with iba-1 and caspase-3. Quantification was thus impossible in any of these groups.

Galectin-3 affects hypoxic-ischemic injury in male mice

Sub-analysis for gender revealed that KO male mice had suffered reduced tissue loss in areas corresponding to the deep gray matter areas, compared to WT male mice. There was, however, no difference between KO and WT mice when it came to neuropathological scores or total tissue volume loss, although there was a trend towards decreased injury in the male KO mice. Female KO mice did not differ from female WT mice concerning volume loss, tissue area loss or neuropathological scores. Regarding microglial infiltration, male, but not female, KO mice had increased Iba-1-positive area, compared with their WT littermates. No gender difference was, however, seen in inflammatory markers (IL-1β, IL-18 or MMP-9). Male KO mice also had reduced nitrotyrosine formation, compared with WT mice, at 8 h and the difference at 24 h was of borderline significance. Female KO mice did not differ from female WT mice in this respect.

NADPH oxidase does not aggravate perinatal brain injury in mice (Paper I)

Ibotenate increased ROS formation in vitro (in microglial cells) and oxidative stress in vivo (HNE staining at 4 h after the insult). The in vitro effect was completely blocked by gp91ds-tat and the in vivo HNE staining was virtually extinct in apocynin-treated animals. However, local i.c. administration of gp91ds-tat did not reduce excitotoxic lesions in either

gray or white matter, compared with control animals, at 24 h or 120 h after injury. Systemic inhibition by apocynin injection did not reduce excitotoxic brain injury in gray or white matter at 24 or 120 h in any of the studied regimens, or when the dosage was increased to 150%. In contrast, lesion size was significantly increased by apocynin treatment (see Figure 7). The number of active caspase-3-positive cells was significantly increased after ibotenate injection in both white and gray matter and a single dose of apocynin resulted in a significantly increased number of caspase-3-positive cells, although it did reduce markers of oxidative stress in gray matter 24 h after the excitotoxic insult.

No difference in infarction area or in mean tissue loss was detected 7 days after severe HI (60 min) in non-littermate WT and gp91^{phox} KO pups (both female and male). These results were independent of gender. In male littermates, infarction area, injury score and white matter markers all showed a trend towards aggravated injury in the KO mice. Severe HI (60 min) and moderate HI (50 min) did not result in any difference in ROS formation or markers for oxidative stress (nitrotyrosine) between the KO and WT mice at 8 h or 24 h after HI. Microscopically, nitrotyrosine-positive cells were found in MAP-2-negative areas, as previously described (Zhu et al., 2004). No nitrotyrosine-positive cells were found to be

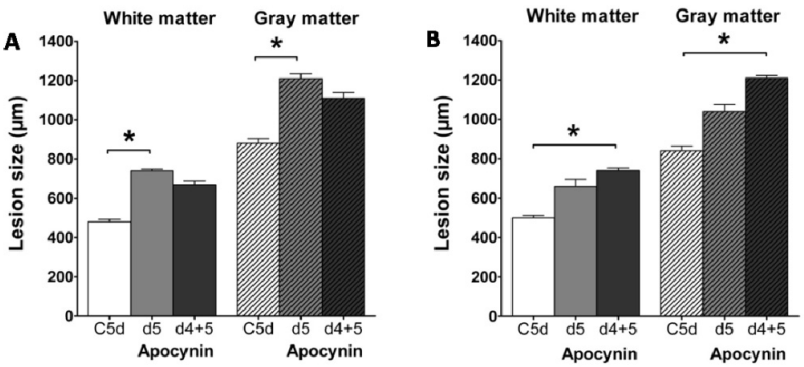

Figure 7. Brain injury was increased after apocynin treatment

Neonatal excitotoxic brain injury, induced in P5 pups, was increased in both gray and white matter after apocynin treatment. Apocynin was injected before and immediately after injury (P4+5) or only after injury (P5). Lesion size was assessed at 24 h (A) and at 120 h (B) after excitotoxic insult (apocynin n=7-10, vehicle n=6-10). Data are presented as mean length of the lesion in the rostro-caudal axis ± S.E.M in apocynin-treated versus vehicle-treated group. Analyses were performed using ANOVA followed by t-tests with Dunnet's correction * $p<0.05$.

co-localised with microglial marker galectin-3; however, very few inflammatory cells were found at these early time-points.

Evaluation of the inflammatory response revealed a difference between moderate and severe injury. After severe injury IL-1β was up-regulated in the injured hemisphere but after moderate injury only KO mice had increased levels of IL-1β in the injured hemisphere. No difference was seen when injured hemispheres from KO and WT mice were compared. Galectin-3, on the other hand, was increased after moderate HI in the injured, compared with the uninjured, hemisphere in gp91^{phox} KO mice as well as in both the injured and uninjured hemispheres in KO, compared with WT, mice (Figure 8). After severe HI, both galectin-3 and IL-1β concentrations were increased in the injured, compared with the uninjured, hemisphere in both KO and WT mice, but concentrations did not differ between the KO and WT groups. In un-operated controls IL-1β was undetectable and galectin-3 levels were the same as in the uninjured hemispheres of study mice. The number of galectin-3-positive microglial cells did, however, not differ between KO and WT mice at any level at either 8 h or 24 h after moderate HI. No significant differences were seen in galectin-3-positive area at 7 days after HI, although a trend towards increased area was observed in the KO mice. The number of neutrophils at 8 h and 24 h after the insult was so low that quantification was impossible.

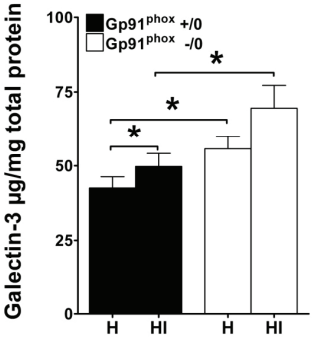

Figure 8. Galectin-3 was increased at 24 h after neonatal HI in the gp91^{phox} -/- mice

Gp91^{phox} -/- (KO) mice had increased protein levels of galectin-3 in both the injured and uninjured hemisphere in homogenates at 24 h after neonatal HI (50 min hypoxia), compared with their WT littermates. Analyses were performed using Student's t-test and paired t-test and results are presented as mean (S.E.M), * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Discussion

Clinical study

Importance of different inflammatory mediators in the amniotic fluid

In Paper III, AF was obtained from women with threatening PTD and from women undergoing planned CS. Amniocentesis can be used to diagnose intrauterine infections in threatening PTD but is not a clinical routine in PTL/pPROM as it has not been proven to improve outcome and it is difficult to justify in healthy pregnancies (Goldenberg et al., 2000; Kliegman et al., 2007). Therefore, AF from women with threatening PTD could not be compared with AF from healthy pregnancies at the same GA, which must be taken into consideration.

Galectin-3, ascorbyl radicals and both rapid and slow AOC could all be measured in all AF samples analysed, regardless of GA at sampling. While ROS and AOC (total) have previously been detected in AF (Burlingame et al., 2003), this is the first study showing the presence of galectin-3 in AF. Galectin-3 and slow AOC were increased in AF from women with both threatening PTD and intrauterine infection, while ascorbyl radicals were decreased in all women with threatening PTD regardless of infection. There were also no differences between the women in PTL and those with pPROM, despite the previous suggestion that ROS formation is of specific importance in pPROM (Woods et al). Also contrary to our original hypothesis, AF containing galectin-3, a known activator of ROS production in inflammatory cells (Karlsson et al., 1998; Liu et al., 1995; Yamaoka et al., 1995), did not induce additional ROS production in neutrophils in vitro; instead, it extinguished the background production as well, suggesting substantial AOC in AF.

Galectin-3 was increased in AF with inflammation/infection, compared with term controls, regardless of classification of inflammation/infection, and although galectin-3 has not been studied before in AF, a few studies have showed increased serum levels with increased inflammatory activation in rheumatoid arthritis and inflammatory bowel disease (Frol'ova et al., 2009; Kang et al., 2009; Ohshima et al., 2003). In the threatening PTD group, only women with MIAC had significantly increased galectin-3, compared with women without

MIAC. This suggests that MIAC induces a more pronounced inflammatory response than IAI or HCA. However, IAI correlates as strongly with PTD as MIAC (Jacobsson et al., 2003b; Shim et al., 2004) and it is generally believed that many women with IAI have undetectable microbial invasion. HCA was studied in a much smaller group of women, and it is possible that a larger group might have revealed minor differences. Furthermore, interpretation is complicated by the fact that AF was sampled at a different time-point, preceding birth and retrieval of the placenta.

In animal studies, galectin-3 is believed to play a developmental role in the placenta, with an increase of both mRNA and protein expression in mid-gestation and a decrease towards term (Iglesias et al., 1998; Lee et al., 1998). In our study, galectin-3 in AF was, however, not affected by GA at sampling and women with threatening PTD but without CA did not differ from term controls, suggesting that galectin-3 is elevated due to infection/inflammation rather than to developmental factors.

MMP-9 was detected using an ELISA with a high detection limit, which may explain the relatively low number of women with infection/inflammation that had detectable levels. In the group with detectable MMP-9, levels were positively correlated with galectin-3, suggesting that both are released in AF during inflammatory activation and that MMP-9, in spite of its potential to degrade galectin-3 (Ochieng et al., 1994), does not actually lower galectin-3 levels.

Excessive ROS formation has been suggested to lead to disruption of membranes through collagen degradation, resulting in pPROM (Buhimschi et al., 2000; Plessinger et al., 2000). Interestingly, term control mothers had higher levels of ascorbyl radicals in AF, compared with samples from women with threatening PTD, regardless of infection. The low levels in women with threatening PTD could be due to developmental factors or to the capacity of PTL and pPROM to induce changes in radical formation and AOC. An unchanged or higher level of AOC in women with threatening PTD, as we found, might also result in lower levels of detectable ascorbyl radicals, since AOC buffers free radicals. The finding that AOC was increased in women with CA indicates that a counteractive, protective response is also activated, together with the infectious/inflammatory response expected to exert increased oxidative stress. Only the slow AOC was affected, suggesting an inflammatorily regulated increase of buffering proteins in the AF. Our findings did not,

however, support any difference between PTL and pPROM when it comes to radical formation or AOC production, as has previously been suggested (Wall et al., 2002; Woods, 2001).

In the group of women with threatening PTD, none of the markers were related to short latency (within 7 days of sampling) or delivery before 34 w GA, indicating that although galectin-3, AOC and ascorbyl radicals are altered in inflammation and infection, they cannot be used as predictors of interval to delivery.

Only 9/44 infants born before 34 w GA were classified as having contracted severe neonatal morbidity associated with intrauterine infection. While galectin-3 and ascorbyl radicals were unaltered, the levels of AOC were significantly higher in mothers of infants with severe morbidity. As the number of infants with severe morbidity in this material was very low and heavily biased by lower GA, no definite conclusion can be drawn from these data. Our data suggest, however, that substantial protective mechanisms are present in AF even in pregnancies where the newborn infant may be affected by exposure to intrauterine infection.

Galectin-3 contributes to neonatal brain injury

mRNA, protein expression and location of galectin-3 after hypoxic-ischemic injury

Galectin-3 is up-regulated at both the mRNA and protein levels after neonatal HI. As early as at 8 h after HI mRNA expression and, to a lesser degree, protein expression is increased. The increase of mRNA is even more pronounced at 24 and 72 h, while protein expression reveals a similar but somewhat delayed pattern with massive staining at 96 h that persists at least up to 7 days after injury. Increased levels of galectin-3 mRNA and protein expression have previously been reported in models of brain infection (Bellac et al., 2007; Riemer et al., 2004). An early increase in protein levels has previously been described in adult mice at 24-48 h after transient ischemia (Lalancette-Hebert et al., 2007; Walther et al., 2000; Yan et al., 2009). The delayed protein expression in one of the studies could be

explained by differences in inflammatory cell infiltration between adult and neonatal animals or in the analysis methods used (Bona et al., 1999; McRae et al., 1995).

The cellular location of galectin-3 seems to be restricted to some of the more active microglial cells as well as to a number of astrocytes. This is in accordance with several studies showing that galectin-3 is mainly detected in microglia/macrophages (Lalancette-Hebert et al., 2007; Pesheva et al., 1998; Walther et al., 2000) and in astrocytes (Yan et al., 2009; Yang et al., 2006) in the brain.

Involvement of galectin-3 in injury and inflammation after hypoxic-ischemic injury

Galectin-3 has not been previously reported to contribute to injury in immature or adult brain injury models. However, antibodies against galectin-3 were not protective in a model for adult stroke, but they were administered starting as late as day two after injury (Yan et al., 2009). In Paper II we present a reduction in the overall tissue volume loss and tissue area loss and injury scores in the hippocampus and striatum. These two areas are of specific interest as they are important in the development of severe neurological deficits after birth asphyxia (Volpe, 2008).

The galectin-3-deficient mice had increased microglial accumulation at later time-points and hardly any apoptotic inflammatory cells. As galectin-3 is involved in pro-inflammatory responses, we expected that the KO mice would have attenuated inflammatory cell response with increased apoptosis, as previously described in peritonitis in mice (Colnot et al., 1998b; Hsu et al., 2000). The number of apoptotic inflammatory cells might have changed later as the cell counts were performed quite early (24 h) after HI. The choice of time-point was based on our observations in which the inflammatory cells were too confluent at later time-points, making it impossible to distinguish between single cells. As the microglial accumulation was increased in the KO mice, increased apoptosis in the inflammatory cells is unlikely. Together with the smaller infarction area observed in the KO mice, this may even indicate that the microglial density was underestimated.

The combined findings of less injury and increased microglial density raise the question of whether there is a more beneficial inflammatory reaction in the KO mice. Microglial cells

have lately been considered to be more protective in the development of adult brain injury (Imai et al., 2007; Kitamura et al., 2004), although neonatal studies are contradictory (Arvin et al., 2002; Dommergues et al., 2003). These studies suggest that microglial cells could shift between a neurotoxic and a neuroprotective phenotype. The reduction in injury in the KO mice was accompanied by increased microglial density in addition to a decreased level of MMP-9, suggesting that galectin-3 might influence the microglia phenotype. The amount of MMP-9 measured in our study was, however, the total MMP-9 and not the much smaller fraction of active MMP-9.

Galectin-3 is a substrate of MMP-9 (Ochieng et al., 1994) and MMP-9 contributes to adult (Asahi et al., 2000) as well as to neonatal brain injury (Leonardo et al., 2008; Svedin et al., 2007). In the immature brain, MMP-9 is found in microglial cells (Svedin et al., 2007). Despite the presence of both galectin-3 and MMP-9 in Iba-1-positive cells, we found hardly any co-localisation between MMP-9 and galectin-3. The aggravating role of MMP-9 after neonatal HI was accompanied by increased loss of white matter markers (Svedin et al., 2007). We found, on the other hand, only a transient reduction in MBP-positive area in the KO mice, which may reflect the severity of the insult in our study.

Galectin-3 is known to induce the formation and release of ROS from neutrophils (Karlsson et al., 1998) and monocytes in vitro (Liu et al., 1995). Although we found that NADPH oxidase did not alter ROS formation and even aggravated injury in the neonatal setting in Paper I, there was a reduction of nitrotyrosine formation in the galectin-3 KO mice. This could, however, be a reflection of the lesser degree of injury in these mice or of a more selective inhibition of NADPH oxidase in the inflammatory cells.

A recent study suggests that galectin-3-positive microglial cells are a possible source of IGF-1 production and that these cells might have protective features in the adult stroke model (Lalancette-Hebert et al., 2007). IGF-1 is also protective when given to mice after induction of neonatal HI (Brywe et al., 2005). However, we found no difference in IGF-1 or in pAkt expression between KO and WT mice, suggesting that galectin-3 does not alter the IGF-1/Akt-dependent trophic response after neonatal HI.

The effect of galectin-3 on apoptosis

Galectin-3 is known to be involved in apoptotic pathways and as it has been detected in some subsets of neurons (Pesheva et al., 2000), we investigated the overall apoptosis through both caspase activity and AIF cell counts, even if we found no galectin-3 protein expression in neurons. Galectin-3 did not seem to influence any of the apoptotic pathways investigated after HI. Neither caspase-8 nor -9 was altered at 24 h after HI. Caspase-3-like activity and the AIF cell count were only increased in the WT mice at 24 h and at one level, which may correspond with the more severe injury seen in these mice. Both the caspase-dependent and the caspase-independent pathways are important in the development of immature brain injury (Blomgren and Hagberg, 2006; Wang et al., 2001; Zhu et al., 2003; Zhu et al., 2007) and different mechanisms behind apoptosis have been shown in female vs. male pups (Zhu et al., 2006). We did, however, not find any gender differences in any of the measurements.

On the other hand, the control animals, not subjected to HI, had an altered caspase-3 and caspase-9 response. Caspase-9- like activity was increased and caspase-3-like activity was decreased in the galectin-3 KO mice, compared with the WT mice. The anti-apoptotic properties of galectin-3 are believed to be partially due to its similarities with the anti-apoptotic protein bcl-2 in cancer cells (Yang et al., 1996), that might affect cytochrome C release (Fukumori et al., 2006; Moon et al., 2001; Yu et al., 2002) as well as its capacity to alter caspase-8 activation (Fukumori et al., 2004). The levels of caspase-3 and caspase-9 were, however, many times lower in the controls, compared with caspase activation at 24 h after HI. It cannot be ruled out that even these small alterations seen under normal conditions might have affected the response after HI.

NADPH oxidase: beneficial or detrimental?

Neutrophils and inflammatory ROS formation have previously been suggested as promising targets for therapeutic interventions after brain injury in both adults (Matsuo et al., 1995; Walder et al., 1997) and neonates (Hudome et al., 1997). On the contrary, we demonstrate in Paper I that targeting NADPH oxidase does not yield any reduction in injury and might even enhance the detrimental effects of HI.

Increase in mRNA expression

As expected, the mRNA of the NADPH oxidase subunits increased as inflammatory cells invaded the blood vessels and the injured areas of the brain. In neonates, studies indicate that the accumulation of neutrophils, most prominent in post-capillary venules, peaks already after 12 h (Bona et al., 1999) and that the subsequent accumulation of inflammatory cells predominantly consists of microglial cells (Bona et al., 1999; McRae et al., 1995). The largest increase of mRNA was seen at 24 h and 72 h after HI, long after the peak of neutrophil expression in the brain, and we could only find very few neutrophils in the brain parenchyma, as supported by earlier findings after neonatal HI (Bona et al., 1999). This suggests that the NADPH oxidase increase was due to microglial activation.

The importance of microglial activation has been proven in the excitotoxic model of brain injury, in which blocking microglial infiltration has been shown to be beneficial (Dommergues et al., 2003). In vitro studies of microglial cells have also previously shown increased expression of NADPH oxidase subunits as well as NADPH oxidase-dependent ROS formation (Sankarapandi et al., 1998).

Genetic and pharmacological inhibition of NADPH oxidase

Neither pharmacological nor genetic inhibition of NADPH oxidase resulted in any protective effects in connection with HI or excitotoxic brain injury. In contrast to adult studies, pharmacological inhibition resulted in unaltered or even aggravated neonatal excitotoxic brain injury. As the pharmacological inhibition was given both locally (gp91ds-tat) and systemically (apocynin) and in different dose regimens (apocynin), these findings are dose-independent and independent of administration route. This test showed that gp91ds-tat reduced ROS formation in vitro and that apocynin reduced oxidative stress in vivo, demonstrating that the inhibitors functioned as expected.

The NADPH oxidase KO mice had up to a 40% reduction in injury in an adult stroke model (middle cerebral arterial occlusion) (Walder et al., 1997), but the same type of KO mice showed no signs of protection in our neonatal HI model, independent of gender or the severity of the insult. This indicates a difference in the role of NADPH oxidase between the adult and the neonatal ischemia-exposed brain. While the neonatal brain demonstrated

increased mRNA expression, the development of neonatal brain injury seemed to be much less affected by NADPH oxidase. These results could reflect the very limited infiltration of neutrophils in the parenchyma after neonatal injury, compared with after adult stroke during which massive infiltration occurs (Matsuo et al., 1995). In addition, neutrophil depletion in adult stroke results in reduced ROS formation as well as reduced injury (Matsuo et al., 1995), while neutrophil depletion in the immature brain is only effective if the depletion is induced long before the insult (Palmer et al., 2004). This indicates that neutrophils contribute to neonatal injury by plugging microvessels and impairing microcirculation, rather than by invading the brain parenchyma (Palmer et al., 2004). This corresponds with our results, indicating very few invading neutrophils and no difference in ROS formation. There were also no differences in oxidative stress, either in the number of nitrotyrosine-positive cells or in direct ROS measurement after HI. The nitrotyrosine-stained cells were investigated at time-points based on previous results in the neonatal HI model (Zhu et al., 2004). These time-points are relatively early if the aim is to detect microglial activation, although a substantial microglial activation is seen at 24 h after neonatal HI (McRae et al., 1995).

Increased inflammatory response in NADPH oxidase KO mice

As we demonstrated in Paper II, galectin-3 contributes to neonatal brain injury. The increase in galectin-3 found in both the uninjured and injured hemispheres after HI in the NADPH oxidase-deficient mice might thus be part of the explanation for the lack of protection against brain injury. Our findings are supported by previous results in patients suffering from a disease resulting from the same genetic defect as the mice in our study; these patients have an increased pro-inflammatory response (Warris et al., 2003). An increased amount of galectin-3 protein, together with an unaltered number of galectin-3-positive cells, indicates increased cellular production in the already present and active cells rather than increased cellular infiltration or local proliferation. The IL-1β response was also altered in the KO mice; protein levels were increased in the injured compared to the uninjured, hemisphere after even a moderate insult.

The inflammatory system is very complex and results obtained in the adult setting often differ from those in neonatal models. For instance, adult (Liu et al., 1999), but not neonatal

(Hedtjarn et al., 2005b), double-KO (for IL-1α and IL-1β) mice sustained reduced brain injury after an ischemic insult.

Gender differences in inflammation

Male mice lacking functional galectin-3 are protected from neonatal hypoxic-ischemic injury

In the galectin-3 KO mice, only males were protected after HI with altered inflammation, while the female KOs had almost identical results as the WT. Several processes have previously been shown to be gender–dependent, such as apoptotic mechanisms (Hagberg et al., 2004; Zhu et al., 2006) and the effect of neuroprotective interventions related to apoptotic processes in the immature brain (Nijboer et al., 2007; Renolleau et al., 2007). Instead of altered apoptosis, we found differences in the inflammatory response, although the groups were too small to allow more detailed evaluation. Gender differences in the inflammatory response have previously been described and both testosterone and oestrogen have anti-inflammatory properties (Fish, 2008; Ganesan et al., 2008). The levels of these hormones are not expected to differ between males and females at this early age but there are genetic differences that may be important (Johnston and Hagberg, 2007).

Gender difference in inflammatory activation in amniotic fluid from women with threatening preterm birth

In Paper III, the gender of the foetus seems to influence production of galectin-3. Galectin-3 was elevated in AF from control women with female foetuses at term as well as in cases of threatening PTD in combination with inflammation/infection. It is already known that foetal tissue can produce pro-inflammatory mediators (Menon and Fortunato, 2004; Romero et al., 2006), but further studies on the interaction between foetal gender and inflammation are needed.

Concluding remarks

Chorioamnionitis affects galectin-3, reactive oxygen species formation and antioxidative capacity

Galectin-3 has been demonstrated in AF for the first time and together with ascorbyl radicals and AOC (rapid and slow) it was consistently found in all samples. Women with threatening PTD and signs of CA had increased levels of galectin-3 and slow AOC but decreased levels of ascorbyl radicals, compared with term controls. AF also displayed a massive AOC as it extinguished background ROS formation in primed neutrophils in vitro. None of the markers were, however, affected by short latency or preterm birth (before 34 w GA) and no differences were seen between women with PTL and pPROM. There also seemed to be a gender difference and further analyses are warranted.

Galectin-3: a promising neuroprotective target?

Galectin-3 is up-regulated at both the mRNA and protein levels after neonatal HI in mice and is mainly located in microglial cells. Galectin-3 contributes to injury specifically in the deep gray matter areas of the brain and alters the inflammatory response by modulating microglial density and the levels of MMP-9. No effects were seen on apoptosis or in the expression of growth factors after HI. Under normal conditions KO mice had altered caspase activity, i.e. decreased caspase-3 and increased caspase-9 activity. Male KO mice were also more affected by galectin-3, with significant reduction of injury in deep gray matter and increased microglial infiltration. The gender differences and the mechanisms of galectin-3 effects on neonatal brain injury require further studies before therapeutic interventions targeting galectin-3 can be suggested.

NADPH oxidase should not be used as a target for brain injury treatment in neonates

Although mRNA is increased after HI and pharmacological inhibition almost abolished the increased ROS formation after excitotoxic injury, brain injury evaluation revealed that

neither pharmacological nor genetic inhibition of NADPH oxidase resulted in decreased brain injury after neonatal excitotoxic or HI insult. This might be due to increased inflammatory response, with elevated galectin-3 levels.

It is also important that data obtained in adult models cannot be applied to neonates as we have demonstrated that protective mechanisms in the adult setting may not be protective in neonatal models.

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