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# Hypoxia in human NT2-N neurons

*The role of acidosis, mitochondria and inflammation*

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Elisabeth Frøyland



Department of Pediatric Research

Faculty Division Rikshospitalet

University of Oslo

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Acidosis during reoxygenation has an early detrimental effect on neuronal metabolic activity.  
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- II Frøyland E, Pedersen ED, Kvissel AK, Almaas R, Pharo AM, Skålhegg BS, Mollnes TE, Rootwelt T.**  
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- III Frøyland E, Skjæret C, Wright M, Dalen ML, Cvancarova M, Kasi C, Rootwelt T.**  
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## Abbreviations

AP-1	activator protein 1
CC	$\beta$ -chemokines
CCR	CC-receptor
CD 35	complement receptor 1
CD 46	membrane cofactor protein
CD 55	decay accelerating factor
CD 59	protectin
CXC	$\alpha$ -chemokines
CXCR	CXC-receptor
CXCR1+2	interleukin-8 receptors
ETC	electron transport chain
HI	hypoxia-ischemia
HIF-1	hypoxia inducible factor 1
HIE	hypoxic-ischemic encephalopathy
Hx	hypoxanthine
IL	interleukin
IP-10	$\gamma$ -interferon-inducible-protein-10
LDH	lactate dehydrogenase
MCP-1	monocyte chemoattractant protein-1
MPT	mitochondrial permeability transition
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF- $\kappa$ B	nuclear factor $\kappa$ B
NMDA	<i>N</i> -methyl- <i>D</i> -aspartate
PBS	phosphate-buffered saline
RA	retinoic acid
RANTES	Regulated on activation normal T cell expressed and secreted
ROS	reactive oxygen species
RQ	relative quantification
TLDA	TaqMan Low Density Array
TNF	tumor necrosis factor



# Introduction

## Perinatal asphyxia

### Definitions

The term *hypoxia* is used when oxygen deficiency results in altered or impaired tissue energy metabolism. *Ischemia* means diminished blood supply to an organ or tissue. *Asphyxia* is defined as a condition of impaired gas exchange, either through the placenta or the lungs, leading to progressive *hypoxemia* (low partial pressure of oxygen in the blood), hypercapnia (high partial pressure of carbon dioxide in blood) and metabolic acidosis, the latter being due to insufficient tissue oxygen supply leading to an anaerobic metabolism with production of lactate. However, according to the ACOG committee (2004), the clinical term *birth asphyxia* is a nonspecific diagnosis that should not be used and a neonate who has had hypoxia proximate to delivery severe enough to result in hypoxic-ischemic encephalopathy (HIE) will show other signs of hypoxic damage, including all of the following:

- Metabolic acidosis in foetal umbilical cord arterial blood obtained at delivery (pH <7 and base deficit  $\geq$ 12 mmol/L).
- Apgar scores of 0-3 for beyond 5 minutes.
- Evidence of neonatal neurological sequelae (e.g. seizures, coma, hypotonia).
- One or more of the following organ or system injuries; cardiovascular, pulmonary, gastrointestinal, haematological, hepatic or renal.

### Epidemiology

Approximately 4 million infants out of 130 million worldwide annual births suffer from birth asphyxia. Of these, approximately 25% die and 25% develop some kind of neurological sequelae (UNICEF 2003). In the western part of the world, 2-6/1000 develop HIE (Levene et al. 1985). While there has been a dramatic reduction in under-five mortality in the past two decades, there has been relatively little change in newborn

mortality (Bhutta et al. 2005), and newborn deaths now constitute over 40% of all deaths in children aged below five years (Bryce et al. 2005). Reducing neonatal mortality may thus be critical for reaching the millennium development goal 4 target of a two-third reduction in deaths among children under five years of age. The number of disability-adjusted life years (DALYs) for birth asphyxia estimated by WHO exceed those due to all childhood conditions preventable by immunization. In addition, asphyxia at birth is the most common cause of neurodevelopmental handicap occurring in full-term infants as a result of a perinatal insult (Levene et al. 1985;Volpe 2001).

### **Risk factors, pathophysiology and mechanisms**

HIE can have different causes, as Badawi *et al* found 70% of all term infants to show HIE without preceding asphyxia (Badawi et al. 1998). Interruption of blood or oxygen supply to the foetus can be caused by maternal factors (hypotension, pre-eclampsia, uterine tetany or rupture), by placental or umbilical cord factors (abruption, infection, inflammation, umbilical cord compression or occlusion) or postnatally due to diseases in the infant (severe respiratory distress syndrome, recurrent apnoeic spells, CNS depression, anomalies or infection) (Nelson 2003;Volpe 2001). The foetal response leads initially to an increase in cerebral blood flow (Jensen et al. 1991;Lehnardt et al. 2003). However, as a result of asphyxia, impairment of cerebral auto regulation occurs, resulting in a linear relationship between blood pressure and cerebral blood flow, leaving the brain vulnerable to damage when blood pressure fluctuates (Fenichel 1983;Volpe 2001). Eventually, cardiac output is reduced, leading to systemic hypotension and a decrease in cerebral blood flow (Volpe 2001). The foetus is then at risk of developing multiple organ damage, including brain damage. The hypoxic-ischemic brain damage in full-term or post-term infants is clinically seen as HIE, while in pre-term infants the clinical findings are more unspecific due to the immaturity of the CNS (Tapia-Rombo et al. 2000).

At the cellular level, cerebral hypoxia-ischemia (HI) initiates a cascade of biochemical events starting with a shift from oxidative to anaerobic metabolism due to insufficient tissue oxygen supply (Calvert et al. 2005;Inder et al. 2000). Anaerobic metabolism results in the accumulation of reduced nicotinamide-adenine-dinucleotide (NADH) and flavin-

adenine-dinucleotide (FADH) and lactic acid, eventually causing metabolic acidosis. Anaerobic glycolysis cannot maintain the cellular energy demands, resulting in a depletion of high-energy phosphate reserves, including ATP. HI stimulates the release of excitatory amino acids, including glutamate, activating glutamate cell-surface receptors, resulting in an influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$ -ions also accumulate as a consequence of its release from mitochondria and endoplasmic reticulum. In addition, energy-requiring transcellular ion pumping fails, leading to the accumulation of intracellular  $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{Ca}^{2+}$ , and water (cytotoxic edema). Within the cytosol, free fatty acids accumulate from an increased membrane phospholipid turnover and undergo peroxidation by oxygen-free radicals that arise amongst others from mitochondrial metabolism and as by-products in the synthesis of prostaglandins, (hypo-) xanthine and uric acid. Moreover, nitric oxide, a free-radical gas, is generated via  $\text{Ca}^{2+}$ -activation of nitric oxide synthase in selected neurons and diffuses to adjacent cells that are susceptible to nitric oxide toxicity. The combined effects of cellular energy failure, acidosis, glutamate,  $\text{Ca}^{2+}$  accumulation, free-radical formation, lipid peroxidation and nitric oxide neurotoxicity serve to disrupt structural components of the cell resulting in its ultimate death.

### **Hypoxic-ischemic encephalopathy**

The hypoxic-ischemic brain damage in full-term or post-term infants is clinically seen as HIE (Tapia-Rombo et al. 2000). HIE has been classified into three grades, and the severity of HIE is a very useful predictor of long-term outcome in asphyxiated newborn infants (Fenichel 1983;Levene et al. 1985;Sarnat et al. 1976). A simplified grading could be:

- Mild/grade I: irritability (“hyperalert”), mild hypotonia and poor sucking.
- Moderate/grade II: lethargic, seizures, marked abnormalities of tone and requires tube feeding.
- Severe/grade III: comatose, prolonged seizures, severe hypotonia and failure to maintain spontaneous respiration.

Grade I is associated with good outcome, grade III with severe handicap or death in almost all infants, while grade II carries a poor prognosis in 15-30% of all infants. The

determination of the severity of an asphyxial exposure is difficult. Although evidence of a significant metabolic acidosis can establish that exposure to hypoxia has occurred, it does not necessarily reflect the severity, the duration or the nature of the asphyxial exposure (i.e., continuous or intermittent), whether the asphyxia during labour and delivery is the last of a series of episodes or the characteristics of the fetal cardiovascular response (Low 1997). The determination of the short-term outcome, as expressed by neonatal HIE and organ system complications, provides both an indicator of the severity of an asphyxial insult (classified into mild, moderate or severe, table 1), and criteria for the prediction of the long-term outcome (Low 1997).

<b>Asphyxia</b>	<b>Metabolic acidosis at delivery*</b>	<b>Encephalopathy</b>			<b>Cardiovascular, respiratory, and renal complications</b>	
		<i>Minor</i>	<i>Moderate</i>	<i>Severe</i>	<i>Minor</i>	<i>Moderate/Severe</i>
<i>Mild</i>	+	±			±	
<i>Moderate</i>	+		+			±
<i>Severe</i>	+			+		+

\*Umbilical artery base deficits  $\geq 12$  mmol/L.

Table 1: **Classification of intrapartum fetal asphyxia, in mild, moderate and severe (Low et al. 1997).**

## **Treatment**

The cascade of deleterious events leading to cell death appears to continue also after the termination of a hypoxic-ischemic insult, and is named “delayed” cell death (Fellman et al. 1997; Wyatt et al. 1989). This gives the possibility of a beneficial intervention during this “therapeutic time window”. However, as postulated by Gluckman *et al*, we should view the perinatal period as a vulnerable one, in which an asphyxial injury does not lead to a set outcome and where a variety of physiological factors impact on the developing brain (Gluckman et al. 2001).

Although many interventions have proven beneficial in animal studies, even when begun in the first hours of reperfusion, only a few appear to be relatively safe clinically and likely to be useful for protection of both neurons and oligodendroglia. Possible intervention-strategies in the prevention or amelioration of perinatal HI brain injury are:

Decrease energy depletion (hypothermia, barbiturates, mild hypercapnia), inhibit glutamate release (calcium channel blockers, magnesium, adenosine/adenosine agonists, hypothermia, free radical scavengers), block glutamate receptors [*N*-methyl-*D*-aspartate (NMDA) receptor antagonists (MK-801, magnesium, ketamine) and non-NMDA receptor antagonists (NBQX, CNQX)], inhibit leukocyte-microglial-cytokine effects (neutropenia, anticytokine antibodies, IL-1 receptor antagonists) and blockade of downstream intracellular events (hypothermia, free radical synthesis inhibitors, free radical scavengers, NOS inhibitors-scavengers and antiapoptotic agents).

Amongst these, only mild hypothermia seems to improve the outcome after perinatal asphyxia both in animal experiments (Bona et al. 1998;Thoresen et al. 1995;Tooley et al. 2003) and in clinical studies (Eicher et al. 2005;Gluckman et al. 2005;Shankaran et al. 2005). The mechanisms of this effect are still somewhat uncertain. A reduction in temperature slows down cerebral metabolic rate and reduces energy demand (Erecinska et al. 2003;Laptook et al. 1995). However, the major neuroprotective effect of post-insult hypothermia seems related to the suppression of mechanisms and cascades responsible for delayed energy failure and delayed cell death hours after the insult. Among the observed findings are a reduced inflammatory response, preventing inflammatory cell activation and reduced infiltration to the ischemic brain (Wang et al. 2002b), as well as a decreased nuclear factor  $\kappa$ B (NF- $\kappa$ B) activity (Yenari et al. 2006). Also a reduced release of NO and excitotoxins is seen (Thoresen et al. 1997) as well as a reduced generation of free oxygen radicals (Lei et al. 1997;Si et al. 1997). Furthermore, post-ischemic hypothermia attenuates damage to mitochondrial function (Canevari et al. 1999), and interferes with apoptotic pathways (Pabello et al. 2005;Van et al. 2003). The incidence of serious adverse events in clinical studies was similar in hypothermia and control groups (Eicher et al. 2005;Gluckman et al. 2005;Shankaran et al. 2005). However, a recent Cochrane review, consisting of eight randomised controlled trials comprising 638 term infants, found an increase in the need for ionotrope support of borderline significance and a significant increase in thrombocytopenia (Jacobs et al. 2007). Mild therapeutic hypothermia is now considered to be safe in an intensive care setting, and is introduced in many countries (Azzopardi et al 2008), including Norway. The current criteria for hypothermia treatment at Rikshospitalet University Hospital are gestational age  $\geq 36$

weeks, no signs of serious malformations, and at least one out of; Apgar  $\leq 5$  at 10 min, pressure ventilation necessary at 10 min or pH  $<7.00$  or BE  $< -16$  mmol/L within 60 min after birth. In addition, the infant must have moderate to serious encephalopathy with symptoms of changed consciousness, and at least one out of; musclehypotonia, pathological reflexes of tendon, eye movement or pupil, weak or missing sucking reflex or seizures.

A recent systematic review in BMJ Clinical evidence studied the effects of interventions in term or near-term newborns with perinatal asphyxia (McGuire 2006). They found limited evidence from three small, weak RCTs suggesting that mortality may be lower in infants treated with antioxidants compared with placebo (Benders et al. 2006;Gunes et al. 2007;van et al. 1998). Further, limited evidence from one small RCT suggested that a magnesium sulphate/dopamine combination may be more effective than no treatment in reducing a combined outcome of mortality, abnormal scans, and failure to feed (Ichiba et al. 2002). Small RCTs with flawed methods suggested that anticonvulsants are of no benefit in reducing mortality or improving neurodevelopmental outcomes (Evans et al. 2007;Singh et al. 2005). Recent meta-analyses show that mortality is lower in newborn infants resuscitated with room air compared to 100% oxygen (Rabi et al. 2007;Saugstad et al. 2005b;Tan et al. 2005). Finally, they were not able to conclude on whether calcium channel blockers (Levene et al. 1990), corticosteroids (Alderson et al. 2005;Altman et al. 1984), fluid restriction (Keckes et al. 2005), hyperventilation (Rosenberg 1992), inotrope support (Hunt et al. 2002), mannitol (Adhikari et al. 1990), or opiate antagonists (McGuire et al. 2004) are helpful in infants with perinatal asphyxia.

To conclude, there is currently evidence of a beneficial effect mainly of therapeutic hypothermia in term newborns with HIE (Jacobs et al. 2007).

## **Acidosis**

During HI, anaerobic metabolism due to insufficient tissue oxygen supply results in two, instead of 38, mol ATP per mol glucose. The subsequent production of lactate causes both extracellular and intracellular acidosis. The acidosis continues for a few hours after the oxygen supply to the tissue has been re-established (Saugstad et al. 2005a) and it can have multiple effects on the neuronal tissue, both protective and detrimental. During an

asphyxial episode, acidosis (accumulation of acid in the tissue) is not synonymous with acidemia (accumulation of acid in the blood). Infants with clinical acute birth asphyxia can be born without acidemia. It has been suggested that these non-acidemic, asphyxiated infants, who are not able to establish a “beneficial acidemia”, are at an increased risk of brain injury compared to infants with mild or moderate acidemia (pH 7.0-7.2) (Hermansen 2003).

The beneficial effect of a mild to moderate acidosis can be explained by decreased cerebral oxidative metabolism (Richardson 1993), a shift in the oxygen dissociation curve to the right (the Bohr effect) promoting oxygen unloading in the tissues (Hermansen 2003) and protection against ischemic injury in the developing heart (Iannettoni et al. 1992). Acidosis act also protective through inhibition of NMDA-mediated calcium currents (Giffard et al. 1990; Tombaugh et al. 1990), through reducing kainate-mediated damage (Giffard et al. 1990) and through inhibition of NO-synthase (Anderson et al. 2000).

These beneficial, protective effects of a mild to moderate acidosis can be lost in the case of a severe acidosis, which causes decreased myocardial contractility and diminished cardiac output (Downing et al. 1966), directly contributing to cerebral ischemia. When pH falls below 7.0, the infant is placed at a substantially increased risk of developing long-term cerebral damage (Andres et al. 1999). Acidosis can be detrimental through increasing lipid peroxidation (Siesjo et al. 1985), exacerbation of oxidative injury by impairing antioxidant enzyme functions and increasing intracellular free iron levels (Buonocore et al. 1998; Buonocore et al. 2003), as well as the release of iron from ferritin and increasing the pool of low molecular weight iron (Bralet et al. 1992). Acidosis can also be detrimental through aggravating AMPA-receptor-mediated cell damage (McDonald et al. 1992), inhibiting astrocyte glutamate reuptake (Swanson et al. 1995) and by activating human neutrophils, delaying neutrophil apoptosis and extending neutrophil functional lifespan (Trevani et al. 1999). In our NT2-N neurons, we have shown that the effect of acidosis on neuronal survival is dependent on the timing of acidosis, as acidosis was protective during oxygen-glucose deprivation and detrimental during reoxygenation (Almaas et al. 2003; Froyland et al. 2005).

## **Mitochondrial dysfunction and secondary energy failure**

The mitochondrial electron transport chain (ETC) completes the oxidation of fuel molecules by oxygen and the concomitant energy transduction into ATP via five complexes embedded in the inner mitochondrial membrane (Munnich et al. 2001). Mitochondrial DNA encodes 13 out of the > 80 subunits of the ETC, while nuclear DNA encodes the remaining majority (Enns 2003). Studies provide evidence that mitochondrial respiratory capacity is impaired during both global and focal ischemia (Blomgren et al. 2006; Hagberg 2004; Kuroda et al. 1996; Nakai et al. 1997; Nelson et al. 1994). This impairment is seen as a decrease in ADP-stimulated and uncoupled respiratory rates, due mainly to a decrease in the activity of complex I, II-III and V, while complex IV activity is unaffected by graded cerebral ischemia (Allen et al. 1995). Substantial recovery of mitochondrial function often occurs during the first hours of reperfusion, however it may deteriorate again as reperfusion is continued (Almeida et al. 1995; Anderson et al. 1999; Folbergrova et al. 1995; Kuroda et al. 1996; Li et al. 2000; Nakai et al. 1997; Wagner et al. 1990a; Wagner et al. 1990b). This dysfunction may reflect an inhibition of one or several of the complexes or a more general “mitochondrial inhibition”. Decreased activity of complex II and IV in hyperglycemic cats (Wagner et al. 1990a) and of complex IV in gerbils (Almeida et al. 1995) has been demonstrated during reoxygenation following transient ischemia. Since ischemia per se gives rise to a decrease in complex I, II-III and V activities (Allen et al. 1995), it has been postulated that recirculation triggers damage to complex IV, which needs a complete boundary layer of phospholipids, susceptible to peroxidation, for full activity (Almeida et al. 1995).

This “delayed” damage can be due to recirculation-enhanced production of reactive oxygen species (ROS), causing membrane damage or enzyme inactivation due to lipid peroxidation and/or protein oxidation (Floyd et al. 1992). Delayed treatment with the free radical spin trap  $\alpha$ -Phenyl-*N*-*tert*-butyl nitron (Folbergrova et al. 1995; Kuroda et al. 1996) and NXY-059 (Yoshimoto et al. 2002) prevents secondary energy failure, strongly indicating that free radicals play an important role in recirculation-induced injury (Floyd et al. 1992). Also, the immunosuppressant drugs FK506 (Nakai et al. 1997) and Cyclosporin A (Li et al. 2000) ameliorate secondary mitochondrial dysfunction through an effect on mitochondrial permeability transition (MPT). The mitochondrial ETC has



been recognized as one of the major cellular generators of ROS. When the ETC is damaged or inhibited, the production of ROS may increase (Enns 2003), therefore contributing to the ROS generated during ischemia and reperfusion (Blomgren et al. 2006). The sites of ROS production in the mitochondria are believed to be ubiquinone of complex III and the FMN group of complex I (Liu et al. 2002). Electrons leak out to molecular oxygen forming superoxide, which is quickly converted to H<sub>2</sub>O<sub>2</sub> by the mitochondrial superoxide dismutase (Liu et al. 2002). On the other hand, free radicals may also damage the mitochondria, although the precise mechanisms remain to be clarified.

Ischemia, together with e.g., free radicals, high calcium and neurotoxins may also affect the mitochondria in another way, as these factors can affect the formation of the MPT pore. MPT is a sudden permeability increase of the inner mitochondrial membrane to solutes  $\leq 1.5$  kDa (Kroemer et al. 1997;Maciel et al. 2001). MPT opening causes an uncoupling of the ETC with the collapse of the mitochondrial transmembrane potential and cessation of ATP synthesis, matrix Ca<sup>2+</sup> outflow, generation of ROS and release of apoptogenic proteins such as apoptosis-inducing factor (Kroemer et al. 1997;Li et al. 2000;Zhu et al. 2007). Both depolarization and increased permeability of the outer membrane have been suggested as a possible mechanism for cytochrome *c* release, but whether mitochondrial depolarization is a prerequisite for apoptosis, is debated. While staurosporine-induced apoptosis in rat hippocampal neurons involves both release of cytochrome *c* and activation of caspases-3, there is no requirement for mitochondrial depolarization (Krohn et al. 1999). Recently, it was suggested that different phenotypes of cell death, varying from cell to cell, is dependent on the energy available to drive the apoptotic pathways to completion (Northington et al. 2007).

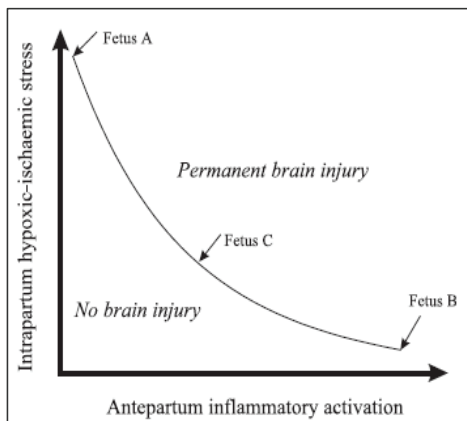
## **Inflammation**

Inflammation is a general term used to describe the many diverse processes that tissues employ in response to infection, injury, or insult of any kind. The initial phase of inflammation was described by Celsus (about 30 BC-38 AD) by four cardinal signs:

rubor (redness), tumor (swelling), dolor (pain), and calor (heat). Later, Virchow (1821-1902) added the fifth, *functio laesa* (loss of function).

Acute inflammation is initiated by the release of inflammatory mediators, comprising among others the products of coagulation, fibrinolysis, the complement system, cytokines, and oxygen derived free radicals (Gilroy et al. 2004). Within seconds after the injury, there is increased blood flow and leakage of fluid into the surrounding tissue, due to dilated capillaries and increased capillary permeability, respectively. Neutrophils, macrophages and mast cells belonging to the “first line defence” (innate immunity), infiltrate through adhesion and migration. Recognition of pathogen associated molecular patterns on micro-organisms by e.g. complement receptors, scavenger receptors and toll-like receptors results in phagocytosis and elimination of invading pathogens and/or repair of the injured tissue. In addition, T and B lymphocytes (adaptive immunity), proliferate clonally and respond highly specifically to antigenic determinants of specific pathogens. Similar responses to infection, injury, or insult of any kind are known to occur in the CNS. However, tight junctions between endothelial cells of the blood brain barrier limit access of peripheral immune cells to the brain, making it generally an immune privileged tissue. In the brain, inflammation is characterized by restricted infiltration of leukocytes, activation of resident cells (e.g., microglia, astrocytes and endothelial cells) and local production of cytokines (Allan et al. 2003).

Inflammation probably plays a significant role in perinatally acquired brain damage, both as a cause in it self and as a contributing mechanism during HI (Greenwood et al. 2005;Hagberg et al. 2002). Both animal experiments and epidemiological studies indicate that if the perinatal brain is exposed to both inflammation and HI, the damaging effect increases markedly (Figure 1) (Eklind et al. 2001;Hagberg et al. 2002;Kendall et al. 2005;Nelson et al. 1998). Inflammatory mediators may therefore represent a "final common pathway" for both infectious and HI brain damage (Shalak et al. 2002b).



**Figure 1: Putative relationship between brain injury, antepartum inflammatory activation and intrapartum HI stress. *Foetus A* can withstand substantial intrapartum stress before permanent injury is initiated. *Foetus B* will develop permanent injury as a result of antepartum infection despite minimal intrapartum stress. *Foetus C* is vulnerable to intrapartum stress and will escape permanent injury provided that the ischemic threshold is not reached. Adapted from (Kendall et al. 2005) with permission from Blackwell Publishing.**

A causal relationship between inflammation and brain damage was strengthened by animal experiments where induction of perinatal inflammation, for example by injecting lipopolysaccharide (LPS) leads to brain damage (Eklind et al. 2001). Epidemiological studies show a correlation between markers of inflammation like chorioamnionitis and cerebral palsy (Grether et al. 1997; Wu et al. 2000). On the other hand, HI can lead to inflammation, as increased level of cytokines are found in the plasma of newborn infants after hypoxia (Silveira et al. 2003). Several studies suggest that cytokines can be produced *in situ* rather than being transported into the brain from the systemic circulation (Cai et al. 2000; Kadhim et al. 2001; Saito et al. 1996). After HI, high levels of interleukin (IL)-6 and tumor necrosis factor (TNF)- $\alpha$  have been found in CSF of newborn infants (Silveira et al. 2003). Elevations after HI were also found in brain sections from rat of IL-1 $\beta$  and TNF- $\alpha$  (Bona et al. 1999), IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 (Hagberg et al. 1996), IL-18 (Hedtjarn et al. 2002) and MIP-1 $\alpha$  (Cowell et al. 2002), and of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in gerbils (Saito et al. 1996).

### **Cytokines and chemokines**

Cytokines are a heterogeneous group of naturally-produced proteins, consisting among others of chemokines, interleukins, inflammatory cytokines, interferons and

hematopoietic growth factors. Although cytokines are produced predominantly by cells of the immune system, there are a number of reports showing the presence of cytokines in the CNS (Bakhiet et al. 2002;Cai et al. 2000;Coughlan et al. 2000;Ellison et al. 2005;Kadhim et al. 2001;Mousa et al. 1999;Savman et al. 1998).

Chemokines, a type of cytokines, are small (8-14 kDa), mainly basic, molecules which are structurally and functionally related to form a family of proteins. They were first discovered in 1987, and there are now more than 50 chemokines that interact with at least 20 receptors. Based on the position of their first N-terminal cysteine residue, chemokines are subdivided into four groups; the CXC ( $\alpha$ -chemokines), the CC ( $\beta$ -chemokines), the CX3C ( $\delta$ -chemokines) and the C ( $\gamma$ -chemokines) (Figure 2). Chemokines are produced in the CNS in neurons, astrocytes, microglia, oligodendrocytes and endothelial cells (Dorf et al. 2000;Gebicke-Haerter et al. 2001).

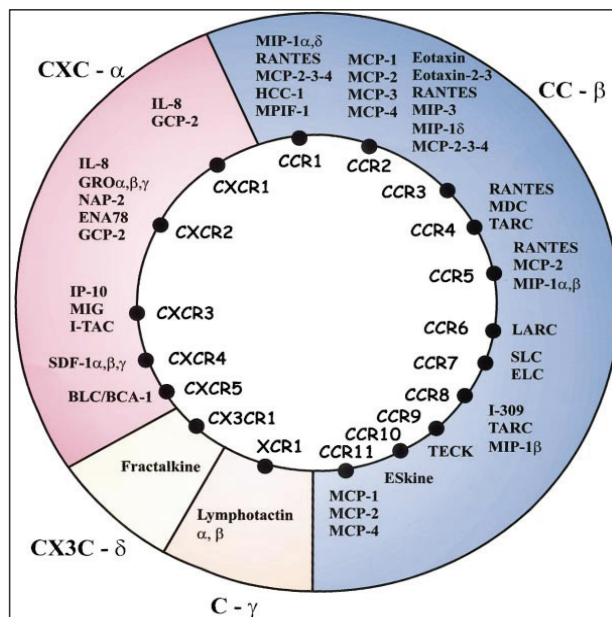


Figure 2: **Chemokine receptor classification and ligand specificity.** The four groups of chemokines; CXC, CC, CX3C and C with their members, and the four classes of chemokine receptors; CXCR, CCR, CX3CR and XCR. Adapted from (Bajetto et al. 2001) with permission from Elsevier.

## ***Receptors***

Cytokines initiate their actions by binding to specific surface receptors on target cells. These receptors exhibit high affinities for their ligand, and very small amounts of cytokines can elicit a cascade of intracellular signals that result in a particular cellular response (Wang et al. 2002a). For example, IL-1 binds to IL-1R1 (Andre et al. 2005;Friedman 2001) and activates two distinct signalling pathways, resulting in the activation of NF- $\kappa$ B and the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated protein kinase (ERK1/2) p38 and c-jun N-terminal kinase (JNK) (O'Neill et al. 1998). IL-6 acts by binding to its high affinity receptor IL-6R (gp80), soluble or cell bound, which results in signal transduction via the JAK/STAT system (Heinrich et al. 1998). IL-10 activity is mediated by its specific cell surface receptor complex, IL-10/IL-10R, resulting essentially in transcriptional activation of several hundred genes, probably through inhibition of NF- $\kappa$ B (Asadullah et al. 2003).

Chemokines exert their activity by binding to surface bound transmembrane G protein-coupled receptors (Nguyen et al. 2001), often through interactions with sulphated proteins and proteoglycans (Bajetto et al. 2002). Chemokine receptor nomenclature is based on the chemokine group to which their ligand(s) belong, since the chemokine-chemokine receptor interactions are almost always restricted within a single subclass (Bajetto et al. 2002). So far, six CXC receptors (CXCR1 to CXCR6), 11 CC receptors (CCR1 to CCR11) and a single receptor for fractalkine (CX3CR1) and lymphotactin  $\alpha/\beta$  (XCR1) have been identified (Figure 2, (Bajetto et al. 2002)).

A large number of chemokine receptors are expressed either constitutively or induced by inflammatory mediators on neurons, astrocytes, microglia, and oligodendrocytes, and they are found in humans, macaque, mouse and rat (Bajetto et al. 2002). The most characteristic response, stimulation of cell migration, appears to require functional coupling of the receptor to  $G\alpha_i$ , resulting in a rapid activation of phospholipase C, inositol-1,4,5-triphosphate generation and a transient elevation of cytosolic  $Ca^{2+}$ -concentrations (Bajetto et al. 2002). Also, regulation of other ion fluxes after chemokine receptor activation has been described (Hegg et al. 2000;Liu et al. 2000).

### ***Function***

Cytokines function as mediators of intercellular communication, as they regulate cell function during development and maintain homeostasis in the mature organism and during defence-responses to infection and inflammatory, autoimmune, traumatic, and ischemic injury (Wang et al. 2002a). Cytokines can be both proinflammatory (e.g., IL-1 and IL-6) and antiinflammatory (e.g., IL-10). Whether cytokines associated with the inflammatory response are neuroprotective or detrimental, depends on the type of cytokine, timing, conditioning and the presence of other molecules (Nelson et al. 2002a). Chemokines were initially recognized to control immune cell trafficking and recirculation by stimulating target-cell-specific directional migration of leucocytes (Baggiolini 1998), the hallmark being their ability to stimulate directed migration of cells toward a chemical gradient (Dorf et al. 2000). Stimulation of their receptors leads to diverse responses, and despite structural similarity and apparent redundancy, the chemokine receptors differ in their capacity to activate signal transduction pathways (Bajetto et al. 2002). In addition to playing a fundamental role in immune system function and homeostasis, chemokines are also shown to be important in angiogenesis, tumor and metastasis progression and in the CNS (Bajetto et al. 2001;Bajetto et al. 2002;Tran et al. 2003). However, the roles for chemokines in neuronal development and in fetal and neonatal CNS inflammation are largely unknown (Coughlan et al. 2000;Nguyen et al. 2001). As discussed previously, chemokines play a crucial role in the leukocyte accumulation into ischemic lesions, making their receptors potential targets for therapeutic intervention after ischemia (Minami et al. 2005).

### **The complement system**

The complement system, first described over a century ago, comprises >30 proteins in plasma and on cell surfaces. It is part of the host defence response, and acts in a specific manner to protect the host against invading organisms and to bridge in the interface between innate and adaptive immunity (Mollnes et al. 2002;Walport 2001). Killing of pathogens is the crucial effect of the complement system, done mainly by opsonization of pathogens, leading most often to phagocytosis. The complement system consists of three

different pathways (Figure 3); the classical (a), the lectin (b), and the alternative (c) pathway, depending on initiators and downstream activation of the different systems.

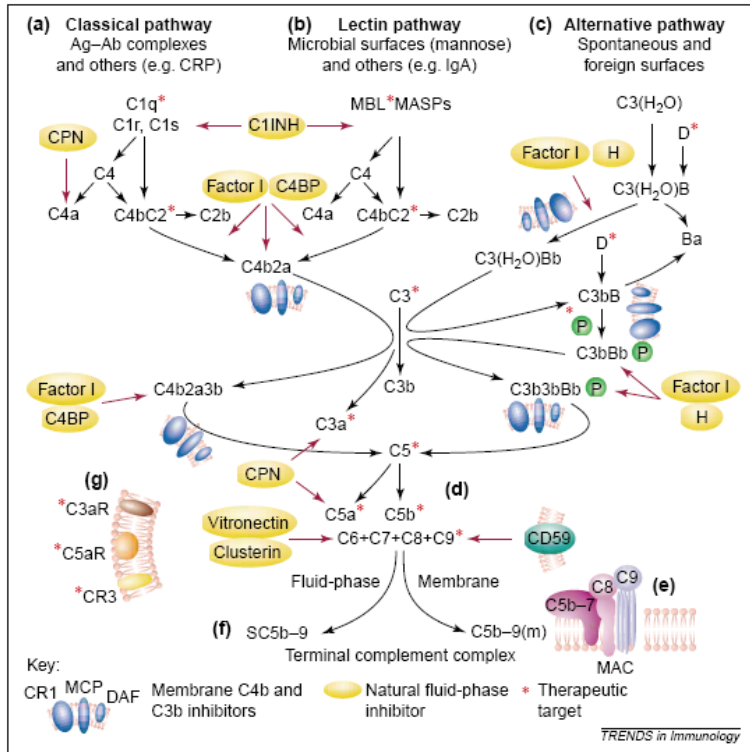


Figure 3: **The complement system, schematic presentation.** Adapted from (Mollnes et al. 2002) with permission from Elsevier.

The *classical* pathway is activated when antibodies bind antigen, and occasionally also by non-antibody mechanism (e.g. C-reactive protein). Activated C1q triggers the serine proteases C1r and C1s, the latter cleaving first C4 to C4a, which binds C2, and then C2 to C4b2a (C3 convertase). C3 convertase cleaves C3 to C3a and C3b. The *lectin* pathway is activated when microbes with terminal mannose groups are recognized by mannose-binding lectin (MBL). MBL is homologous to C1q and triggers MBL-associated serine proteases (MASPs). Downstream activation of the lectin pathway is virtually identical to classical-pathway activation. The *alternative* pathway is activated by different bacteria,

fungi, viruses and tumours which hydrolyze C3, leading to binding of factor B and formation of C3 convertase [(C3(H<sub>2</sub>O)Bb)]. The three pathways merge at the common pathway of the complement system with activation of C3 and C5, leading to the terminal pathway activation with C5a and TCC as final products.

Complement activation is regulated strictly by inhibitory proteins, and there are as many regulators as ordinary components of the cascade. Deficiency of any of the regulatory proteins is associated with substantially disturbed homeostasis and serious conditions (Mollnes et al. 2002). The membrane regulators complement receptor 1 (CR1/CD35), membrane cofactor protein (MCP/CD46) and decay accelerating factor (DAF/CD55) regulate complement activation by acting as cofactors for factor-I-mediated cleavage of C4b and C3b (CR1 and MCP), or accelerating the decay of the C3 and C5 convertases (CR1 and DAF). Protectin (CD59), also a membrane regulator, prevents the binding of C9 to the C5b-8 complex in the terminal pathway.

### **Hypoxia regulated gene-expression**

Hypoxia alters the expression of several genes through both transcriptional activation and post-transcriptional mechanisms. Genes that become activated are taking part in intracellular metabolic adaptations to hypoxia, e.g., transcription factors (e.g., hypoxia inducible factor 1 (HIF-1), activator protein 1 (AP-1) and NF- $\kappa$ B), glucose transporters and glycolytic enzymes. Also, genes encoding factors acting locally to save hypoxic tissue, e.g., vascular endothelial permeability growth factor, and factors affecting the entire organism, e.g., erythropoietin and tyrosine hydroxylase, are activated.

**HIF-1** is an oxygen-regulated transcription factor that functions as a master regulator of oxygen homeostasis (Semenza 2001). It is a heterodimer composed of HIF-1 $\alpha$  and HIF-1 $\beta$  (Semenza 2001). Whereas HIF-1 $\beta$  is constitutively expressed, HIF-1 $\alpha$  expression is induced upon decreases in oxygen tension. Upon removal of a normoxic-induced degradation, an exponential increase in expression occurs when the cells are subjected to decreasing oxygen concentrations (Jiang et al. 1996). HIF-1 $\alpha$  translocates to the nucleus where it dimerizes with HIF-1 $\beta$ ; this complex binds to hypoxia-responsive elements (HRE) in the promoters of target genes and activates their transcription (Eguchi et al.



1997;Pollenz et al. 1994). Several dozen HIF-1 regulated target genes have been identified that play essential roles in cellular and systemic responses to hypoxia, including glycolysis, erythropoiesis, angiogenesis and vascular remodeling (Semenza 2000;Willam et al. 2006), the main goal of these is to facilitate cell survival (Greijer et al. 2005).

*AP-1* is a transcription factor made up of Fos- and Jun-related protein dimers (c-Fos, FosB, Fra1, Fra2, c-Jun, junB, and junD). It transduces signals from biological mediators (e.g., cytokines, growth factors and neurotransmitters) to stress-response genes by binding to their response elements. AP-1, together with NF- $\kappa$ B, are examples of early-response genes encoding transcription factors that couple changes in ambient levels of intercellular biological signal molecules (e.g., cytokines and growth factors) or genotoxic signals (e.g., free radicals) to specific and coordinated patterns of gene expression. Activator proteins play a complex role in the modulation of apoptosis (Tong et al. 1998) and ischemia causes an increase in AP-1 proteins.

The transcription factor *NF- $\kappa$ B* is a key regulator of inflammation and cell survival. It consists of five subunits; p50, p52, p65 (RelA), RelB, and c-Rel, forming homo- and heterodimers (Zhang et al. 2005). In the inactive state, NF- $\kappa$ B dimers are sequestered in the cytoplasm by the specific inhibitors I $\kappa$ B ( $\alpha$ ,  $\beta$  and  $\gamma$ ). NF- $\kappa$ B can be activated in cells by a variety of stimuli, including glutamate, endotoxin, ROS, TNF- $\alpha$ , IL-1 $\beta$ , mitogens, viral proteins, ionizing radiation, UV light, and certain chemical agents (Blackwell et al. 1997;Cheah et al. 2005;Scholzke et al. 2003;Siebenlist et al. 1994). On stimulation, I $\kappa$ B is phosphorylated by the I $\kappa$ B kinase (IKK) complex, ubiquitinated, and degraded by the 26S proteasome. In addition, there are also pathways of NF- $\kappa$ B activation that are independent of IKK or the proteasome (Scholzke et al. 2003). When the I $\kappa$ B inhibition has been removed, the NF- $\kappa$ B dimer (p50/p50 or p50/p65) translocates to the nucleus and binds to specific promoter sites. This results in the transcription of an array of genes, including those for proinflammatory cytokines and chemotactic factors that drive and amplify the inflammatory response, e.g., cytokines (TNF- $\alpha$ , TNF- $\beta$ , IL-1 $\beta$ , IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), MIP-1, Regulated on activation normal T cell expressed and secreted (RANTES), IFN- $\beta$ , G-CSF), growth factors, adhesion molecules, immunoreceptors, and acute-phase proteins (Blackwell et al. 1997). These

early response genes are rapidly induced because NF- $\kappa$ B activation does not depend on *de novo* protein synthesis, like AP-1 transcription factors do. In cerebral ischemia, NF- $\kappa$ B is activated in neurons (Huang et al. 2001;Schneider et al. 1999;Stephenson et al. 2000;Zhang et al. 2005). Neuronal, but not astrocytic, expression of a NF- $\kappa$ B inhibitor reduced both infarct size and cell death 48 hours after permanent median cerebral artery occlusion (MCAO) in mice (Zhang et al. 2005). In p50 knockout mice, ischemic damage is significantly reduced (Schneider et al. 1999). Activation of NF- $\kappa$ B can be inhibited *in vivo* by treatment with e.g., antioxidants (Stephenson et al. 2000).

In conclusion, due to amongst others compartmentalization and co-activation of a lot of different genes, it is difficult to foresee the overall effect of stimulation or inhibition of NF- $\kappa$ B on hypoxic-ischemic cell death.

## Aims of the papers

- Paper I**
- i) Explore whether the detrimental effect of acidosis during reoxygenation is mediated through a deleterious effect on mitochondrial function.
  - ii) Study the effects of hypoxia, reoxygenation and acidosis on mitochondrial respiratory complexes.
  - iii) Study the timecourse of cell death throughout 21 h of reoxygenation after 3 h of hypoxia.
- Paper II**
- i) Study the release of cytokines in NT2-N neurons during hypoxia and reoxygenation.
  - ii) Investigate the effect of acidosis on this cytokine release.
  - iii) Study the impact of addition of IL-8, MCP-1 and antibodies to these on cell death.
- Paper III**
- i) Explore whether hypoxia and reoxygenation in NT2-N neurons change the expression of cytokine receptors and other relevant inflammatory genes.
  - ii) Investigate whether acidosis influences this expression.
- Paper IV**
- i) Investigate the presence of complement receptor 1 (CD 35), membrane cofactor protein (CD 46), decay accelerating factor (CD 55), protectin (CD 59), and complement C3a and C5a receptors (C3aR and C5aR) in human NT2-N neurons.
  - ii) Evaluate the effects of hypoxia-reoxygenation on these proteins.
  - iii) Examine the effect of hypoxia-reoxygenation on the C3d-deposition on NT2-N neurons and endothelial cells.

## Methods

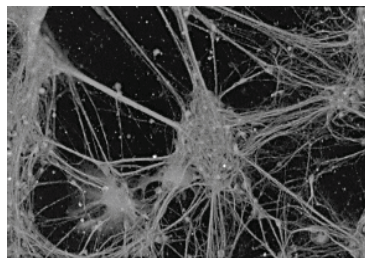
### NT2-N neurons

Upon treatment with retinoic acid (RA), NTera2/clone D1 (NT2) human teratocarcinoma cells can be induced to terminally differentiate into postmitotic neuronal cells (NT2-N) (Pleasure et al. 1992). Before culturing, the NT2 cells are undifferentiated, without markers of astrocytes (GFAP), oligodendrocytes (CNPase), or neurons (MAP-2) (Ferrari et al. 2000).

RA is given for 4-5 weeks, mimicking the *in vivo* situation in which high levels of RA, retinoic receptors and RA-binding proteins are found in the embryonic CNS (Horton et al. 1995; Rossant et al. 1991). RA initiates expression of neuronal markers resembling developing human neurons (Pleasure et al. 1992), induces cholinergic differentiation in a subpopulation of NT2 cells (Zeller et al. 1995) and increases apoptosis (Zigova et al. 2001). Already after 3 days of RA-treatment, the cells show increased expression of neurofilament proteins and by 10-14 days processes are observed (Pleasure et al. 1993). The cells are replated after the treatment with RA, and judged by the presence of neuron-specific markers, around 5 % of the cells are now neurons (Pleasure et al. 1992). 2 days later, the upper layer of neuronal cells is mechanically dislodged from a bottom layer of non-neuronal cells and plated in wells.

The cells receive then mitotic inhibitors for 4 weeks to eliminate non-neuronal cells. This treatment has no evident effect on the NT2-N cells, and in the end the culture consists of > 95% differentiated NT2-N neurons. The rest of the cells resemble glia cells.

Morphologically, the NT2-N neurons develop a rich network of processes; dendrites containing ribosomes and RNA and axons lacking ribosomes. They express neuronal markers appropriate for human neurons including functional dopaminergic (Sodja et al. 2002) and muscarinic acetylcholine (Squires et al. 1996) receptors, various voltage-



dependent calcium channels (Neelands et al. 2000) and NMDA and non-NMDA

glutamate receptor channels (Hardy et al. 1994;Squires et al. 1996;Younkin et al. 1993). While 2-week-old NT2 cells are resistant to glutamate, the 6-week-old NT2-N cells are damaged (Hardy et al. 1994;Munir et al. 1995). This gradual increase in sensitivity to glutamate, parallels the increased expression of NMDA receptors, resulting in a density of approximately 1/10 th of that found in mature rat hippocampal membranes, but resembling that found in human neurons (Munir et al. 1995). Recently, the expression of large-conductance calcium-activated potassium channels (BK<sub>Ca</sub>) was found (Chapman et al. 2007). The cells also have GABA<sub>A</sub> receptors (Matsuoka et al. 1997), with alteration of their receptor function and subunit expression by hypoxic exposure (Gao et al. 2004). Finally, the expression of the CNS specific protein  $\alpha$ -internexin, suggests likeness to “late embryonic human CNS neurons” (Younkin et al. 1993).

NT2-N neurons survive, mature and integrate when transplanted into the CNS of rodents (Croitoru-Lamoury et al. 2006;Ferrari et al. 2000;Kleppner et al. 1995;Watson et al. 2003). Positive effects have been demonstrated in animal models of stroke (Borlongan 1998), brain and spinal cord injury (Saporta et al. 2002;Watson et al. 2003), Parkinson’s disease (Baker et al. 2000) and familial amyotrophic lateral sclerosis (Garbuzova-Davis et al. 2002). Implantation of NT2-N cells into the human brain in a phase I clinical trial for stroke showed that the cells survived for 27 months and did not revert to a neoplastic state (Nelson et al. 2002b).

A recent publication showed that a co-culture of NT2-N neuronal and NT2-A astrocytic cells was more resistant to toxicity than a NT2-N mono-culture, suggesting that the use of astrocytes in an *in vitro* neurotoxicity test-system may be more relevant to human CNS structure and function than neuronal cells alone (Woehrling et al. 2007). In addition, this is an *in vitro* model with many limitations compared to the *in vivo* situation (see “Discussion”). However, although this system has weaknesses due to the fact that it is a neoplastic cell line that is not derived from the brain, it is of human origin, and has been widely and successfully used in physiological and pathophysiological studies.

## **The hypoxia model**

The hypoxia model was the same for paper I-III and for the 0.1% hypoxia in paper IV. The experiments were always started at day three or four after the last change of medium. Each well was examined microscopically at least 30 min before use. The different pH groups and treatments were randomly placed on the different 12-well plates. 100 IU/mL penicillin and 100 µg/mL streptomycin were added to Dulbecco's modified Eagle medium (DMEM) without glucose and the medium was bubbled for 5 minutes with 95% N<sub>2</sub> and 5% CO<sub>2</sub>, while heated to 37°C. Immediately before the experiment, the wells were washed twice with 0.5 mL phosphate-buffered saline (PBS), and 0.5 mL of deoxygenated medium was added to each well.

The cells were then placed in a preheated, humid anaerobic chamber. Vacuum was applied to the chamber for 25 s (20-25 inches of Hg) and the chamber was filled with 95% N<sub>2</sub> and 5% CO<sub>2</sub>. This procedure was performed four times. Inside the chamber, hydrogen was generated with a Gaspak Plus envelope containing a palladium catalyst, to remove trace amounts of oxygen. After the last gas exchange, the pressure was confirmed using a water lock, and the chamber was placed in an incubator at 37°C for 3 h. We have previously shown that these procedures reduce oxygen concentration to 0.1-0.2% after the fourth gas exchange, it further decreases to <0.1% within 30-60 min and remains at this level thereafter (Rootwelt et al. 1998). We reconfirmed this recently.

In paper I-III, some experiments were stopped immediately after 3 h of hypoxia, named 3+0 h. In the remaining experiments, and in paper IV, the medium was removed and new medium containing glucose to a final concentration of 5.5 mM/L was added. The cells were then returned to the normoxic incubator for 1, 4, 9, or 21 h in paper I, for 3, 6, 12 or 21 h in paper II and for 21 h in paper III and IV. The experiments were ended with aspiration of the supernatants and lysis of the cells. The control groups received exactly the same treatment as the hypoxia groups, except that the initial DMEM contained glucose before bubbling for 5 min with 21% oxygen and 5% CO<sub>2</sub>, and that the chamber was flushed with 21% oxygen and 5% CO<sub>2</sub> after each vacuum. In paper I-III these control experiments were terminated at 3+0 h or 3+21 h.

## pH

In our hypoxia-reoxygenation model (Almaas et al. 2003), we are able to separate the effects of a treatment given during hypoxia only, from the effects of a treatment given during reoxygenation only or during both periods. In paper I, II and III we studied the effect that lowering of the pH during either hypoxia and/or reoxygenation had on different measurements, using four different experimental pH groups (Table 2). In the first paper, HCl was added to adjust pH to 5.9 in the acidotic experiments (Paper I). However, we were not fully content with the stability of the pH, as it could vary with especially medium batch and bubbling with 95% N<sub>2</sub> or 21% oxygen and 5% CO<sub>2</sub>. In paper II and III, we therefore added 10 mM of the buffer PIPES (Piperazine-N,N'-bis[2-ethanesulfonic acid]) from a 0.8 mM stock and then adjusted pH to 6.1-6.3 in the acidotic experiments and to pH 7.4-7.6 in the neutral experiments, by titration with HCl.

<b>NN</b> = Neutral medium during hypoxia and Neutral medium during reoxygenation
<b>NA</b> = Neutral medium during hypoxia and Acidotic medium during reoxygenation
<b>AN</b> = Acidotic medium during hypoxia and Neutral medium during reoxygenation
<b>AA</b> = Acidotic medium during hypoxia and Acidotic medium during reoxygenation

Table 2: **Four experimental pH groups**, *NN*, *NA*, *AN* and *AA*, with neutral (*N*) or acidotic (*A*) medium during hypoxia and/or reoxygenation.

## Biological markers, analyses and imaging techniques

### MTT

To assess cell viability we used an MTT assay, a frequently used method which has been shown to correlate well with direct estimates (double staining) of neuronal cell survival in NT2-N neurons (Itoh et al. 1998). The soluble yellow tetrazolium dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide is intracellularly reduced to an insoluble purple product. The formed crystals are dissolved with DMSO and measured spectrophotometrically. The reduction of MTT has been shown to occur both inside the

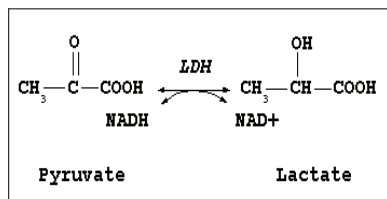
mitochondria (at coenzyme Q and cytochrome *c* in the ETC) and outside the mitochondria (in endosomes and lysosomes) (Liu et al. 1997).

### **Hypoxanthine**

Hypoxanthine (Hx) is a breakdown product from ATP. Hx-accumulation is therefore an indicator of cellular energy failure (Saugstad 1975;Saugstad 1988). Hx has been shown to react abruptly to changes in oxygenation status (Almaas et al. 1997). There is a high correlation between ATP depletion and delayed cell death after free radical damage (Aito et al. 1999). We found a similar high correlation between Hx and lactate dehydrogenase (LDH) release after combined hypoxia and glucose deprivation (paper I and (Almaas et al. 2000)). Hx is elevated prior to LDH-release, suggesting that cell membrane disruption is not a prerequisite for Hx-accumulation (Rootwelt et al. 1998).

### **Lactate dehydrogenase**

LDH is a stable cytoplasmic enzyme present in all cells. It catalyses the interconversion of pyruvate and lactate, with concomitant interconversion of NADH and NAD<sup>+</sup>:



Damage of plasma membrane causes rapid release of LDH. We measured LDH in the supernatant and lysate with a colorimetric cytotoxicity detection kit. In the first step, NAD<sup>+</sup> is reduced to NADH/H<sup>+</sup> by the LDH-catalyzed conversion of lactate to pyruvate. In the second step, the catalyst (diaphorase) transfers H/H<sup>+</sup> from NADH/H<sup>+</sup> to the tetrazolium salt INT which is reduced to formazan. The amount of colour formed in the assay, measured in an ELISA reader at 492 nm with blanks read at 660 nm and subtracted, is proportional to the amount of LDH. LDH release in percentage of total LDH was calculated as supernatant/(supernatant + lysate). We found that acidosis



markedly affected the stability of LDH in the supernatant. Therefore, LDH was only measured in the two groups that were neutral during reoxygenation (the NN and AN groups). In the current model, LDH release is very well correlated to Hx-measurements (paper I) and to the number of dead cells using a viability staining method of hypoxic and control cultures (Rootwelt et al. 1998).

### **Mitochondrial respiratory complexes IV and II + III**

We measured the activity of the respiratory complexes IV and II + III to study the effects of hypoxia and acidosis and to explore whether the detrimental effect of acidosis during reoxygenation was mediated through a deleterious effect on mitochondrial function. Cells from three wells were scraped off, and the pellets were frozen at  $-70^{\circ}\text{C}$ . The cells were later resuspended in ice-cold STE buffer and disrupted with a tight-fitting Teflon-glass homogenizer. Enzyme assays were performed at  $30^{\circ}\text{C}$  in a final volume of 0.2 mL, using a SPECTRAMax PLUS<sup>384</sup> Micro plate Spectrophotometer. COX (complex IV) activity was determined by monitoring the initial rate of ferrocytochrome *c* (200  $\mu\text{M}$ ) oxidation at the wavelength pair 510-535 nm (Wiedemann et al. 2000) in a medium containing 50 mM potassium phosphate (pH 7.0) and 0.02 % n-dodecyl- $\beta$ -D-maltoside. Succinate-cytochrome *c* reductase (complex II + III) was measured by following the reduction of ferricytochrome *c* at 550 nm with 580 nm as reference wavelength (Birch-Machin et al. 2001). Enzyme activities were calculated as nanomoles cytochrome *c* oxidized (complex IV) or reduced (complex II + III) per minute per milligram of cell protein. We also calculated the ratio of complex IV/(II + III) to reduce the effect of well to well differences, and to demonstrate the different effect of hypoxia-reoxygenation on these complexes (Munnich et al. 2001).

### **Cytometric bead array**

To elucidate the contribution of locally produced cytokines during HI in humans, we used cytometric bead array to measure the cytokine-release to the supernatant during hypoxia and reoxygenation. The assay principle is briefly as follows: Mixed particles dyed with different fluorescence intensities, separately detected by flow cytometry, are coated with

specific antibodies directed against different analytes. Thus, the different capture beads will bind their specific analytes, which then is detected again by free antibodies against the same chemokine and conjugated to phycoerythrin. 25 $\mu$ L of bead solution was added to 25 $\mu$ L of sample and then mixed with 25 $\mu$ L of a mixture of the corresponding detection antibodies. The samples were incubated for 3 h, washed once and resuspended in 50 $\mu$ L washing buffer before analysis in a FACS Calibur flow cytometer. All data were analyzed using cytometric bead array software. Standard curves were generated for each analyte using the mixed analyte standard provided with the kit. The concentration of each of the analytes was determined by interpolation from the corresponding standard curve.

### **Indirect immunofluorescence**

Cells were grown on cover slips with a density of 1 million cells per well in 12-well plates. They underwent 3 h of hypoxia and glucose deprivation followed by 15 h of reoxygenation. The cells were then washed with PBS and fixed/permeabilized with ice cold methanol, followed by washing for 2 x 5 min with PBS containing 0.01% TWEEN 20 ® (PBST). The cover slips were blocked with PBST containing 2% bovine serum albumin (PBST-BSA) for 15 min. Primary antibodies were diluted in PBST-BSA; mouse IgG anti-Neurofilament 200 (NF-200, 1:100) together with rabbit anti-human IL-8 (1:50) or rabbit anti-human MCP-1 (1:50) and incubated for 30 min. Cells were then washed 3 x 5 min with PBST-BSA before incubation with fluorochrome-conjugated secondary antibodies for 30 min; anti-rabbit-FITC and anti-mouse-TRITC diluted 1:250 in PBST-BSA. Finally, cells were washed 4 x 5 min in PBST-BSA with 0.5 $\mu$ g/mL Hoechst added in the last wash for DNA staining. The cover slips were mounted in Citifluor AF1 mounting medium and examined using an Olympus BX61 microscope equipped with an F-VIEW digital camera. Photos were acquired on the computer using analySIS®. As negative controls the primary antibody was omitted.

### **IL-8 and MCP-1 addition**

Recombinant human IL-8 and MCP-1 were reconstituted in deionized water to a final concentration of 0.2 mg/ml and 0.16 mg/ml, respectively, as well as antigen-affinity

purified polyclonal antibodies, anti-human IL-8 and anti-human MCP-1, both to a final concentration of 0.2 mg/ml. All solutions were stored in small aliquots at -20°C. Recombinant human IL-8 (40, 400 and 4000 ng/mL), recombinant human MCP-1 (400 and 4000 ng/mL), polyclonal antibody anti-human IL-8 (0.048 µg/mL) or polyclonal antibody anti-human MCP-1 (4 µg/mL) were added to both the acidotic and the neutral medium before hypoxia. The medium was not changed at the beginning of the reoxygenation period lasting 21 h, however, 1% glucose was added to the medium at reoxygenation. For these studies we therefore only had two experimental groups, the NN and AA group.

### **TaqMan Low Density Array**

Total RNA was extracted from  $3 \times 10^6$  cells, quantified using a ND-1000 spectrophotometer and the RNA quality was analyzed with a Bioanalyzer. Total RNA (18–75ng/µl) was reverse transcribed into cDNA employing a cDNA kit (High capacity cDNA Archive kit) in a Gene Amp PCR system 9700 thermal cycler. Real-time-PCR was performed using pre-coated gene expression TaqMan Low Density Array (TLDA) cards. Each card has eight channels (for eight different RNA samples) and each channel services 48 wells (in paper III for 24 genes in duplicate). Each card had been previously coated and contained the primers for 24 different genes in duplicate including two housekeeping genes (peptidylprolyl isomerase A (PPIA) and 18S rRNA). The cDNA was diluted and an appropriate amount was mixed with Nuclease-free water and TaqMan 2X universal mastermix. The cDNA concentration corresponded to 70-300 ng RNA per channel. After applying the cDNA samples, the cards were centrifuged, sealed and loaded onto the 7900HT instrument. The 7900HT sequence detection system allows real-time-PCR quantification of nucleic acids by employing the comparative  $C_t$  method of relative quantification (RQ). The arithmetic formula for RQ is  $2^{-\Delta\Delta C_t}$ , where  $\Delta\Delta C_t$  is the normalized signal level in a sample relative to the normalized signal level in the corresponding calibrator sample (Livak et al. 2001).

### **Real-time-PCR**

Total RNA from  $3 \times 10^6$  cells was prepared, the RNA concentration was determined using a Nanodrop spectrophotometer and reverse transcribed into cDNA before real-time-PCR was performed. 10 ng cDNA for all target genes (CXCR1, CXCR2, CCR1 and CCR2) and housekeeping gene (PPIA) was used, employing a SYBR Green PCR Master mix in an ABI PRISM® 7000 Sequence Detection System. Sample concentrations were analyzed employing the same Comparative  $C_t$  method of RQ (see above).

### **Cellular enzyme-linked immunosorbent assay**

A cellular enzyme-linked immunosorbent assay (CELISA) technique was used to investigate the presence of CD 35, CD 46, CD 55, CD 59, and C3aR and C5aR on the NT2-N neurons. Fixed NT2-N cells were incubated for 60 min with un-conjugated antibodies before a secondary antibody (sheep anti-mouse IgG horseradish peroxidase linked F(ab')<sub>2</sub>) was applied. O-phenylenediamine in citrate buffer was used as a peroxidase substrate solution, and the colour reaction was read with a MRX ELISA Reader at 490 nm. The cells were subsequently stained with 0.05% crystal violet and a nuclear stain eluted in 33% acetic acid before the reading of OD at 540 nm. OD-ratio was corrected to the number of cells present in each well. Concentration-matched isotype antibodies served as negative controls.

## Summary of results

### Paper I

#### **Acidosis during reoxygenation has an early detrimental effect on neuronal metabolic activity.**

We recently showed that acidosis is protective during hypoxia and detrimental during reoxygenation. To study the effects of hypoxia and acidosis on metabolic activity and mitochondrial respiratory complexes, human NT2-N neurons were exposed to 3 h of hypoxia and glucose deprivation and reoxygenated for 0, 1, 4, 9, or 21 h. Acidotic reoxygenation had an early detrimental effect on metabolic activity, shown by a significantly higher MTT reduction and a decreased Hx accumulation in cells reoxygenated with neutral compared to acidotic medium, both after acidotic hypoxia (MTT:  $p=0.006$ , Hx:  $p<0.001$ ) and after neutral hypoxia (MTT:  $p=0.005$ , Hx:  $p=0.001$ ). During hypoxia and reoxygenation, there was an earlier reduction in the activity of complex IV compared to the complexes II+III, and the ratio between these complexes fell during the first hour of reoxygenation. The reduction in complex IV activity was alleviated with acidotic hypoxia. Acidosis during reoxygenation, however, had no effect on the activity of either complex IV or complexes II+III. We concluded that acidosis during hypoxia increases neuronal survival and preserves complex IV activity, while acidosis during reoxygenation has an early detrimental effect on metabolic activity, but this is not mediated through an effect on the mitochondrial complexes IV or II+III.

### Paper II

#### **Effect of acidosis on IL-8 and MCP-1 during hypoxia and reoxygenation in human NT2-N neurons.**

NT2-N neurons were exposed to 3 h of hypoxia and glucose deprivation and reoxygenated for 21 h to study the contribution of locally produced cytokines, the effect on cell death of addition of IL-8 and MCP-1 or antibodies to these, as well as the impact of acidosis. Compared to controls, both neutral hypoxia (IL-8:  $p=0.023$ ) and neutral reoxygenation (IL-8:  $p=0.042$ , MCP-1:  $p=0.007$ ) led to increased IL-8 and MCP-1 production. Acidotic compared to neutral hypoxia led to lower IL-8 ( $p=0.002$ ) and MCP-1 ( $p=0.008$ ) levels. Acidosis during reoxygenation, however, significantly increased IL-8 release, while MCP-1 release was diminished. The cells also secreted RANTES and  $\gamma$ -interferon-inducible-protein-10 (IP-10), but not any of the 8 other cytokines tested. We found no effect on cell death, measured by MTT assay, of addition of IL-8, MCP-1, or antibodies to these. We concluded that human NT2-N neurons release IL-8 and MCP-1 during 21 h of reoxygenation after 3 h of hypoxia. Acidosis during reoxygenation led to a differential effect on IL-8 and MCP-1, with increased IL-8 and decreased MCP-1. IL-8 and MCP-1 had however, no detectable effect on cell death in our model.

### Paper III

#### **Inflammatory receptors and pathways in human NT2-N neurons during hypoxia and reoxygenation. Impact of acidosis.**

We studied RNA expression of cytokine receptors and members of inflammatory pathways in human NT2-N neurons during 3 h of hypoxia and glucose deprivation followed by 21 h of reoxygenation, as well as the impact of acidosis. Right after acidotic hypoxia, RNA of IL-10RA and CXCR4 were significantly increased relative to the acidotic control, while the anti-apoptotic proteins Bcl-2 and Bcl-xL were significantly decreased. After 21 h of neutral reoxygenation after neutral hypoxia, there was a significant increase in RNA of CXCR1 (RQ=4.1,  $p<0.05$ ), CXCR2 (3.6,  $p<0.05$ ), CCR2 (3.8,  $p<0.05$ ), Hsp70 (2.4,  $p<0.05$ ), HIF-1 $\alpha$  (1.5,  $p<0.001$ ), TRAF6 (1.3,  $p<0.05$ ) and TNFR1 (1.6,  $p<0.05$ ). After 21 h of acidotic reoxygenation after acidotic hypoxia, we found a significant increase in RNA of IL-1R1, IL-10RA, CXCR4 and Hsp70 compared to control, and a significant decrease in FAS and TRAF6 expression. There was a significant increase in Bax expression and a significant decrease in Bcl-2 and Bcl-xL expression in three out of four pH groups at 21 h of reoxygenation. Acidotic, relative to neutral, hypoxia and reoxygenation also influenced the expression of various genes. We conclude that inflammatory receptors and pathways are differentially activated during hypoxia and reoxygenation in NT2-N neurons, and that this activation is pH dependent.

### Paper IV

#### **Expression of complement regulators and receptors on human NT2-N neurons - effect of hypoxia and reoxygenation.**

By using cellular enzyme-linked immunosorbent assay and immunofluorescence microscopy, NT2-N neurons were shown to express significant amounts of proteins of CD59 (Clone H19/Clone BRIC229:  $p<0.001/p<0.001$ ), CD46 ( $p<0.001$ ), CD55 ( $p<0.01$ ) and C3aR ( $p<0.001$ ). CD35 and C5aR were not significantly expressed. There was no effect of 3 h of 1% hypoxia and 21 h of reoxygenation with 50% AB-serum on any of the regulators or receptors. However, CD55 ( $p=0.02$ ) was down-regulated after 0.1% hypoxia and subsequent reoxygenation with AB-serum. No difference in C3d-deposition was observed during hypoxia-reoxygenation in neither neurons nor endothelial cells.

## **Discussion**

The overall aim for the present work was to understand more about the mechanisms behind hypoxic-ischemic brain damage, so that treatment for this important condition hopefully can be improved. We focused our research on acidosis, mitochondrial function and inflammation, all important but not fully understood phenomena in hypoxic-ischemic brain damage and all possibly amendable to interventional treatment.

When we started with this project, the mechanisms behind the beneficial, protective effects during HI of mild to moderate acidosis, which was lost with severe acidosis, were discussed. Mitochondria were considered both targets and mediators of the damage during HI. Inflammation was thought to play a significant role in perinatally acquired brain damage, both as a direct cause and as a contributing mechanism during HI. Moreover, complement activation had been shown to cause tissue damage during HI through the release of biologically potent activation products and by impaired function of regulatory proteins.

### **Acidosis**

Acidosis has diverse and opposing effects on neuronal function and survival. The NT2-N hypoxia-reoxygenation model enables us to separate the effects of a treatment given during hypoxia only, from the effects obtained with a treatment given during reoxygenation only or during both periods. In paper I-III we studied the impact of lowering the pH during either hypoxia and/or reoxygenation on cell death. We confirmed and extended the previous finding in our lab (Almaas et al. 2003), that the effect of acidosis during HI is dependent on timing, as acidosis was protective during hypoxia and detrimental during reoxygenation.

We found that the detrimental effect of acidotic reoxygenation on metabolic activity was evident already after 1 h of reoxygenation and that it was maintained during 21 h of reoxygenation, as MTT reduction (in percentage of normoxic controls) was significantly enhanced in cells reoxygenated with neutral compared to acidotic medium, both after acidotic and neutral hypoxia. We considered the possibility that acidosis negatively

influenced the MTT assay itself, but the cells were washed with buffer before the assay and the assay itself was performed in a buffered solution. Furthermore, MTT reduction was unaffected by acidosis in the normoxic control experiments. We therefore concluded that this was a real effect of acidotic reoxygenation.

This finding of an early detrimental effect of acidosis during reoxygenation makes it important to ask the question whether early buffering could be advantageous in the treatment after a hypoxic-ischemic insult. A MR spectroscopy study in piglets showed that low cerebral pH is a continuous problem throughout 48 h of resuscitation (Penrice et al. 1997). Hyperchloremic acidosis induced by HCl infusion significantly reduces MAP in normotensive, septic rats (Kellum et al. 2004). However, although a low pH has a negative inotropic effect in isolated hearts, the whole-body response in patients is less clearly detrimental (Forsythe et al. 2000). It has been postulated that the important correlate of successful resuscitation is an improved coronary perfusion and not pH (Kette et al. 1990). Also, it is difficult to accurately assess the pH at the cellular level through the clinician's window on acid-base status, the arterial blood pH (Forsythe et al. 2000). A Cochrane review on sodium bicarbonate treatment concluded that there is insufficient evidence from randomised controlled trials to determine whether infusion of sodium bicarbonate reduces mortality and morbidity in infants receiving resuscitation at birth (Beveridge et al. 2006). Furthermore, an intravenous infusion of sodium bicarbonate can elevate blood pH but may fail to reliably augment the intracellular pH, indeed intracellular pH falls in most animal models (Forsythe et al. 2000). This is due to a rise in CO<sub>2</sub> content when bicarbonate is given, because only CO<sub>2</sub> enters into the cells, and not bicarbonate. Also, no controlled study has shown an improved hemodynamic state with sodium bicarbonate infusion (Forsythe et al. 2000), and it adversely affects cardiac resuscitation in pigs (Kette et al. 1991). The use of sodium bicarbonate infusion during resuscitation of newborn is now not recommended (Niermeyer et al. 2000). One could therefore speculate whether other buffers with intracellular effects may be more suitable during resuscitation. The weak base, Trimethylamine, prevented functional damage in isolated rat dorsal spinal roots after hyperglycaemic hypoxia (Euchner-Wamser et al. 1994). Tris-hydroxymethyl-aminomethane (THAM) combined with mild hypothermia was cerebroprotective in rats with acute subdural hematomas (Okauchi et al. 2002). To



conclude, although acidosis has a detrimental effect during reoxygenation, there are at present no buffers which have been proven effective clinically, and more work has to be done.

We further studied the mechanisms behind the differential effect of acidosis. The protective effect of acidosis during hypoxia and the detrimental effect during reoxygenation were confirmed by MTT measurements, hypoxanthine accumulation and LDH release. NMDA receptor stimulation is central to hypoxic cell death in our model (Almaas et al. 2000;Almaas et al. 2002;Almaas et al. 2003;Rootwelt et al. 1998) and blockade of NMDA receptors confers no added protection compared to that given by acidosis alone (Almaas et al. 2003). We can therefore conclude that the protective effect of acidosis during hypoxia to a large extent is mediated through inhibition of NMDA receptor-mediated damage (Almaas et al. 2003;Froyland et al. 2005;Giffard et al. 1990;Tombaugh et al. 1990).

A possible aggravating effect of acidosis during reoxygenation, as postulated in paper I, could be mediated through a deleterious effect on mitochondrial function. We found an early detrimental effect on MTT reduction and a progressive accumulation of hypoxanthine with acidotic reoxygenation, suggesting impaired mitochondrial function due to acidosis. However, MTT reduction is not specific for mitochondrial function, as some reduction may occur in living cells outside the mitochondria (Berridge et al. 1993;Liu et al. 1997). Furthermore, the pH-level during reoxygenation did not significantly affect the activities of complex II+III or IV, and changes in complex II+III and IV could therefore not explain the detrimental effects of acidosis during reoxygenation. Another possible aggravating effect of acidosis during reoxygenation could be an increase in free radical formation (Almaas et al. 2003;Siesjo et al. 1985), but we have not found oxygen radicals to be an important mediator of cell death in our model (Almaas et al. 2002). To conclude, acidosis during reoxygenation has a detrimental effect on metabolic activity, which may be mediated through a deleterious effect on mitochondrial function, although we were not able to demonstrate a specific mitochondrial effect of acidosis.

### **Mitochondrial function**

Complex IV activity was affected earlier by hypoxia and reoxygenation than complex II+III. This was confirmed by calculating the ratio of complex IV to complex II+III (Munnich et al. 2001). This is in accordance with the findings of an early onset and a progressive reduction in the partly mitochondrial DNA encoded complex IV mRNA and activity, in contrast to a more stable nuclear DNA encoded complex II (Abe et al. 1995). Other animal studies have also found an early decrease of complex IV activity (Dimlich et al. 1990; Nelson et al. 1994). This early onset and progressive disturbance of complex IV activity may contribute to the failure of energy production, i.e. the secondary energy failure observed in animal and clinical studies (Iwata et al. 2007; Lorek et al. 1994; Northington et al. 2001b).

We have previously shown that a combination of energy failure and glutamate stimulation is toxic to NT2-N neurons (Rootwelt et al. 1998). After 21 h of reoxygenation, we detected a reduction in complex IV comparable to the level that previously has been shown to impair ATP production, as complex I, III, and IV activities in brain mitochondria had to decrease by approximately 25%, 80%, and 70%, respectively, before major changes in oxygen consumption and ATP synthesis occurred (Davey et al. 1998). However, the reduction at previous time points was more modest and by itself not enough to explain the observed changes in energy status. It is therefore still unclear whether the decline in mitochondrial function after HI in this and other studies contributes to the final neuronal damage, or if it is a secondary event in the developmental injury (Wagner et al. 1990a). Irrespectively, the early influence on complex IV is of importance since treatment started in this “therapeutic time window” after HI might reduce the damage.

### **Inflammation**

There is growing evidence that inflammation plays a significant role in perinatally acquired brain damage, both as a direct cause and as a contributing mechanism during HI (Hagberg et al., 2002), in both preterm and term infants (Ferriero, 2004). Furthermore, both animal experiments and epidemiological studies indicate that if the perinatal brain is

exposed to both inflammation and HI, the damaging effect increases markedly (Eklind et al., 2001; Kendall and Peebles, 2005; Nelson and Grether, 1998).

Although cytokines are predominantly produced by cells of the immune system, it has been postulated that brain cells can contribute to inflammation in response to brain injury by producing chemokines *in situ* (Cai et al. 2000;Kadhim et al. 2001), and, since they carry receptors, also respond to inflammatory cytokines produced either by cells in the CNS or transported into the CNS from the periphery. NT2-N neurons have previously been shown to constitutively release MCP-1 (Coughlan et al., 2000). We confirmed and advanced this finding in paper II. During hypoxia and reoxygenation production of the chemokines MCP-1, IL-8 and RANTES was markedly increased, while IP-10 was unaffected by hypoxia. These findings are in accordance with a recent study of human preterm infants which showed IL-8 to be significantly higher in CSF than in plasma, both in infants with and without white matter injury, showing that locally produced IL-8 contributes significantly to CNS IL-8 levels (Ellison et al. 2005). Also, increased production of IL-8 has been demonstrated after hypoxia in granulosa-lutein cells from human ovarian follicles (Yoshino et al. 2003) and in human monocyte derived macrophages (Hirani et al. 2001). However, neither Lu *et al* nor Coughlan *et al* detected release of IL-8 from unstimulated NT2-N neurons (Coughlan et al. 2000;Lu et al. 2005), using different sensitivity and cell density, respectively. However, by using *in situ* hybridization and immunohistochemistry, Bakhiet *et al* found low levels of cytokines in unstimulated human foetal astrocytes and neurons, and that in the presence of LPS and other inflammatory mediators, both astrocytes and neurons were major sources of IL-8, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES (Bakhiet et al. 2002). Studies with asphyxia or HI have showed an increase in IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-18 and MIP-1 $\alpha$  in rat brain tissue (Bona et al. 1999;Cowell et al. 2002;Hagberg et al. 1996;Hedtjarn et al. 2002) and high levels of cytokines in newborn infants; e.g. IL-6 and TNF-  $\alpha$  in CSF (Silveira et al. 2003) and IL-6, IL-8 and RANTES in plasma (Shalak et al. 2002a;Silveira et al. 2003).

We further found that IL-8 and MCP-1 were affected by acidosis, while RANTES and IP-10 were unaffected. Acidosis during hypoxia was associated with lower IL-8 and MCP-1 levels and reduced damage. In contrast, acidosis during reoxygenation was associated with higher IL-8 levels but lower MCP-1 levels and increased damage (paper

II). This differential effect of acidosis during reoxygenation may be related to the fact that these two inflammatory mediators belong to two different families of chemokines, and therefore are regulated differentially. Acidosis during normoxia has previously been shown to stimulate IL-8 production in astrocytes, but not in neurons (Watanabe et al. 1998).

We found, however, no significant effect on cell death, measured by MTT assay, of addition of recombinant IL-8, MCP-1, anti-human IL-8 or anti-human MCP-1. This is in contrast with Matsumoto *et al.*, who found significant reduction of brain edema and infarct size after treatment with a neutralizing anti-IL-8 antibody in an *in vivo* rabbit model of transient focal ischemia (Matsumoto et al. 1997). Previous studies have given conflicting results concerning the effect of MCP-1 on neuronal death, as both protection from NMDA-induced apoptosis (Eugenin et al. 2003) and increased apoptosis (Kalehua et al. 2004) have been reported. An explanation for the lack of effects in our model could be that cytokine production reflects a response to, rather than active involvement in, neurodegeneration (Allan et al. 2001). Also, when administered separately, cytokines tend not to evoke cell death directly (Allan et al. 2001). Finally, cell-culture systems usually comprise immature cells. They are an isolated system and lack e.g. glia, endothelia, white cells and synaptic connections that might be essential for cytokine action (Allan et al. 2001).

By binding to different receptors on target cells, cytokines act differently and cause, exacerbate, mediate and/or inhibit cellular injury and repair (Allan et al. 2001). We therefore extended our study to include cytokine receptors, other inflammatory receptors and their downstream inflammatory genes in paper III. NT2-N neurons have previously been shown to constitutively express receptors for IL-8 (CXCR1 and CXCR2, Coughlan et al., 2000; Lu et al., 2005), MCP-1 (CCR2, Coughlan et al., 2000) and RANTES (CCR1, Valerio et al., 2004). We confirmed and extended these findings. During hypoxia and reoxygenation we further presented data on the expression of the receptors CXCR1, CXCR2, CXCR4, CCR1, CCR2, IL-1R1, IL-6R, IL-10RA and TNFR1, in addition to the expression of Bax, Bcl-2, Bcl-xL, FAS, Hsp70, HIF-1alpha, GLUT1 and TRAF6 (paper III). Hypoxia, reoxygenation and acidosis were found to influence the expression of these receptors and their downstream inflammatory genes. We first studied receptors for

interleukins and found an increased expression of IL-1R1, IL-6R and IL-10RA after 21 h of reoxygenation. This is in accordance with previous findings in rats where increased mRNA for IL-1R was found after HI (Wang et al. 1997). Up-regulation of IL-6 (the ligand; not the receptor) is seen in the hippocampus after kainic acid-induced seizures and in the striatum between 4 and 24 h after a mechanical lesion (Lehtimäki et al. 2003; Thier et al. 1999). Furthermore, a reduction in the number of apoptotic cells is observed after addition of recombinant IL-10 to cerebellar granule cells together with or 1 h after glutamate (Bachis et al. 2001). These results suggest that IL-1R1 and IL-6R are important for the progressive neurodegeneration that ensues subsequent to a mild HI injury, while in our study, the increase in IL-10RA during acidotic hypoxia-reoxygenation may be a protective mechanism.

We did not detect expression of the cytokine receptors CXCR1, CXCR2, CCR1 or CCR2 in the NT2-N neurons with the TLDA assay. However, these receptors were detected with a manual quantitative real-time PCR, that was carefully set up in regard to the choice of primer sequences, primer concentrations and amount of template used. Under neutral conditions we found hypoxia to increase the amount of these receptors, but we did not detect any significant pH-effect. CXCR4 increased in accordance with previous studies showing transient up-regulation of CXCR4 in response to HI in cerebral mice cortical neurons (Stumm et al. 2002) and in rat penumbra-like areas (Felszeghy et al. 2004).

After 21 h of reoxygenation we found a significant increase in the proapoptotic Bax and a significant decrease in the antiapoptotic Bcl-2 and Bcl-xL in the three pH groups (NA, NN and AA) which in this model show most damage (Almaas et al. 2003; Froyland et al. 2005). This gave a similar increase in the Bax-to-Bcl-2 ratio as a previous study on focal cerebral ischemia in mice (Matsushita et al. 1998), following MCA occlusion in rats (Gillardon et al. 1996) as well as in cell cultures of the neocortex subjected to prolonged hypoxia (Tamatani et al. 1998). Apoptotic mechanisms have, however, previously been shown to play a minor role after oxygen and glucose deprivation in our model (Almaas et al. 2003), and the implications of these findings for the cell death observed in our model are therefore uncertain.

We also detected the expression of FAS, TNFR1 and TRAF6. In accordance with previous findings of increased Fas protein expression in neonatal rat brain after HI (Felderhoff-Mueser et al. 2000;Northington et al. 2001a), we found increased expression of FAS after 21 h of reoxygenation. However, we do not have any good explanations for the significant increase only seen in the most protected (AN) group, while models of HI both in rats and neonatal mice showed neuroprotection of a lack of functional FAS (Martin-Villalba et al. 1999;Rosenbaum et al. 2000). Studies on TNF- $\alpha$  have detected both a protective role, as neuronal damage after focal ischemia-reperfusion is significantly increased in mice lacking TNFR1 receptors compared with wt-mice (Gary et al. 1998), as well as a detrimental role, as TNF binding protein was neuroprotective in focal cerebral ischemia in mice (Nawashiro et al. 1997). Previous studies on TNFR1 and TNFR2 have shown induction of both of these receptors after ischemia in e.g. rat and human neurons (Botchkina et al. 1997;Dziewulska et al. 2003), while we only detected TNFR1, being significantly increased in the most damaged groups after 21 h of reoxygenation. We detected a marked increase in RNA for Hsp70 during acidotic hypoxia. This may contribute to the protective effect of acidosis during hypoxia seen in our model, as over-expression of Hsp70 has been shown to provide neuroprotection against cerebral ischemia both *in vitro* (Kelly et al. 2001;Lee et al. 2001) and *in vivo* (Matsumori et al. 2005;Rajdev et al. 2000;Tsuchiya et al. 2003). HIF-1 $\alpha$  was significantly increased compared to controls following 21 h of reoxygenation in the two most damaged groups, while acidosis during either hypoxia or reoxygenation led to a decrease in HIF-1 $\alpha$  after 21 h of reoxygenation. Previously, inconsistent effects of acidosis on HIF-1 $\alpha$  protein and its target genes have been found, suggesting yet undefined pH-sensitive factors to be involved in the regulation of these genes (Willam et al. 2006).

Interpretation of cell death and inflammation in an *in vitro* model is difficult due to an isolated system of usually immature neuronal cells that lacks e.g. glia, endothelial cells, white cells and extensive synaptic connections that may be essential for cytokine action (Allan et al. 2001). Also, changes in cytokines of both protective and damaging character occur, which together affect the final cell death. We found no effect on cell death of addition of IL-8, MCP-1 or antibodies to these in our NT2-N model (paper II). Explanations for the lack of effects in our model as mentioned above could be that

cytokine production reflects a response to, rather than active involvement in, neurodegeneration, and that when administered separately, cytokines tend not to evoke cell death directly (Allan et al. 2001). This illuminates the fact that testing in a pure neuronal system only will not give all relevant information.

However, we have shown that neurons clearly play a part in the production of cytokines and that they express the relevant receptors. In addition, we have described the influence of hypoxia, reoxygenation and acidosis on cytokine production and receptor expression. Other studies have shown that this interplay can affect the extent of cell death. However, the system is quite complex with many different players, and includes additional important actors *in vivo*. Still, these pathways represent important pharmacological targets.

In paper IV, we demonstrated for the first time the expression of complement regulatory proteins and receptors on human NT2-N neurons, as these cells constitutively expressed C3aR, CD46, CD 55 and extensive amounts of CD59, but no C5aR or CD35 (Pedersen et al. 2007). CD59, which protects the cell against sublytic stimulation and cell lysis by inhibiting the insertion of membrane-attack complexes, has previously been found in a human neuroblastoma cell line (Cole et al. 2006), fetal human neuronal cells (Singhrao et al. 2000) and cerebellar Purkinje cells (Storstein et al. 2004). Loss of CD59 probably increases the susceptibility of the cells to complement-mediated injury, as found in damaged tissue after myocardial infarction (Vakeva et al. 2000). It is reasonable to suggest a role for CD59 in protecting the brain during a stroke, since CD59 deficiency leads to enhanced tissue damage in experimental autoimmune encephalomyelitis (Mead et al. 2004) and ischemia-reperfusion injury in mice (Turnberg et al. 2004).

Although we found no effect of hypoxia and reoxygenation on CD59 expression (our *in vitro* data do therefore not support the importance of loss of CD59 for ischemia-reperfusion), the loss of this regulator may have serious consequences for cell survival *in vivo*. We found a slight down-regulation of CD55 after severe hypoxia with 0.1% oxygen, which may render the cells more susceptible to complement damage, and thereby, contribute to brain tissue damage during a stroke. Although we found the NT2-N cells to express substantial amounts of C3aR (anaphylatoxin), which previously has also been shown to play a considerable role in the development of experimental autoimmune

encephalomyelitis (Boos et al. 2004), the role of C3aR receptors in the case of a stroke, still remains unclear and needs to be further investigated.

To conclude, the mechanisms for HI damage and its effectors are very complex. We have studied some of them. Many of these can be important, but we have not found any single factor that can be targeted in clinical practice at present. Research on hypothermia shows that the mechanisms and cascades responsible for delayed energy failure and cell death develops in the first hours after the insult. Hypothermia is now used as a treatment for asphyxiated full-term newborns, hopefully prolonging the “therapeutic time window” and improving prognosis. Most likely, we need a combination of different treatments. These should be tested both *in vivo* and in isolated cell-culture systems.



## Conclusions

- Paper I**
- i) The detrimental effect of acidosis during reoxygenation was not mediated by an effect on complex II+III or IV.
  - ii) Complex IV activity was affected earlier by hypoxia and reoxygenation than complex II+III. Whereas acidosis during hypoxia preserved complex IV activity, pH during reoxygenation did not significantly affect the activities of complexes II+III and IV.
  - iii) Acidotic reoxygenation was found to have an early detrimental effect on metabolic activity, measured as MTT reduction, and to cause an increased accumulation of Hx.
- Paper II**
- i) During 21 h of reoxygenation after 3 h of hypoxia, human NT2-N neurons released IL-8, MCP-1, RANTES and IP-10, but none of the 8 other cytokines tested.
  - ii) Acidosis led to a differential effect on IL-8 and MCP-1, with increased IL-8 and decreased MCP-1, both during reoxygenation and in normoxic controls.
  - iii) Addition of IL-8, MCP-1 or antibodies to these during hypoxia and reoxygenation had no apparent effect on cell death. This lack of effect was seen both with acidosis and with neutral medium.
- Paper III**
- i) Cytokine receptors and other relevant inflammatory genes were differentially activated during hypoxia and reoxygenation.
  - ii) This differential activation was pH dependent.
- Paper IV**
- i) Human NT2-N neurons constitutively expressed C3aR, CD 46, CD 55 and, in particular, CD 59.
  - ii) Severe hypoxia-reoxygenation down-regulated CD 55 expression, while the other proteins were not affected.
  - iii) Hypoxia-reoxygenation did not influence C3d-deposition.

## Erratum

Figures 1 and 3 appearing in article I, “Acidosis during reoxygenation has an early detrimental effect on neuronal metabolic activity”, were not typeset correctly. The correct figures appear below.

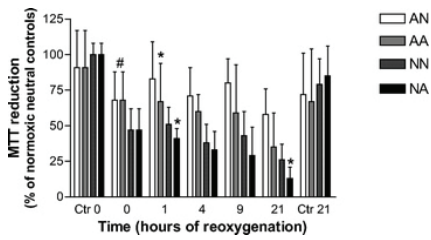


Fig. 1

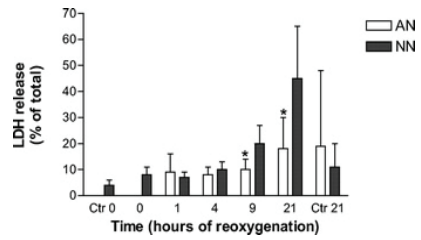


Fig. 3

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