Neuroprotective strategies for the neonatal brain after hypoxia-ischemia: pharmacological interventions and hypothermia

Xiyong Fan

Author: Xiyong Fan
Cover image: Zhenyu Yun & Zhipu Fan, Hohhot, China
Print: Wöhrmann Print Service, Zutphen
ISBN: 978-90-393-5348-6

Neuroprotective strategies for the neonatal brain after hypoxia-ischemia: pharmacological interventions and hypothermia

Neuroprotectie van het neonatale Brein na hypoxie en ischemie: farmacologische interventies en hypothermie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr.J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 1 juli 2010 des middags te 4.15 uur

door

Xiyong Fan

geboren op *19 november 1980*
te *Hohhot, China*

Promotoren: Prof.dr. F.van Bel
Prof.dr. C.J.Heijnen

Co-promotor: Dr. F.Groenendaal

Contents

Chapter 1

Introduction: Pharmacological neuroprotection after perinatal hypoxic-ischemic brain injury

Xiyong Fan[1,2,3], Annemieke Kavelaars[2], Cobi J. Heijnen[2], Floris Groenendaal[1], Frank van Bel[1]*

[1]Department of Neonatology and [2]Laboratory for Neuroimmunology and Developmental Origins of Disease (NIDOD), University Medical Center, Utrecht, the Netherlands.

[3]Department of Paediatrics, Peking University First hospital, Beijing, China

***Correspondence**

Frank van Bel, MD, PhD

Department of Neonatology

University Medical Center Utrecht

KE 04.123.1

PO Box 85090

3508 AB Utrecht, the Netherlands

telephone: +31-88-75 5 4545

fax: +31-88-75 5 5320

Email: f.vanbel@umcutrecht.nl

Current Neuropharmacology, 2010, accepted

Abstract

Perinatal hypoxia-ischemia (HI) is an important cause of neonatal brain injury. Recent progress in the search for neuroprotective compounds has provided us with several promising drugs to reduce perinatal HI-induced brain injury. In the early stage (first 6 hours after birth) therapies are concentrated on prevention of the production of reactive oxygen species or free radicals (xanthine-oxidase-, nitric oxide synthase-, and prostaglandin inhibition), anti-inflammatory effects (erythropoietin, melatonin, Xenon) and anti-apoptotic interventions (nuclear factor kappa B- and c-jun N-terminal kinase inhibition); in a later stage stimulation of neurotrophic properties in the neonatal brain (erythropoietin, growth factors) can be targeted to promote neuronal and oligodendrocyte regeneration. Combination of pharmacological means of treatment with moderate hypothermia, which is accepted now as a meaningful therapy, is probably the next step in clinical treatment to fight post-asphyxial brain damage. Further studies should be directed at a more rational use of therapies by determining the optimal time and dose to inhibit the different potentially destructive molecular pathways or to enhance endogenous repair while at the same time avoiding adverse effects of the drugs used.

Key words

brain, hypoxia, ischemia, neonate, neuroprotection, pharmacology

Introduction

Despite important progress in obstetric and neonatal care during the last decades, perinatal hypoxia-ischemia (HI) or birth asphyxia is still one of the most important causes of neonatal brain injury and the associated adverse developmental outcome (Glass et al., 2007; Gonzalez et al., 2008). Currently, treatment options for post-asphyxial reperfusion/reoxygenation injury of the brain are largely supportive with prompt recognition and treatment of seizures, normalization of blood glucose levels, optimizing blood gases and blood pressure (Scafidi et al., 2008). Recently, experimental and clinical studies have shown that moderate hypothermia with a reduction of body temperature to 33-to-34 ^{0}C, if started within the first 6 h after birth, provides moderate neuroprotection (Azzopardi et al., 2009; Hoeger et al., 2006; Ohmura et al., 2005; Robertson et al., 2008). This therapy has now been accepted in clinical practice as a strategy to fight post-asphyxial brain damage (Hoehn et al., 2008). It is conceivable that outcome can further improve when hypothermia is combined with other (pharmacological) means of neuroprotection or with stimulation of regenerative processes in the neonatal brain. Damage due to perinatal HI in the premature infant is different from that in the full term newborn. In the premature infant, HI brain injury mainly involves pre-myelinating oligodendrocytes and white matter injury. However, this review will not discuss post-HI damage to the brain of preterm infant.

We will first summarize and briefly discuss the potentially destructive molecular pathways set in motion upon reperfusion and reoxygenation of the brain of the asphyxiated full-term neonate (Badr Zahr et al., 2006). Subsequently, we will describe which pharmacological interventions can be considered to inhibit these destructive pathways including the most optimal postnatal moment to do so.

Molecular mechanisms of reperfusion injury to the brain after perinatal HI

Post-asphyxial reperfusion injury to the brain is caused by a cascade of molecular reactions as summarized below in order of their occurrence:

1) During HI, ATP formation is impaired due to hypoxia and immediately after reperfusion and reoxygenation reactive oxygen species are produced. Together, these processes cause an excessive influx of calcium stimulated by opening of voltage-regulated calcium channels which lead to release of neurotransmitters such as glutamate. Glutamate can activate receptor-regulated (N-methyl-D-aspartate [NMDA])

calcium channels thereby further increasing calcium. This triggers enhanced production of free radicals and activation of lipases, proteases, and endonucleases. As a consequence, releasing of free fatty acids, especially arachidonic acid, will activate cyclooxygenase and will catalyse the formation of prostaglandins which will liberate among other things superoxide free radicals. In addition, formation of oxygen free radicals is also enhanced via metabolizing hypoxanthine formed during the actual period of HI to uric acid. Collectively, these processes will lead to a surge of the superoxide free radical, which plays a central role in further production of free radicals and other toxic compounds, see also below (Girard et al., 2008; Hilton et al., 2006; Kumar et al., 2008).

2) During the actual period of HI, protein-bound iron within the neuronal and microglial cells will be liberated from its binding proteins, especially because of the low intracellular pH.Non-protein bound iron (NPBI) or free iron will accumulate, which is an important pro-radical. Upon reperfusion and reoxygenation, NPBI will react with superoxide-derived hydrogen peroxide (see above) to form the very toxic hydroxyl free radical, the so-called Fenton reaction (Ferriero, 2001). NPBI has been related to excessive brain damage in the immediate post-HI period in both experimental (Papazisis et al., 2008) and clinical (Ogihara et al., 2003) studies.

3) Nitric oxide (NO) is a free radical produced by nitric oxide synthases (NOS) that are expressed in the brain in neurons, astrocytes, and endothelial cells and can be induced in microglia. There are three isoforms of NOS (van den Tweel et al., 2005a): endothelial or eNOS, neuronal or nNOS, which are constitutionally expressed forms of NOS that produce NO in moderate amounts in response to increased intracellular calcium. An inducible form or iNOS can also be expressed in the brain. eNOS is thought to have a neuroprotective function via enhancing perfusion of the brain if necessary (Cimino et al., 2005). Upon reperfusion and reoxygenation after perinatal HI, however, eNOS and nNOS are activated leading to excess production of NO. Moreover, in a later stage (see below), continuous production of NO occurs via upregulation of iNOS in infiltrating neutrophils, macrophages and microglia. NO then can react with superoxide (see above) to form the toxic peroxynitrite which can contribute to further damage to brain tissue (Chang et al., 2009; Fabian et al., 2008; Suzuki et al., 2002; Yang et al., 2005).

4) Three-to-12 hours after reperfusion and reoxygenation an inflammatory response, probably induced by excessive free radical production and high levels of extracellular glutamate, will be activated and pro- and anti-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8 and IL-10 are produced (Leonardo et al., 2009). The activation of two

transcription factors, *i.e.* Nuclear Factor kappa B (NFκB) and c-Jun N-terminal kinase (JNK) play a central role in the post-HI inflammatory process. In addition, these transcription factors can regulate expression of pro- and anti-apoptotic proteins and thus can contribute to damage (see below) or neuroprotection (Nijboer et al., 2008a; Nijboer et al., 2008b; Waetzig et al., 2005).

5) As indicated above, apoptotic activity contributes to brain damage in the neonate and is an important pathway in the process of delayed neuronal death (Northington et al., 2001). Apoptosis is an energy-dependent process and ATP is required for apoptosome formation and subsequent caspase activation (Blomgren et al., 2007). Caspases and especially the executioner caspase-3 are activated in this process and bring about most of the changes that characterize apoptotic cell death (Wang et al., 2001). Activated caspase-3 is expressed at higher levels in the developing brain after perinatal HI, giving rise to the assumption that apoptotic mechanisms of neuronal cell death seem to be more important in neonatal brain injury compared to adults (Gill et al., 2002).

6) It has been proposed that inflammatory activity in the brain, together with the excessive production of toxic compounds like peroxynitrite and free radicals induce downregulation of the formation of neurotrophic factors and neurogenesis. Since the ability of the developing brain to recover after perinatal HI may well depend on production of neurotrophic and neurite-outgrowth promoting factors, downregulation of these growth factors may play an important role in delayed brain damage (up to several weeks) after perinatal HI (Scheepens et al., 2003).

Figure 1 shows proposed injuring mechanisms induced upon and after reperfusion / reoxygenation after perinatal hypoxia-ischemia.

Pharmacological neuroprotective strategies

Based on the mechanisms of perinatal HI brain injury, current therapeutic studies mainly focus on the pharmacologic targets we mentioned above. Since moderate hypothermia is developing into a clinical therapy, studies to investigate the effect of pharmacological interventions in combination with hypothermia are now also initiated. These pharmacologic therapies can start at different points of time after the actual HI insults with or without hypothermia depending on to the mechanism of action (neuroprotection versus repair mechanisms). In addition, treatment with stem cells to enhance repair is a promising intervention (van Velthoven et al., 2010; van Velthoven et al., 2009) to repair HI brain damage.

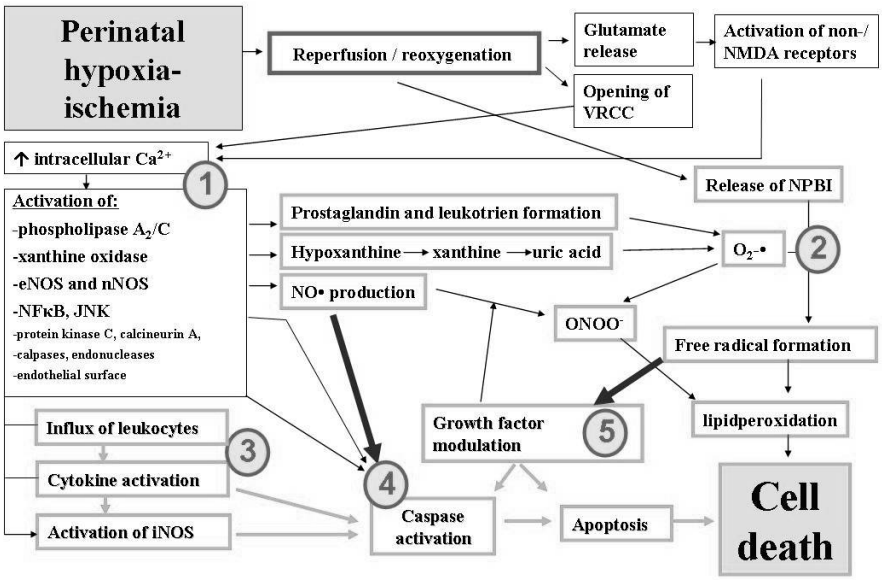

Figure 1. **Possible injuring mechanisms induced upon and after reperfusion / reoxygenation after perinatal hypoxia-ischemia.**

Reperfusion/reoxygenation induces opening of ion channels which leads to influx of calcium into neurons and subsequent excessive neurotransmitters producing and activation of enzymatic reactions (1). The actions give rise to subsequent metabolisation of hypoxanthine, fatty acids originating from neuronal cell membranes, and of nitric oxide production and the Fenton reaction (2). This leads to excessive free radicals formation, lipidperoxidation and ultimate neuronal cell death. Somewhat later (6-12h after reperfusion/reoxygenation), the pro-inflammatory response occurs with activation of cytokines and iNOS production (3), leading to caspases activation (4), growth factor modulation (5) and apoptosis. *JNK=c-Jun N-terminal kinase; NMDA= N-methyl-D-aspartate; NO= nitric oxide; NOS= nitric oxide synthase; eNOS= endothelial NOS; nNOS= neuronal NOS; iNOS=inducible NOS, NPBI=non-protein bound iron; NFκB=Nuclear Factor kappa B; VRCC=voltage-regulated calcium channel.*

(1)Early post-HI period (up to 6h after reperfusion/reoxygenation):

The early post-HI period contains an important therapeutic window (Peeters et al., 2003) and the main pharmacological interventions are dependent on anti-oxidative, anti-inflammatory, and/or anti-apoptotic properties.

- *voltage-regulated and receptor-regulated ion channel blockers:* Reduction of calcium influx into neuronal cells by blockage of voltage-regulated ion channels has extensively been investigated. Calcium blockers including nicardipine and flunarizine

gave encouraging results in experimental studies (Berger et al., 1998; Kittaka et al., 1997). However, a clinical study with nicardipine in term neonates suffering from perinatal asphyxia had to be discontinued because of severe hypotension (Levene et al., 1990). Blocking NMDA-receptors (*i.e.* glutamate regulated ion channels) with magnesium was investigated in clinical trials, but without beneficial effects and severe hemodynamic adverse effects were sometimes reported (Groenendaal et al., 2002; Maroszyńska et al., 1999; Whitelaw et al., 2002). A recently advocated approach is the use of the gaseous anesthetic Xenon, which is thought to act as an NMDA-receptor antagonist. Experimental studies in newborn animals show efficient neuroprotection, especially when administered in combination with moderate hypothermia (Dingley et al., 2006; Hobbs et al., 2008). A major disadvantage of this intervention is that Xenon is very expensive and administration is rather complicated, requiring intubation and ventilation of the patient, and a high percentage of Xenon thereby reducing the maximum FiO_2.

- *Anti-oxidative therapies:* The production of superoxide, the superoxide-derived hydrogen peroxide and hydroxyl radicals are mainly related to xanthine-oxidase, prostaglandin and NPBI production (Mink et al., 2007; Shouman et al., 2008; Windelborn et al., 2008). Allopurinol is a xanthine-oxidase inhibitor that at higher dosages is thought to chelate free iron molecules and to be a direct scavenger of hydroxyl radical (Saugstad et al., 1996). This should be also true for oxypurinol, the even more efficient active metabolite of allopurinol (Moorhouse et al., 1987). Allopurinol was first recognized to have neuroprotective properties by Palmer et al. in a neonatal rat model of HI brain injury (Palmer et al., 1993). The data of this study were promising, although another study was less positive on the neuroprotective potential of allopurinol (Peeters et al., 2003). Human studies in asphyxiated term newborns are not very convincing concerning the neuroprotective effects of allopurinol and oxypurinol, showing at best a moderate reduction of free radical production. One study reported virtually no positive effect on neurodevelopmental outcome (Benders et al., 2006), whereas one study in asphyxiated term newborns reported an improvement of neurodevelopmental outcome after allopurinol treatment (Gunes et al., 2007). Most probably, in most clinical studies therapy is started too late to expect a reduction of xanthine-oxidase production (Benders et al., 2006). A pilot study of our group on maternal allopurinol treatment in those mothers who were on the brink of delivery with fetal hypoxia, showed a reducing effect on biomarkers of neuronal damage and NPBI after allopurinol administration to the mother (Torrance et al., 2009). We are currently examining the effect of maternal allopurinol treatment on neonatal outcome in a similar set-up.

With respect to the pro-free radical properties of NPBI, several experimental studies have been performed in newborn animals of various species including pigs and rats with positive results (Papazisis et al., 2008; Sävman et al., 2005). Up to now, however, free ion chelators, have never been tested in newborn babies to treat reperfusion/reoxygenation injury after perinatal HI.

Due to the toxic effects of excessive formation of NO free radical in the early reperfusion/reoxygenation phase, inhibition of NOS production may ameliorate perinatal brain damage after HI. Non-selective NOS inhibitors such as nitro-L-arginine administered during the early post-HI period have been reported to reduce free radical-mediated reperfusion injury to the neonatal brain (Dawson et al., 1993; Hamada et al., 1994). However, an increasing number of studies showed that non-selective NOS inhibitors, especially those with prominent inhibitory effects on eNOS, prevent adequate post-HI brain perfusion, eventually leading to increased production of free radicals and thus aggravating brain damage (Ashwal et al., 1994; Groenendaal et al., 1999; Marks et al., 1999). Selective inhibition of nNOS and iNOS with the nNOS inhibitor 7-nitroindazole and the iNOS inhibitor aminoguanidine proved to be more promising as a neuroprtective strategy as has been shown in several studies in neonatal rats (Ishida et al., 2001; Tsujiet al., 2000; van den Tweel et al., 2002). The compound 2-iminobiotin (2-IB), which has inhibitory effects on nNOS and iNOS in vitro, did have strong neuroprotective effects in a neonatal rat model of neonatal HI brain damage. Notably, however, only female and not male animals were protected against post-HI reperfusion damage to the brain (Nijboer et al., 2007a). Moreover, the existing evidence suggests that the in vivo neuroprotective effect of 2-iminobiotin was not dependent on nNOS/iNOS inhibition (Nijboer et al., 2007b; van den Tweel et al., 2005b). The exact mechanism of action of 2-IB remains to be determined, but it is clear that in females neuroprotection by 2-IB is associated with reduced activation of apoptotic pathways.

Finally, prostaglandin inhibition has been another important target to fight post-HI brain damage in the newborn. Indomethacin, a cyclooxygenase inhibitor, has been shown to reduce neonatal brain damage after perinatal HI in experimental studies (Taskin et al., 2009). Although indomethacin is currently used in preterm babies to reduce or prevent the occurrence of periventricular/intraventricular hemorrhages (Kumar Nair et al., 2004; Ment et al., 2004) and can reduce white matter injury in these tiny infants (Miller et al., 2006; Torres et al., 2004), it has not been used yet in the term infant to reduce reperfusion/reoxygenation injury of the brain after perinatal HI.

- *Anti-inflammatory therapies*: As mentioned above, the inflammatory pathway is activated after perinatal HI and therefore anti-inflammatory strategies are another meaningful tool to fight reperfusion injury to the newborn brain.

Erythropoietin (EPO), which was first recognized as a humoral mediator involved in the maturation and proliferation of erythroid progenitor cells (van der Kooij et al., 2008), has recently been recognized as a neuroprotective agent in the brain of a variety of mammals including humans (Gonzalez et al., 2009; Iwai et al., 2007; Juul et al., 2009; van der Kooij et al., 2008; Zhu et al., 2009). Mostly recombinant Human EPO (rhEPO) has been used in these studies. One of the possible mechanisms of its neuroprotective effects is an anti-inflammatory effect after binding to its receptor (EPOR) which is expressed on several types of brain cells including astrocytes and microglial cells (Nagai et al., 2001; Sun et al., 2005). Intraperitoneal and subcutaneous administration of EPO in newborn animals after HI improved neurobehavioral outcome (Demers et al., 2005; Kumral et al., 2004) and administration to humans also had neuroprotective effects (Zhu et al., 2009). Also negative results are reported in both clinical and experimental studies (Ehrenreich et al., 2009; van der Kooij et al., 2009). An ongoing study in mice pups of our own group suggests gender dependent neuroprotection in females (see Chapter 3).

Melatonin, the major secretory product of the pineal gland, mainly mediates circadian rhythmicity and seasonality (Claustrat et al., 2005). Recent studies reported that melatonin has a neuroprotective effect during perinatal HI-induced brain injury. Using a P7 rat HI model, it was shown that sensorimotor asymmetry and learning deficits were significantly reduced after administration of melatonin before or up to 10 min after HI (Welin et al., 2007). Histologically, brain injury was significantly attenuated in the melatonin-treated group. In a fetal sheep model with umbilical cord occlusion, melatonin had anti-inflammatory effects as it reduced microglial cell activation (Carloni et al., 2008). The anti-inflammatory effect of melatonin may be mediated by preventing the translocation of NF-κB to the nucleus, thus reducing the upregulation of pro-inflammatory cytokines (Reiter et al., 2000). To date no human studies are known investigating its neuroprotective effects after perinatal HI.

Moreover, our own recent studies have shown that treatment of neonatal rats after HI with etanercept, a soluble TNF-α receptor functioning as a TNF-α inhibitor, also has neuroprotective effects (Nijboer et al., 2009).

-Anti-apoptotic strategies: As we mentioned above, NFκB is a ubiquitously expressed transcription factor that regulates expression of inflammatory genes and of genes

involved in apoptosis. TAT-coupled NFκB essential modulator Binding Domain (NBD)-peptide, a specific NFκB inhibitor, was reported to rapidly distribute to the brain and inhibit cerebral NFκB activation when administered intraperitoneally after neonatal HI in p7 rats. TAT-NBD treatment prevented upregulation of p53 and activation of caspase-3 after HI (Nijboer et al., 2008a). TAT-NBD treatment strongly reduced histological damage when administered within 6 h after HI. Our most recent data demonstrate that at this histological improvement was associated with restoration of sensorimotor and cognitive abnormalities. Surprisingly, NFκB treatment did not reduce cerebral cytokine production despite the marked protective effects. Activation of the c-Jun N-terminal kinase (JNK) pathway is also involved in neonatal brain injury (Waetzig et al., 2005). The specific JNK inhibitor TAT-JBD, which is the TAT coupled JNK binding domain of JNK-interacting protein-1 (also known as L-JNK-I) also has neuroprotective effects. Recent studies reported that JBD improved both short term and long term histological and behavioral outcomes in neonatal rats after HI (Nijboer et al., 2009; Nijboer et al., 2010). Similar to what was observed for the NFκB inhibitor, the protective effect of JBD occurred independently of inhibition of cytokine production (Nijboer et al., 2010). Notably, prolonged inhibition of the JNK pathway as induced by administration of the D-isomer of JNK-I or of the NFκB pathway by repeated administration of NBD at 0, 6 and 12h after HI did not improve outcome or even had some adverse effects (Ginet et al., 2009; Nijboer et al., 2008b; van den Tweel et al., 2006).

-*Hyperbaric oxygen therapy:* The earliest insight into the therapeutic effect of hyperbaric oxygen (HBO) originated from the observation that HBO was capable of decreasing edema and necrosis in ischemic skeletal muscle (Strauss et al., 1983). Although the optimal application and therapeutic effect of HBO therapy in neonatal HI brain injury remains controversial (Zhang et al., 2005), studies on the effect of HBO are still being carried out (Matchett et al., 2009). In a P7 rat model, administration of HBO for 1h after HI significantly improved sensorimotor function during behavioral test and reduced the loss of brain volume (Calvert et al., 2002). The detailed mechanisms of neuroprotection by HBO are not clear yet. Existing studies indicated that neuroprotection was associated with reducing polymorphonuclear leukocytes (PMNL) adhesion (Zamboni et al., 1993), downregulating endothelial cell adhesion molecules (CAM) expression (Buras et al., 2000), inhibiting iNOS production while inducing eNOS production (Buras et al., 2000; Kurata et al., 1995), upregulating antioxidant enzyme activity (Li et al., 2008) and improving cellular energetics (Chen et al., 1998). Furthermore, HBO is capable of suppressing mitochondrial apoptotic pathways via

reducing cytoplasm cytochrome c levels, decreasing caspase enzyme activity and upregulating the ratio of Bcl-2 and Bax expression (Li et al., 2009). HBO also contributes to brain cell proliferation (Yang et al., 2008). In a P7 rat hypoxic-ischemic model, the treatment of HBO could be delayed until 12 h after HI, while the effect decreased 24 h after HI (Wang et al., 2008).

(2) Treatment modalities later in the post-HI period:

The intrinsic ability of the immature brain to reduce (post) HI-induced damage is also dependent on production of trophic factors and endogenous regenerative activity. In view of the capacity of the neonatal brain to repair damage, stimulation of endogenous repair processes has also been suggested as a possible intervention in neonatal HI-brain damage.

-*Trophic factor therapy*: Several trophic or growth factors have been examined in the context of neonatal HI brain injury: epidermal growth factor (EGF), insuline-like growth factor 1 (IGF-1) and brain-derived neurotrophic factor (BDNF) among other factors are very important for the appropriate development of the developing brain (Binder, 2007). Downregulation of the expression (neurotrophic) growth factors is thought to play a pivotal role in (delayed) damage to the brain of the neonate after perinatal HI. Suppletion of deficient growth factors may therefore reduce or prevent delayed HI-induced brain damage. Trophic factors also stimulate neurogenesis by activation of the endogenous neural stem cells (NSC) residing in the subventricular zones and subgranular zone of the dentate gyrus, as shown in studies in rodent pups models (Alagappan et al., 2009; Kanagawa et al., 2006).

EPO has, besides its anti-oxidative and anti-inflammatory effects (Sifringer et al., 2009; Sun et al., 2005), also quite strong neurotrophic abilities as was shown in experimental animal and in vitro studies: a 17-mer peptide sequence called epopeptide AB has been identified in the structure of EPO, and this peptide is considered to contain the neurotrophic properties (Campana et al., 1998). In vitro this peptide can induce neural progenitor cell proliferation and prevents neuronal cell death (Chen et al., 2007). In an adult rat stoke model, administration of EPO promoted neurogenesis in the subventricular zone via upregulation of BDNF expression (Wang et al., 2004). By using embryonic a neural stem cell culture, studies showed that EPO enhanced formation of neurons (Shingo et al., 2001). In a P10 rat stroke model, EPO increased the percentage of newly generated neurons as well (Gonzalez et al., 2007).

Other neurotrophic factors have also been investigated. IGF-1, an anabolic pleiotrophic factor, is essential for postnatal brain development (Peeters et al., 2001). It has been shown that IGF-1 is induced in neonatal brain after HI (Satar, et al., 2004). In a P7 rat model, intranasal administration of IGF-1 improved neurobehavioral performance, inhibited apoptotic cell death, and enhanced proliferation of neuronal and oligodendroglial progenitor cells after HI (Lin et al., 2009). Basic fibroblast growth factor (bFGF), a polypeptide growth factor, has been shown to prevent NMDA-induced neurotoxicity in neonatal rats after HI (Kirschner et al., 1995). Continuous intracerebroventricular injection with BDNF resulted in an increased number of surviving neurons in P8 rat brains after HI (Galvin et al., 2003). An important role with respect to neurotrophic reduction and repair of brain damage has been supposed for hypoxia-induced-factor 1 (HIF-1), which will be activated during hypoxia-ischemia and gives rise to the production of a series of transcriptional targets, of which EPO and vascular endothelial growth factor (VEGF) are important ones with respect to brain repair (Fan et al., 2009; Weidemann et al., 2009; Zhang et al., 2009).

Neuronal stem cell transplantation is a potentially important therapy to reduce long-term brain damage after perinatal HI and have shown to improve motor- and behavioral outcome in adolescent and adult periods (van Velthoven et al., 2010; Yasuhara et al., 2008; Yasuhara et al., 2006). It appears from these studies that NSCs can differentiate into neurons and oligodendrocytes and stimulate angiogenesis (Schmidt, et al., 2009; van Velthoven et al., 2010).

Recent evidence suggests that stroke or HI-induced global brain damage can also be treated with stem cells from other sources than brain, such as mesenchymal stem cells (MSCs) (Toyama et al., 2009; Wei et al., 2009). MSCs can easily be obtained from the bone marrow of healthy adults, placental tissue, umbilical cord stroma (Wharton's Jelly) and even from cord blood. Since MSCs are hardly immunogenic, these cells can also be used for allogeneic transplantation. After administration in the brain, these MSCs can express neuronal markers like NeuN and MAP-2, the astroglial marker GFAP, the microglial marker IB4 (Chen et al., 2001; Shen et al., 2007). Furthermore, It is known that MSC secrete several trophic factors that are known to contribute to neuroprotection including, colony-stimulating factor-1, stem cell factor, VEGF, basic fibroblast growth factor (bFGF), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Li et al., 2002; Rivera et al., 2006). Notably, recent findings indicate that intracranial administration of MSC as late as 3-10 days after the HI insult reduces histological damage and improves sensorimotor outcome in both neonatal mouse and rat HI model

(van Velthoven et al., 2010; Yasuhara et al., 2008; Yasuhara et al., 2006). We also showed that MSC treatment stimulated formation of new neurons and oligodendrocytes and that newly formed cells were not of transplant origin. These findings indicate that MSC treatment enhances endogenous repair mechanisms in the brain (van Velthoven et al., 2009).

(3) Combination therapy: more effective than single therapy?

Although studies in rodents have identified many different molecular pathways as potential targets for neuroprotective strategies after perinatal HI, intervening in one particular pathway may not be sufficient to completely prevent brain injury because of the complex mechanisms of hypoxia-ischemia. Rather than a single therapy directed at one of the potentially destructive pathways, combinations of therapies intervening at different levels in the cascade might lead to more prominent reduction of brain injury.

There is already some evidence that pharmacolgoical interventions combined with hypothermia have a stronger neuroprotective effect after HI than either one alone (Barks et al., 2008; Hobbs et al., 2008). Since it is conceivable that hypothermia postpones secondary energy failure, application of hypothermia immediately after the hypoxic event could prolong the window for pharmacotherapeutic intervention (Guan et al., 2000; Ohmura et al., 2005). In a P7 rat HI model, the hypothermia/Xenon combination conferred more protection as determined in behavioral tests and at the level of histology than either treatment alone (Hobbs et al., 2008). In addition, protection was observed even when Xenon administration was delayed or applied for shorter period of time (Thoresen et al., 2009). The hypothermia/EPO combination is used in out-of-hospital cardiac arrest and this combination can improve the survival rate in adults, but to date there are no data on combined EPO/hypothermia treatment in neonatal HI (Cariou et al., 2008). Administration of melatonin to adult rats during hypothermia promotes tissue oxygenation and enhances the body's resistance to hypothermia (Hlutkin et al., 2008). However, this combination has only been tested in adult studies, and there is no experience in neonatal models. An extremely important issue here is that each pharmacologic compound directed at a specific molecular pathway has its own optimal dose and optimal point in post-ictal time to achieve its optimal neuroprotective effect. It will be a major challenge to define the optimal time of application for each individual intervention and to design the best schedule to let them act in concert.

Figure 2 shows the possibilities for combination therapy and the most optimal point of time to start a specific treatment modality.

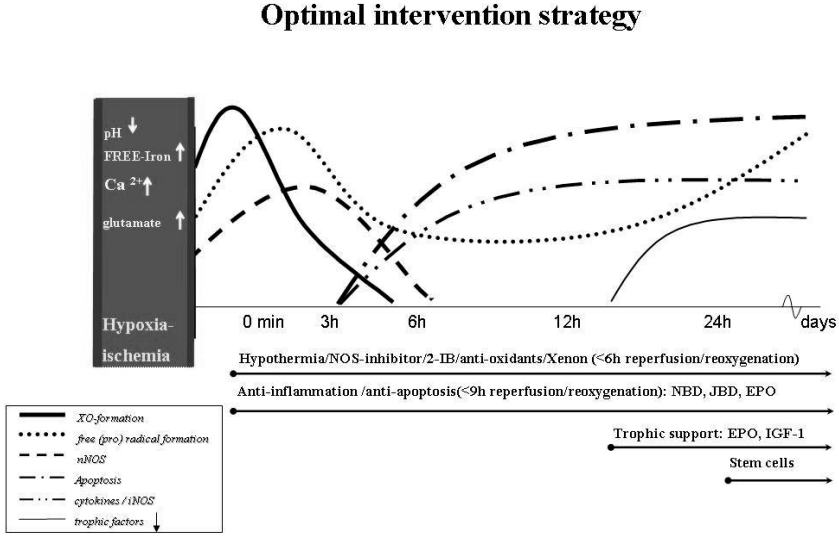

Figure 2. Possibilities for combination therapy and the most optimal point of time to start a specific treatment modality. *XO=xanthine-oxidase; NOS= nitric oxide synthase; nNOS= neuronal NOS; 2-IB=2-iminobiotin; TAT-NBD=TAT-coupled NFκB essential modulator binding domain; TAT-JBD= TAT coupled JNK binding domain; EPO=erythropoietin; IGF-1=insuline-like growth factor 1.*

Conclusions and recommendations

In conclusion, rapid progress in neuroprotective pharmacology has already provided us with a wide selection of pathways that could be targeted for treatment of perinatal HI-induced brain injury. Combination of therapies may lead to a larger neuroprotective effect on the brain than single compound treatment and this possibility should be pursued further.

Further research of promising pharmacologic interventions should be intensively performed and major attention should be given to reducing side effects and toxicity so that more and more therapies can be carried from animal experiments to clinical trials. Recent findings indicating that gender differences in sensitivities to both HI injury and drug treatment should be taken into account (Hurn et al., 2005) and gender-specific therapies may provide more promising interventions for perinatal HI-induced brain injury. Finally, recent findings indicating that stimulation of endogenous repair mechanisms can have potent effects on both histological and behavioral are promising. These findings are

especially important since repair mechanisms could be effectively activated even days to weeks after the insult.

Outline of the thesis

In this thesis, we chiefly focused on the neuroprotective strategies based on moderate hypothermia combined with promising pharmacological interventions in an animal model of neonatal HI brain injury. We aimed to assess the neuroprotection not only by immunochemical histology but also by long-term behavioral and motor outcome.

Chapter 1 discusses the concept of hypoxic-ischemic injury of the immature brain after perinatal HI (birth asphyxia) and summarizes possible neuroprotective strategies, including neonatal HI animal models and clinical neuroprotective actions related to brain injury after perinatal HI.

Chapter 2 summarizes the role and regulation of hypoxia-inducible factor-1α (HIF-1α) expression in brain development and in neonatal hypoxic-ischemic brain injury. Unravelling of the complex functions of HIF-1α may be important to the design of neuroprotective therapies for hypoxic-ischemic brain injury.

Chapter 3 investigates the beneficial effect of erythropoietin (EPO) in a neonatal mice model of HI brain injury. The effect of EPO will be investigated in both short-term and long-term studies, using immunohistology and behavioral tests. Effects of gender and dose effects are evaluated involved in the protective properties of EPO.

Chapter 4 discusses the neuroprotective effect of hypothermia in neonatal rats after HI-induced brain injury. Behavioral tests are performed and the neurodevelopmental outcome and gender effects are assessed after hypothermia treatment.

Chapter 5 discusses the combined therapies of hypothermia and EPO. The histological and behavioral changes are further investigated in rats after neonatal HI brain injury, assessing the synergic effect of this combination.

Apoptotic-induced brain damage after perinatal HI plays a substantial role in neurological outcome. In Chapter 6, pifithrin-μ, a p53 inhibitor, will be administrated to neonatal rats after HI. The biochemical and histological changes in the brain are investigated, and the behavioral outcome is also assessed. New information has been provided for the neuroprotective intervention to inhibit apoptotic activity.

Chapter 7 summarizes the relevant promising therapies to reduce amount of neonatal HI brain injury due to perinatal asphyxia and further discusses the optimal interventional strategies.

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Chapter 2

The role and regulation of hypoxia-inducible factor-1α expression in brain development and neonatal hypoxic-ischemic brain injury

Xiyong Fan[1,2,3], Cobi J. Heijnen[2], Michael A. van der Kooij[1,2], Floris Groenendaal[1], Frank van Bel[1*]

[1]Department of Neonatology and [2]Laboratory for Neuroimmunology and Developmental Origins of Disease (NIDOD), University Medical Center Utrecht, the Netherlands.

[3]Department of Paediatrics, Peking University First Hospital, Beijing, P.R. China

[*]Correspondence

Frank van Bel, MD, PhD

Department of Neonatology

University Medical Center Utrecht

KE.04.123.1

3584EA, Utrecht, The Netherlands.

Tel: +31 88 7554544.

Fax: +31 88 7555521.

E-mail: f.vanbel@umcutrecht.nl

Brain Research Reviews, 2009, 62(1): 99-108

Abstract

During neonatal hypoxic-ischemic brain injury, activation of transcription of a series of genes is induced to stimulate erythropoiesis, anti-apoptosis, apoptosis, necrosis and angiogenesis. A key factor mediating these gene transcriptions is hypoxia-inducible factor-1α (HIF-1α). During hypoxia, HIF-1α protein is stabilized and heterodimerizes with HIF-1β to form HIF-1, subsequently regulating the expression of target genes. HIF-1α participates in early brain development and proliferation of neuronal precursor cells. Under pathological conditions, HIF-1α is known to play an important role in neonatal hypoxic-ischemic brain injury: On the one hand, HIF-1α has neuroprotective effects whereas it can also have neurotoxic effects. HIF-1α regulates the transcription of erythropoietin (EPO), which induces several pathways associated with neuroprotection. HIF-1α also promotes the expression of vascular endothelial cell growth factor (VEGF), which is related to neovascularization in hypoxic-ischemic brain areas. In addition, HIF-1α has an anti-apoptotic effect by increasing the expression of anti-apoptotic factors such as EPO during mild hypoxia. The neurotoxic effects of HIF-1α are represented by its participation in the apoptotic process by increasing the stability of the tumor suppressor protein p53 during severe hypoxia. Moreover, HIF-1α plays a role in cell necrosis, by interacting with calcium and calpain. HIF-1α can also exacerbate brain edema via increasing the permeability of the blood brain barrier (BBB). Given these properties, HIF-1α has both neuroprotective and neurotoxic effects after hypoxia-ischemia. These events are cell type specific and related to the severity of hypoxia. Unravelling of the complex functions of HIF-1α may be important when designing neuroprotective therapies for hypoxic-ischemic brain injury.

Key words

hypoxia-inducible factor; hypoxia; ischemia; brain injury; neonate

Introduction

Neonatal brain injury may result from a variety of conditions, including hypoxia-ischemia, intrauterine infection, and perinatal cerebral hemorrhage. The most common cause of neonatal brain damage is hypoxia-ischemia (HI) (Bracci et al., 2006). HI can disturb brain development and can lead to a variety of serious neurological disorders such as motor and learning disabilities, cerebral palsy, epilepsy and seizures. Although advances in obstetric and neonatal care have reduced substantially neonatal morbidity and mortality, subjects suffering from neonatal HI still experience life long cognitive, sensory and motor disabilities (Northington et al., 2001). Many studies have been performed investigating the mechanisms of hypoxic-ischemic brain damage in the past years, such as free radical formation (Kumar et al., 2008), excitotoxicity (Papazisis et al., 2008) and inflammation (Pleasure et al., 2006; Nijboer et al., 2009). Hypoxia-inducible factor-1α (HIF-1α), which was first discovered in human hepatoma cells as a key factor mediating target gene transcriptions in 1988 (Goldberg et al., 1988), has been intensively investigated for its role in the modulation of hypoxic-ischemic brain injury since 1995(van den Tweel et al., 2006; Chen et al., 2009).

In the present article, we first review the current state of knowledge about the role and regulation of HIF-1α expression in brain development and neonatal hypoxic-ischemic brain injury, and then discuss the possible focus for future research in order to design suitable targets for neonatal brain injury therapy.

The structure and paralogs of HIF-1α

HIF-1α is a member of the Hypoxia Inducible Factor (HIF) family. HIF-1α was discovered in 1988 as the 3' enhancer of the erythropoietin (EPO) gene (Goldberg et al., 1988). The structure of HIF-1α contains two transactivation domains: N-terminal domain (N-TAD) and C-terminal domain (C-TAD). The C-TAD in particular has been shown to regulate the activity of gene transcription (Lando et al., 2002). HIF-1α also contains an oxygen-dependent degradation domain (ODDD) that mediates oxygen-regulated stability.

Other alpha subunits of the HIF family such as HIF-2α and HIF-3α share a number of structural and biochemical similarities with HIF-1α (Wiesener et al., 2002; Gu et al., 1998). HIF-1α has been more extensively studied, whereas research on HIF-2α, HIF-3α and other HIF isoforms is relatively scarce.

HIF- 1α degradation and blockade of target genes transcription in normoxia

During normoxia, HIF-1α is bound to the chaperone molecule Hsp90. In the presence of oxygen and 2-oxoglutarate, HIF-1α is hydroxylated by prolyl hydroxylases (PHD). In addition, HIF-1α is acetylated by an acetyltransferase named arrest-defective-1(ARD1). The hydroxylated, acetylated HIF-1α protein is easily recognized and bound by the von Hippel Lindau protein (pVHL). The binding of pVHL leads to ubiquinatination of HIF-1α. The ubiquinated pVHL/HIF-1α complex targets the HIF-1α protein to the proteasome where it is degraded (Ran et al., 2005). Although the transcription and synthesis of HIF-1α are constitutive and seem not to be affected by oxygen (Wiesener et al., 1998), HIF-1α has a short half-life (several minutes), and is rapidly degraded in normoxia, resulting in essentially no detectable HIF-1α protein under these circumstances (Wang et al., 1995).

Furthermore, during normoxia, the transcriptional activities of HIF-1α target genes are also inhibited. This inhibition effect is completed by hydroxylation of HIF-1α. Oxygen together with 2-oxoglutarate activate asparaginyl hydroxylase (also known as factor-inhibiting HIF), which hydroxylates an asparagine on the HIF-1α protein. The hydroxylation prevents HIF-1α binding to hypoxia response elements in the promoters of HIF target genes. As a result, the transcription of target genes is prevented (Sharp et al., 2004a).

HIF-1α stabilization in hypoxia

Hypoxia leads to an almost immediate shut down of general protein translation to decrease energy consumption during hypoxic energy starvation (Liu et al., 2006). Simon (Simon, 2006) reported that PHD activity in the murine embryonic cells was inhibited when the concentration of O_2 was lower than 5%,. In addition to enzymatic inhibition of the PHD, hypoxia causes perturbations in the mitochondrial electron-transport chain, increasing the levels of cytoplasmic reactive-oxygen species (ROS). ROS can alter the oxidation state of Fe^{2+} (a cofactor for PHD activity) to Fe^{3+}, which cannot be utilized. The alteration from Fe^{2+} to Fe^{3+} also inhibits PHD activity (Simon, 2006).

Moreover, hypoxia appears to activate mitogen-activated protein kinase (MAPK) that phosphorylates HIF-1α and thus stabilizes the molecule (Minet et al., 2001). Another aryl hydrocarbon nuclear translocator (ARNT) called HIF-1β is also phosphorylated at the same time. These two proteins are dimerized to form HIF-1. HIF-1 acts on hypoxia

response elements (HRE) in the promoter of a variety of hypoxia-responsive genes (O'Rourke et al., 1997; Ratcliffe et al., 1998; Semenza et al., 1996; Vaux et al., 2001) (figure 1).

HIF-1α is also involved in ischemia (Welsh et al., 2006). Kalesnykas et al (Kalesnykas et al., 2008) reported that HIF-1α increased in neurons of rats after unilateral occlusion of a common carotid artery. The authors suggested that decreased blood flow and ischemia resulted in cellular hypoxia during the common carotid artery occlusion, leading to stabilization of HIF-1α.

Studies have shown that HIF-1α protein levels increased immediately after the hypoxic exposure, peaked at 3–4 h after hypoxic–ischemic injury, and the elevated level of HIF-1α persisted up to 24 h after the insult (van den Tweel et al., 2006; Calvert et al., 2006).

The extent of HIF-1α expression differs in various conditions of hypoxia. In a rat pup model (hypoxia for 2.5 h with 8% O_2), Li et al. (Li et al., 2007) found that HIF-1α expression was stronger with hypoxia alone, which suggests that the stimulation of hypoxia alone can induce more HIF-1α expression than the stimulation of both hypoxia and ischemia. The latter may mean that HIF-1α is important in maintaining the integrity of the brain during hypoxic conditions.

To date, there are more than 100 target genes of HIF-1α identified with varying functions (Ke and Costa, 2006), including erythropoiesis/ iron metabolism, angiogenesis, vascular tone, matrix metabolism, glucose metabolism, cell proliferation/survival, necrosis, anti-apoptosis and apoptosis (figure 1). For the purpose of this review, we will evaluate the role of HIF-1α in brain development and discuss the effect of HIF-1α in erythropoiesis, anti-apoptosis, apoptosis, necrosis, angiogenesis and other properties after neonatal HI. Furthermore we will summarize the regulatory factors of HIF-1α expression related to the nervous system.

HIF-1α and brain development

The role of HIF-1α in brain development has been investigated for several years. In normal mouse embryos, HIF-1α expression increases between embryonic days 8.5 and 9.5 (Iyer et al., 1998). In neonatal conditional knockout mice after hypoxia (6% O_2 of 3h), neuron-specific HIF-1α-deficiency led to hydrocephalus accompanied by a reduction in neuronal cells and an impairment of spatial memory at the age of 10 weeks (Tomita et al., 2003). Endogenous hypoxia-inducible mechanisms are crucially involved in early

physiologic brain development. In a study with murine midbrain-derived neural precursor cells (mNPCs), HIF-1α conditional knock-out (HIF-1α CKO) mNPCs showed specific impairment of survival and proliferation of neurons in the midbrain after hypoxia treatment in 3% O_2 for two weeks (Milosevic et al., 2007). Meanwhile, expression of vascular endothelial cell growth factor (VEGF) mRNA was reduced in HIF-1α CKO mNPCs, and treatment of HIF-1α CKO mNPCs with VEGF partially recovered proliferation of neurons in the midbrain. The proliferative effect of HIF-1α may be related to the upregulation of VEGF.

Because of the important role of HIF-1α during brain development, therapeutic studies have been carried out. FG-4497, a prolyl-4-hydroxylase inhibitor (PHI), was reported to have protective effects by regulating HIF-1α expression in a rat model of hypoxic kidney injury (Rosenberger et al., 2008). In a study with P7 mice, FG-4497 was injected intraperitoneally in a dose of 100mg/kg (Schneider et al., 2009). Six hours after injection, HIF-1α protein strongly accumulated in the brain, and the expression of specific target genes such as VEGF and erythropoietin (EPO) was significantly upregulated. Since EPO and VEGF are known to be involved in cell proliferation and differentiation (see below), we suggest that the PHI FG-4497 activated HIF-1α expression at an early stage of brain maturation appears to modulate the processes involved in brain development.

HIF-1α and erythropoiesis, role of erythropoietin

In response to hypoxia, the capacity of red blood cells to transport oxygen is up-regulated by the expression of genes involved in erythropoiesis. Hypoxia increases the expression of EPO, which is required for the formation of red blood cells. EPO has been originally recognized as a humoral mediator involved in the maturation and proliferation of erythroid progenitor cells (van der Kooij et al., 2008). Recent studies have shown that EPO mRNA and the actual EPO protein are found in the brain of a variety of mammals including humans (Marti et al., 1996). The EPO receptor (EPOR) is widely expressed in most cerebral cell types, including astrocytes, neurons, endothelial cells and microglial cells (Nagai et al., 2001). In particular, the astrocytes surrounding the capillaries strongly express EPOR (Marti, 2004). In vascular endothelial cells, a recent in vitro report showed that during hypoxia the EPOR is regulated by HIF-1α (Yeo et al., 2008). Another important property of EPO is its neuroprotective effect during neonatal hypoxia (van der Kooij et al., 2008; Mu et al., 2003). In P7 rats HI model, EPO induces several pathways

associated with anti-apoptosis, neuroregeneration and anti-inflammation after binding to its receptor (Sola et al., 2005). Finally, EPO also contributes to neurovascular remodeling after HI, leading to improved neurobehavioral outcomes (Iwai et al., 2007).

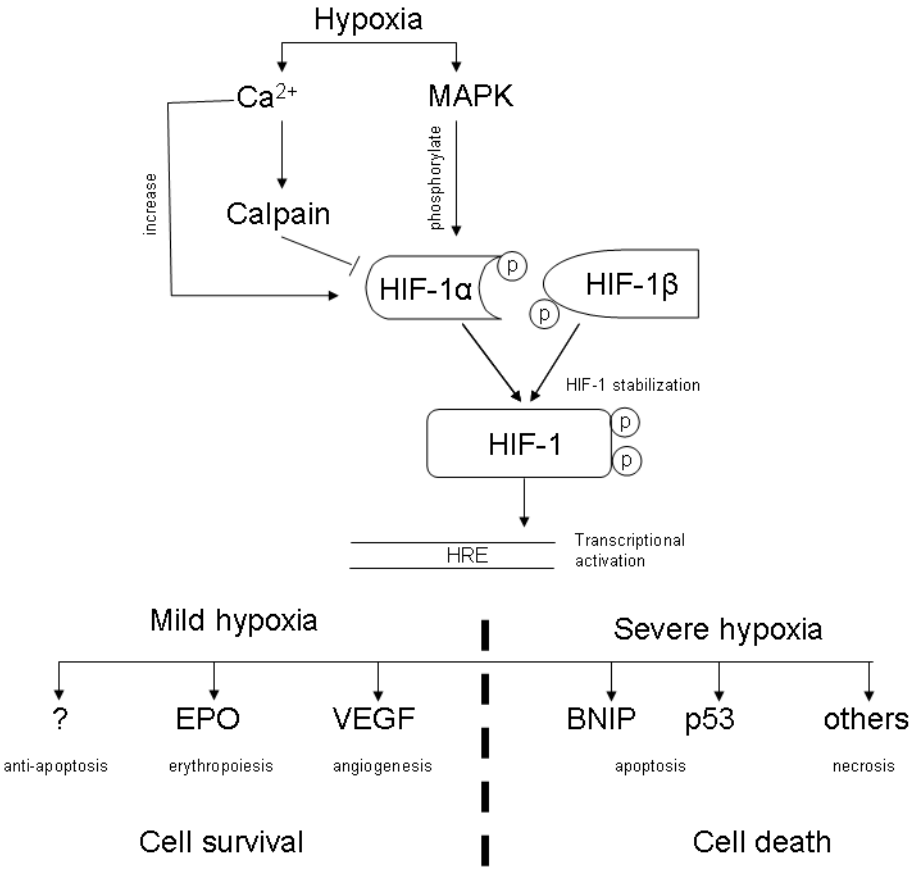

Figure 1. **HIF-1α stabilization and the transcription of its target genes during hypoxia.**

Hypoxia activates MAPK which phosphorylates HIF-1α. Then HIF-1α and HIF-1β are dimerized to form HIF-1. HIF-1 acts on HRE in the promoter of a variety of hypoxia-responsive genes. During mild hypoxia, the transcriptional activations mainly promote cell survival by erythropoiesis, angiogenesis and anti-apoptosis. During severe hypoxia, the transcriptional activations mainly lead to cell necrosis and apoptosis which is induced by BNIP3 and p53. Hypoxia can also induce a calcium influx which increases expression of HIF-1α and activation of calpain. Calpain is involved in HIF-1α degradation. HIF = hypoxia-inducible factor; P = phosphorylation; MAPK = mitogen-activated protein kinase; HRE = hypoxia-responsive elements; EPO = erythropoietin; VEGF = vascular epidermal growth factor; BNIP3 = BCL2/adenovirus E1B interacting protein 3.

As we have mentioned before, EPO is a target gene of HIF-1α (Sharp et al., 2004b). By using a 6-week-old mice model, Stroka et al. (Stroka et al., 2001) described an increase in HIF-1α protein in brain tissue after 1 h of hypoxia (FiO_2 of 6%) with maximum levels observed after 4 to 5 h. The EPO serum level was fourfold higher in mice exposed for 6 h to hypoxia vs. normoxic mice. In a neonatal stroke model with P10 rats, HIF1α expression peaked at 8h after ischemia, and EPO expression was also significantly increased at 8h after ischemia alone (Mu et al., 2005). In contrast to the data mentioned above, Yeo et al. (Yeo et al., 2008) reported that in a mouse model under hypoxic conditions, renal EPO transcription was induced equally by HIF-1α and HIF-2α, but that brain EPO transcription was induced mainly by HIF-2α in spite of HIF-1α and HIF-2α were present in nuclei after 1 h of hypoxia. However, based on ample evidence it has been shown that in the brain HIF-1α plays an important role in EPO transcription (Sharp et al., 2004b; Manalo et al., 2005).

HIF-1α and apoptosis

Neuronal cells undergo apoptosis during and after neonatal HI. HIF-1α is involved in hypoxia-induced apoptosis (Greijer and van der Wall, 2004). There are two possible pathways. First, HIF-1α increases the stability of the tumor suppressor protein p53, which induces cell apoptosis (Chen et al., 2003). Using herpes amplicon-mediated gene transfer in cortical neuronal cultures, expressing a dominant-negative form of HIF-1α (HIFdn) capable of disrupting hypoxia-dependent transcription, reduced delayed neuronal death (46h) that followed oxygen glucose deprivation. In contrast, hypoxia-resistant p53-null primary cultures were not protected by HIFdn expression (Halterman et al., 1999). Moreover, another in vitro study showed that during hypoxia, HIF-1α abrogated the degradation of p53, suppressed p53 ubiquitination, and blocked nuclear export of p53, leading to an increased expression of apoptotis-associated genes (Chen et al., 2003; Vogelstein et al., 2000). Second, overexpression of HIF-1α also induces increased expression of the BCL2/adenovirus E1B interacting protein 3 (BNIP3), which can induce apoptosis in neonatal rat brain (Sandau et al., 2007; Chen et al., 1997; Boyd et al., 1994). Although HIF-1α can increase the expression of both p53 and BNIP3, these molecules are involved in two independent pathways of apoptosis. However, in an in vitro study by Guo et al. (Guo et al., 2001) using Hela cells, it was shown that overexpression of p53 without HIF-1α could not induce the transcription of BNIP3, but the mechanisms involved were unclear.

Meanwhile, HIF-1α may also have an anti-apoptotic function because cells with high expression of HIF-1α are more resistant to hypoxia-induced apoptosis (Greijer and van der Wall, 2004). In a chronic hypoxic (10% O_2 for two weeks) rat model, there was a marked and sustained response of HIF-1α in rat brain, while no apoptosis was detected (Bianciardi et al., 2006). In an acute hypoxia (1%O_2 for 24h) in vitro model, the cells with constitutive expression of HIF-1α protein were more resistant to apoptosis than wild type cells (Akakura et al., 2001). The detailed mechanisms of the anti-apoptotic effect are not clear yet. It is supposed that sustained expression of HIF-1α has an anti-apoptotic effect by increasing the expression of anti-apoptotic factors such as EPO during chronic hypoxia (Bianciardi et al., 2006) and increasing anaerobic metabolism during acute hypoxia (Akakura et al., 2001).

Because HIF-1α is involved in both pro-apoptotic and anti-apoptotic processes, the relationship between these two contradictory effects need to be studied. Induction of apoptosis in neonatal brain depends on the severity of hypoxia (Chen et al., 2009): under mild hypoxic conditions, p53 levels are low, and HIF-1α is phosphorylated and dimerized with HIF-1β, leading to transcriptional activation of anti-apoptotic genes such as EPO (Halterman et al., 1999; Suzuki et al., 2001). However, more sustained or severe hypoxic conditions result in HIF-1α dephosphorylation and an overall increase in p53 levels. This leads to formation of a new transcriptional complex involving both proteins involved in the transactivation of pathological genes such as bax (Halterman et al., 1999; Piret et al., 2002; Calvert et al., 2006). A recent study using a mouse ischemia model described that there are two phases of HIF-1α activation after cerebral ischemia (Baranova et al., 2007). The first phase occurs immediately after ischemia lasting until 24 h, and correlates with the upregulation of most pro-apoptotic genes, while in the second phase of HIF-1α activation from 48h till 8d after ischemia, mostly anti-apoptotic genes are induced. (figure 1)

HIF-1α and necrosis

Neuronal cell death after HI occurs via two pathways. One is apoptosis, as we mentioned above, the other is necrosis, an earlier and rapid pathway of neuronal cell death (Hossain, 2008). During studies with a P7 rat HI model, necrotic neuronal cell death predominates in the ischemic core whereas apoptotic neuronal cells distribute mainly in areas with milder ischemic injury (Nakajima et al., 2000).

Neuronal cells are oxygen-sensing cells, which means that the cells respond to hypoxia with an increase in cytoplasmic free calcium derived from the combined influx of extracellular calcium and the release of calcium present in the endoplasmic reticulum (ER) (Seta et al., 2004). A calcium influx activates several proteins related to cell necrosis, such as calpain (Zong and Thompson, 2006; Liu et al., 2008). In a P7 rat model, calpain was significantly increased 30min after HI (Carloni et al., 2007). By using renal cell carcinoma 4 (RCC4) cells and human embryonic kidney 293 (HEK293) cells, in vitro studies have shown that activation of calpain participates in regulating the stability of HIF-1α (Zhou et al., 2006). Activated calpain is also involved in degradation of HIF-1α during hypoxia (Zhou et al., 2006). This degradation is provoked by raising intracellular calcium, and the calpain pathway is activated when PHD activity is blocked. The calpain-mediated HIF-1α degradation may be relevant to understand the low concentration of HIF-1α during periods of long lasting and/or severe hypoxia associated with calcium fluctuations. Although calcium influx activates calpain-mediated HIF-1α degradation, Hui et al. (Hui et al., 2006) reported that in carotid body type I cells, an increase in the intracellular concentration of calcium during hypoxia stimulated translation of HIF-1α proteins. Nanduri et al. (Nanduri et al., 2009) further reported that calpain had no degradating activity to HIF-1α in pheochromocytoma 12 (PC12) cells under intermittent hypoxia. With respect to glycogen synthase kinase-3 (GSK3), Ginouvès et al. (Ginouvès et al., 2008) reported that stabilization of HIF-1α led to cell necrosis during hypoxia (figure 1). However, these effects may be limited to tumor cells whereas in neurons stabilization of HIF-1α may lead to survival. Nonetheless, the relationship between HIF-1α and necrosis is still not fully elucidated.

HIF-1α and angiogenesis, role of vascular endothelial cell growth factor

Angiogenesis is a complex process that involves multiple gene products expressed by different cell types. A large number of molecules serve as positive regulators of angiogenesis (vascular endothelial cell growth factor VEGF, fibroblast growth factors FGFa and FGFb, transforming growth factors TGFα and TGFβ, hepatocyte growth factor HGF, tumor necrosis factor TNFα, angiogenin, interleukin-8, and angiopoietins) and have been shown to increase by hypoxic challenge (Karamysheva, 2008). However, not all of these molecules are specific for endothelial cells, and only some of them are capable of influencing directly endothelial cells in culture. Among them, the VEGF is the most potent mitogen for endothelial cells under in vitro and in vivo conditions (Conklin et

al., 2002; Feng et al., 2008). VEGF directly participates in angiogenesis by recruiting endothelial cells to hypoxic and a-vascular areas and by stimulating their proliferation (Saito et al., 2008).

During hypoxia, HIF-1α plays an important role in expression of VEGF (Ke et al., 2006; Forsythe et al., 1996). It has been demonstrated that there is a HIF-1 binding site within the VEGF enhancer. After HIF-1α dimerize with HIF-1β to form HIF-1, HIF-1 binds to VEGF enhancer and mediates transcriptional activation (Forsythe et al., 1996). When hypoxic exposure is mild to moderate, upregulation of VEGF probably enhances survival of surrounding cells in the hypoxic area (Yeo et al., 2008; Nanka et al., 2006). However, a rapid and massive increase in VEGF occurs during severe and sustained hypoxia, and it has an overall negative effect on surrounding vasculature causing increased permeability and vascular remodeling which results in blood-brain barrier (BBB) disruption (Yeh et al., 2007; Sivakumar et al., 2008; Zhang et al., 2002). Although BBB disruption can occur at any age, the severe damage associated with BBB permeability mainly happened in immature animals (P7 rats) (Muramatsu et al., 1997).

During ischemia without hypoxia, levels of HIF-1α mRNA and its protein also increase in brain in both mice and humans (Bergeron et al., 1999). Increased HIF-1α mRNA is associated with expression of VEGF in brain tissue surrounding the site of ischemia (Marti et al., 2000). In a P10 rat stroke model with middle cerebral artery occlusion for 1.5h, VEGF expression was detected as early as 4 h after reperfusion and was maintained for at least 24 h (Mu et al., 2003). In another ischemic rat model, upregulation of VEGF in different areas had different effects (Zhang et al., 2002). From 2h till 6h after ischemia, upregulation of VEGF mainly occurred in astrocytes in the ischemic core and penumbra, which may imply that VEGF interacts with VEGF receptors on the ischemic vessels, inducing BBB leakage and central nervous system edema formation. During 2 to 28 days after ischemia, upregulation of VEGF mainly occurred at the boundary zone, and VEGF regulated neovascularization after binding to VEGF receptors at the border of the infarction (Zhang et al., 2002).

However, there might be other pathways that are probably mediated independently of HIF-1α, and are involved in the VEGF activation as well. In an in vitro study with neonatal rat astrocytes, hypoxic elevation of VEGF occurred in the absence of detectable HIF-1α stabilization, suggesting that a HIF-1α-independent regulatory mechanism also exists during mild oxygen deprivation (Schmid-Brunclik et al., 2008). By using gliosarcoma 9 line (GS9L) cells, Damert (Damert et al., 1997) reported that VEGF mRNA can be stabilized during hypoxia through an element in the 3'-untranslated region

of the VEGF transcript. This procedure has no involvement of HIF-1α in the transcriptional activation of VEGF gene expression. So hypoxia-induced VEGF expression in neonatal astrocytes does not fully depend on HIF-1α, assuming that additional regulation via HIF-1α-independent mechanisms are of importance as well during mild hypoxic injury.

Other properties of HIF-1

Recent in vitro work suggests that selective loss of HIF-1α function in astrocytes provides neuroprotection after hypoxia, whereas loss of neuronal HIF-1α increases neuronal susceptibility to hypoxia-induced damage (Vangeison et al., 2008). The latter suggests that the HI-induced effects of HIF-1α might be cell type specific. Astrocytes can contact the brain vasculature with their end feet processes and this interaction is thought to induce endothelial tight junction formation and to decrease permeability of the BBB.In response to hypoxia and ischemia, astrocytes retract their end feet from vessels, resulting in increased permeability of BBB (Mani et al., 2005). In conclusion, acute HIF-1α inhibition may contribute to neuroprotection after HI via preservation of the BBB with a subsequent reduction in brain edema and attenuation of neuronal cell death (Chen et al., 2008).

HIF-1α and hypoxic/ischemic preconditioning

As we have mentioned before, HI is an important cause of neonatal brain injury. However, a sublethal hypoxic/ischemic exposure can improve tolerance of tissue or of cells to a subsequent lethal hypoxic/ischemic insult. This phenomenon is called hypoxic/ischemic preconditioning (H/IPC), actually occurring during perinatal hypoxia (Wang et al., 2008; Murry et al., 1986).

Studies have indicated that HIF-1α is a key factor involved in H/IPC. In a P7 rat model, the expression of HIF-1α was significantly increased in brain tissue and the infarction area was reduced after pre-treatment with 8% O_2 of 3h (Bergeron et al., 2000). When mice were pre-treated with hypoxia (8% O_2 of 1h, 3h, or 6h duration) before permanent occlusion of the left middle cerebral artery, the nuclear content of HIF-1α was rapidly increased in brain tissue and the infarction volume was significantly reduced (Bernaudin et al., 2002). Furthermore, EPO and VEGF were upregulated 24 hours after hypoxia pre-treatment. Another study of hypoxic preconditioning was carried out with

cell-specific neural HIF-1α-deficient mice (Taie et al., 2009). Hypoxic preconditioning was performed in 8% O_2 for 3h. Subsequently the mice were exposed to 14% or 8% O_2 for 30 min after the hypoxic pre-treatment. The results showed that hypoxic preconditioning improved the oxygen partial pressure (pO_2) of brain tissue during subsequent hypoxia, whereas in contrast no detectable effect in the HIF-1α-deficient mice could be observed. These studies support the hypothesis that HIF-1α plays an important role in H/IPC, and that the protective effects of H/IPC may be partially mediated by improving tissue oxygenation via HIF-1α and upregulation of the target genes.

Regulatory factors of HIF-1α expression in neonatal hypoxia-ischemia

Many factors can regulate the expression and stabilization of HIF-1α. PHD, an oxygen related enzyme, may suppress HIF-1α function. As we mentioned before, PHD can hydroxylate HIF-1α which will lead to the subsequent degradation of HIF-1α. After P6 rats were exposed to preconditioning with hypoxia (3h, 8% O_2), HIF-1α significantly increased in brain tissue immediately while PHD activities were inhibited simultaneously (Jones et al., 2006). PHD can also be inhibited by other factors without hypoxia. By using embryonic cortical neurons of rats, another study showed that inhibition of PHD by deferoxamine (DFO) and 3,4-dihydroxybenzoate (3,4-DHB) can stabilize HIF-1α and up-regulate its target genes such as VEGF and EPO (Siddiq et al., 2005). In a P10 rat stroke model, the similar effect of DFO in upregulation of HIF-1α was also demonstrated (Mu et al., 2005).

ROS are involved in stabilization of HIF-1α, so the role of antioxidants in the regulation of HIF-1α expression may be a useful research focus. Using mouse fetal cortical neurons, it has been shown that HIF-1α expression in wild type neurons increased after hypoxia compared to the expression in fetal cortical neurons deficient in Copper/zinc–superoxide dismutase (SOD). Therefore the antioxidant SOD may downregulate HIF-1α expression by scavenging superoxide radicals (Liu et al., 2005). Another in vitro study with murine hippocampal cells also demonstrated that antioxidants (N-acetylcysteine, butylated hydroxyanisole) destabilized HIF-1α during oxidative stress, but this destabilization abrogated the pro-apoptotic effects of BNIP3 pathway which was induced by HIF-1α overexpession (Aminova et al., 2008).

Cytokines play an important role during inflammation, and they are also involved in the regulation of HIF-1α expression. The local production of proinflammatory cytokine interleukin-1β (IL-1β) has been well documented as an early feature of the brain

response to injury (Rothwell, 2003; Herx et al., 2000). During primary human fetal astrocyte cultures, HIF-1α was upregulated at 6 h following cytokine IL-1β treatment and peaked at 24 h, and VEGF was also upregulated at the same time (Argaw et al., 2006). These data showed that IL-1β induced both HIF-1α and its target gene VEGF, and further investigation demonstrated that this effect was significantly potentiated by the presence of another cytokine interferon- γ (IFN-γ).

Another cytokine involved in the regulation of HIF-1α stability is TNF-α. In proximal tubular LLC-PK1 cells or human embryonic kidney cells, the concentration of HIF-1α protein increased after TNF-α treatment, and this increase in HIF-1α was prevented by blocking nuclear factor (NF)-κB (Zhou et al., 2003). These results indicated that TNF-α induce the accumulation of HIF-1α, and this induction demanded the NF-κB pathway. In a murine microglial cell line BV-2, addition of TNF-α resulted in an increase in cellular levels of HIF-1α (Yao et al., 2008). TNF-α did, however, not induce stabilization of HIF-1α in MO3.13 cell line (which expresses the phenotypic characteristics of primary oligodendrocytes). In conclusion, many factors are involved in regulation of HIF-1α, which can be either oxygen-dependent or oxygen-independent.

Conclusions and recommendations

HIF-1α plays an important role in brain development and hypoxic-ischemic brain injury, and the molecule exhibits both neuroprotective as well as neurotoxic properties. A myriad of factors are involved in the regulation of HIF-1α expression. Despite the recent rapid advance in the understanding of molecular mechanisms in response to HI in neonatal brain injury, many questions about HIF-1α remain to be answered. The regulation of HIF-1α expression and the distinct roles of HIF-1α during neuronal cell death or survival is not completely clear, especially the question which condition directs cell death or survival. To elucidate this issue we will also have to identify the additional target genes of HIF-1. The function of the paralogs of HIF-1α (such as HIF-2α and HIF-3α) has not been fully studied as well. Advances in recent research provide us with some directions for further studies. For example, the decision whether HIF-1α induces pro-apoptosis or anti-apoptosis after hypoxia may be determined by the duration of the HI (Halterman et al., 1999), the type of pathological stimuli (Aminova et al., 2005) and the cerebral cell type involved (Vangeison et al., 2008). We may also search for new pathways to promote the expression of neuroprotective target genes such as EPO and VEGF by regulating the production of HIF-1α with PHD inhibitors. Another approach may

be to inhibit the dephosphorylation of HIF-1α or to downregulate its expression with antioxidants so that e.g. p53- and BNIP3-induced apoptosis is blocked. Preconditioning is another important direction for preventing hypoxic-ischemic brain injury via HIF-1α stabilization. From a therapeutical viewpoint, we may pay more attention to the asphyxic newborns who do not receive prenatal hypoxia preconditioning before the occurrence of neonatal HI. Most of the properties of HIF-1α have only been tested experimentally and need further validation through translational research. Unraveling such questions should provide us with new insights into cellular adaptation to hypoxia and will aid to discover new therapeutic approaches for (neonatal) hypoxic-ischemic brain damage.

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Chapter 3

Beneficial effect of erythropoietin on sensorimotor function and white matter after hypoxia-ischemia in neonatal mice

Xiyong Fan[1,2,3], Cobi J. Heijnen[2], Michael A. van der Kooij[1,2], Floris Groenendaal[1], Frank van Bel[1*]

[1]Department of Neonatology and [2]Laboratory for Neuroimmunology and Developmental Origins of Disease (NIDOD), University Medical Center Utrecht, Utrecht, the Netherlands

[3]Department of Paediatrics, Peking University First Hospital, Beijing, China

*Correspondence

Frank van Bel, MD, PhD

Department of Neonatology,

University Medical Center Utrecht

KE 04.123.1

PO Box 85090

3508 AB Utrecht, the Netherlands

telephone: +31-88-75 5 4545

fax: +31-88-75 5 5320

Email: f.vanbel@umcutrecht.nl

Submitted

Abstract

There are mixed reports on the neuroprotective properties of erythropoietin (EPO). We investigated the effect of EPO on the short- and long-term outcome after neonatal hypoxia-ischemia (HI) in mice and compared the effect of two different dose regimens of EPO. Nine-day-old mice were subjected to HI, and EPO was injected at 0h, 24h and 48h after HI in a dose of either 5kU/kg or 20kU/kg. Cylinder rearing test was used as a measure of sensorimotor function. Only in female mice administration of EPO at 5kU/kg but not 20kU/kg improved sensorimotor function and enhanced myelin basic protein staining. Additionally, at 72 h after HI, more Ki67 cells were found in the subventricular zone after EPO 5kU/kg treatment, indicating an increase in progenitor cell proliferation. In conclusion, EPO improves sensorimotor function after neonatal HI and protects against white matter loss. The protective effect of EPO is dose-dependent and only present in females.

Key words

neonate, hypoxia-ischemia, erythropoietin, protection, white matter, gender

Introduction

Neonatal hypoxic-ischemic brain injury occurs in 1- 4 per 1000 live-born infants and is an important cause of cerebral palsy, epilepsy and adverse developmental outcome (Ferriero, 2004; Hagberg et al., 1993). Experimental studies in newborn animals with HI showed that anti-oxidative, anti-inflammatory and neurotrophic agents are neuroprotective (van Bel et al., 2008; Gonzalez et al., 2008). However, in clinical studies with newborns with HI encephalopathy, only mild hypothermia showed a modest neuroprotective effect if started within 6 hours after birth in asphyxiated term newborns with moderate encephalopathy (Edwards et al., 2010; Gluckman et al., 2005; Azzopardi et al., 2007).

Erythropoietin (EPO), a glycoprotein primarily recognized as a mediator promoting maturation and proliferation of erythroid progenitor cells (Carnot et al., 1906; van der Kooij et al., 2008), is an attractive drug for this purpose. EPO has been proven to cross the blood brain barrier (BBB) after systemic administration at a high dose and to reduce free radical formation as well as pro-inflammatory and apoptotic activity in models of brain damage (van der Kooij et al., 2008; Ehrenreich et al., 2004; Sun et al., 2005; Kellert et al., 2007). EPO has also been shown to stimulate formation of new neurons due to its actions as a neurotrophic factor (Shingo et al., 2001; Wang et al., 2004). The effects of EPO are mediated by binding of EPO to its receptor (EPOR), which is present in the brain at relatively high levels in regions known to be sensitive to hypoxia (Digicaylioglu et al., 1995; Rabie et al., 2008). Most studies in neonatal animals treated with EPO after HI showed indeed improved histological outcome (van der Kooij et al., 2008; Sola et al., 2005), although our study in rats with neonatal HI brain damage showed no histological improvement (van der Kooij et al., 2009a). However, Spandou et al. reported that in a similar rat model (Spandou et al., 2005), using a shorter duration of hypoxia, EPO induced effective regeneration of brain tissue.

In human studies no adverse effects of EPO treatment have been reported indicating that EPO is a safe drug for application in neonates (Juul et al., 2008). EPO has been increasingly used in preterm infants to treat neonatal anemia or to improve neurodevelopmental outcome, at a low (0.4kU/kg) or a relatively high dose (3kU/kg) (Juul et al., 2008; Bierer et al., 2006; Fauchère et al., 2008). Furthermore, EPO treatment (0.3-0.5kU/kg) of perinatally asphyxiated term infants resulted in improvement of neonatal behavioral neurologic function (Zhu et al., 2009).

In the present study a moderate HI brain injury was induced by unilateral carotid artery ligation followed by 10% oxygen for 45 min leading to reproducible brain injury (van der Kooij et al., 2009b). The mice were treated with 5kU/kg or 20kU/kg EPO or vehicle at 0h, 24h and 48 h after the insult. We investigated the contribution of EPO on short and long-term sensorimotor function and histological outcome in neonatal mice with a special focus on gender.

Methods

Animals

Experiments were performed in accordance with international guidelines and approved by the Experimental Animal Committee of the University Medical Center Utrecht. C57Bl/6 J mice were bred at the animal facility of Utrecht University and surgery was performed at nine days of age. Mice were housed at 21–23°C in an automatic 12-h light-dark cycle and weaned at the age of weeks. Food and water were available *ad libitum*. All analyses were performed in a blinded set-up.

Experimental model

On postnatal day 9 (P9), 101 mouse pups (51 males, 50 females) underwent HI. The right common carotid artery was isolated and electrocauterized under anesthesia (isoflurane: 5% induction, 1.5% maintenance in O_2:N_2O; 1:1). Pups were allowed to recover for 1.5 hours, followed by 45 minutes hypoxia (humidified 10% O_2, 90% N_2, 35°C). SHAM controls underwent anesthesia and skin incision only. Five mice (3 males, 2 females, 5.0%) died from hypoxia before randomization and treatment, and no mortality occurred after treatment.

EPO treatment

To examine the efficacy of EPO (EPREX, Janssen-Cilag B.V., Netherlands) treatment in P9 mice after HI, mice were randomly assigned to three groups and treated intraperitoneally (ip.) with EPO (5kU/kg or 20kU/kg) or vehicle at 0h, 24h and 48h after hypoxia.

Histology

For the short-term study, mice were terminated by 300 mg/kg pentobarbital at 72h after HI, and perfused with 4% paraformaldehyde in phosphate buffered solution (PBS). Brains were paraffin-embedded and coronal sections (8µm) were cut at both ventricular

level and hippocampal level. Deparaffinized sections were incubated with mouse-anti-Microtubule-associated protein 2 (MAP-2) (1:1000, Sigma-Aldrich, Steinheim, Germany), rabbit-anti-α-cleaved-caspase-3 (1:800, Cell Signaling, USA), rabbit-anti-Ki67 (1:400, Abcam, USA) followed by biotin-labeled secondary antibodies and revealed using Vectastain ABC kit (both Vector-labs, Burlingame, CA) and diaminobenzamidine (Sigma-Aldrich, USA).

MAP-2 loss was quantified using photoshop CS 4 (Adobe Systems, Mountain View, CA). Both hemispheres were outlined on full section images and the ratio of ipsilateral / contralateral areas was calculated (Nijboer et al., 2007). MAP-2 loss=1-(ipsilateral area/contralateral area)×100%.

Cleaved-caspase-3 staining was scored using a semi-quantitative approach in the hippocampal area with the following scale: 0=90-100% caspase-3^+ cells, 1=50-90% caspase-3^+ cells, 2=10-50% caspase-3^+cells, 3=0-10% caspase-3^+cells. Scoring was divided into four regions: cornu ammonis (CA) 1, CA2, CA3 and CA4. The scoring ratio of ipsilateral / contralateral areas was also calculated. Scoring ratio = (ipsilateral score/contralateral score)×100%.

Progenitor cell proliferation was determined by Ki67 staining of the ipsilateral subventricular zone (SVZ).

For the long-term histological study, mice were terminated at 10 weeks of age as described for the short-term study. Brains were mounted in paraffin from which coronal sections (8 µM) were cut. Brain sections were stained with hematoxylin–eosin (HE). Deparaffinized sections were then incubated with mouse-anti-myelin basic protein (MBP) (1:1600, Sternberger Monoclonals, Lutherville, MD) followed by biotin-labeled secondary antibodies and staining was revealed using Vectastain ABC kit (Vector-labs, Burliname, CA) and diaminobenzamidine (Sigma-Aldrich, USA). Both hemispheres were outlined on full section images and the ratio of ipsi- and contralateral areas was calculated with photoshop CS4.

Cylinder rearing test

The cylinder rearing test (CRT) was used to assess sensorimotor function (van der Kooij et al., 2009b; Schallert et al., 2000). At 18 days, 5 weeks and 10 weeks of age, animals were individually placed in a Plexiglas transparent cylinder between 9 am and 10 am (18 days and 5 weeks: 7.5 cm ø×15 cm height; 10 weeks: 11 cm ø× 30 cm height) and observed for 3 min in the housing room. Initial forepaw (left/right/both) preference of weight-bearing contacts during full rear was recorded. The relative proportion of right

(ipsilateral) forepaw contacts was calculated as: Paw preference = (right - left)/(right + left + both)× 100 (Chang et al., 2005).

Statistical Analysis

Statistical analysis was performed using SPSS 15.0 and Graphpad Prism 4.02 software. Data are presented as means ± SEM and a p-value <0.05 was accepted as statistically significant. One-way ANOVA with Bonferroni post tests was used to analyze group differences.

Results

MAP-2 loss

Loss of ipsilateral MAP-2 staining as determined at 72 h after HI was used as a marker of early grey matter area loss. In our P9 model of neonatal HI brain damage, at the ventricular level, the loss of MAP-2 chiefly occurred in ipsilateral striatum. At the hippocampal level, there was a loss of MAP-2 staining in the hippocampal area of the ipsilateral hemisphere of 25% (figure 1A). Fig. 1B shows that there is no significant neuroprotective effect of EPO treatment (5kU/kg or 20kU/kg) compared with vehicle treatment on the level of MAP-2 staining at this early time point after treatment.

Active-caspase-3 staining

To determine whether EPO treatment had an effect on apoptotic cell death, we scored active caspase-3 staining in the hippocampal area and calculated the scoring ratio of ipsilateral / contralateral areas. There was a significantly lower ratio after HI (either vehicle or EPO-treated animals) compared with the SHAM-treated animals in CA1 and CA3 areas of the hippocampus (P<0.05), indicating that apoptotic cell death was ongoing in these regions in the ipsilateral hemisphere at 72h after the insult. We did not detect a significant difference in CA2 and CA4 areas when comparing HI and SHAM control animals. I.p. administration of EPO (5kU/kg or 20kU/kg) at 0h, 24h and 48 h after the insult had no significant anti-apoptotic effect (figure 1C).

Progenitor cell proliferation

Ki-67, which is expressed during mitosis, is considered as a reliable marker for proliferating cells (Scholzen et al., 2000). In the ipsilateral SVZ, the number of Ki67-positive cells was reduced at 72 h after HI. At this time point, more Ki67-positive cells

were observed after EPO 5ku/kg treatment compared with the vehicle group, but no effect was found after EPO 20ku/kg administration (figure 1D).

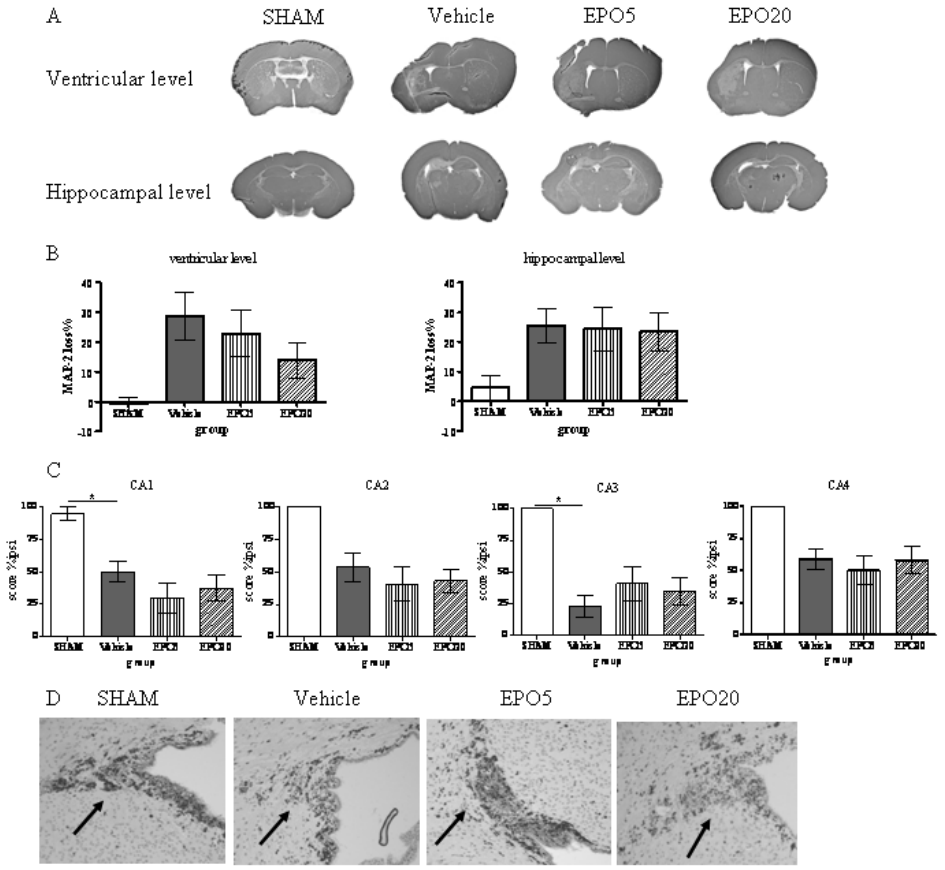

Figure1. **Microtubule-associated protein 2 (MAP-2) loss, semi-quantitative analysis of α-cleaved-caspase-3 activity and Ki67 immuno-positive cells.**

P9 mice were exposed to hypoxia-ischemia (HI) and treated with EPO 5 kU/kg, EPO 20 kU/kg or vehicle. At 72h after HI brains were collected and analyzed immunohistochemically.

-A) Representative examples of MAP-2 staining at the ventricular and at the hippocampal level of the brain.

-B) Mean MAP-2 loss expressed as ratio of ipsilateral/contralateral MAP-2 positive areas at the ventricular level and hippocampal level.

-C) The immuno-positive cell scoring ratio of ipsi- and contralateral areas after α-cleaved-caspase-3 staining (CA1,CA2,CA3,CA4) was calculated as described in the section methods.

-D) Representative examples of Ki67 positive cells in the subventricular zone (see arrow) as a measure of neural progenitor cell proliferative activities

* SHAM vs vehicle $P<0.05$; n= 12 per group (SHAM group n=5)

Paw preference (cylinder rearing test)

At 18 d, 5 w and 10 w of age we assessed sensorimotor function using the CRT. SHAM-treated animals did not show any paw preference during rearing in the CRT. HI caused a preference (~15% at 18d, ~30% at 5w, ~25% at 10w) to use the unimpaired forepaw. EPO treatment did not improve performance in the CRT of male or female animals when assessed at 9 days after the insult. However, EPO-treated female but not male animals showed a significant improvement of sensorimotor function at 5w.Interestingly, the improvement obtained in females of the EPO 5kU/kg group was ~10% stronger that that obtained in females of the 20ku/kg treatment group (P<0.001, P<0.01, respectively).

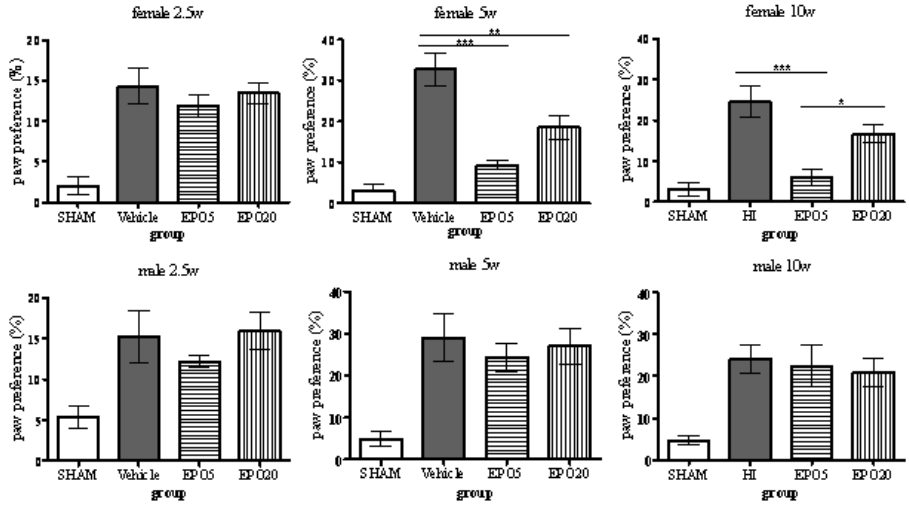

Figure 2. **Effect of EPO treatment on preference to use the non-impaired (ipsilateral) paw in the cylinder rearing test**

Male and female P9 mice were exposed to HI and treated with EPO or vehicle as described in the legend to Fig. 1. At 2.5w, 5w and 10 w old, sensorimotor function was assessed in the cylinder rearing test.

* EPO5 vs EPO20 P<0.05

** EPO20 vs vehicle P<0.01

*** EPO5 vs vehicle P<0.001

n=10 per group (SHAM group n=5)

At 10w, the EPO 5ku/kg-treated female mice still showed improvement of sensorimotor function (P<0.001). Surprisingly, we no longer detected sensorimotor improvement in the 20kU/kg EPO-treated female animals at this late time point. (figure

2). Sensorimotor function in males was not improved by EPO treatment at any of the time points tested.

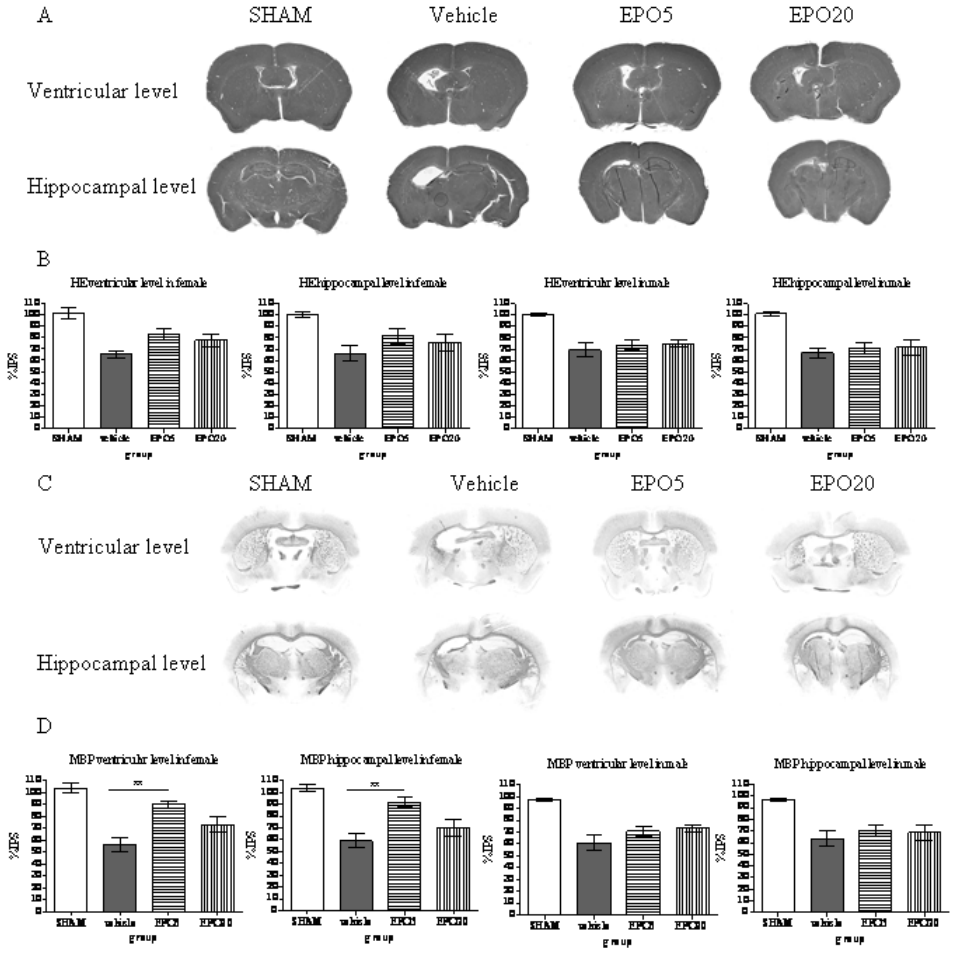

***Figure 3*. Effect of EPO treatment on brain area loss and white matter loss as determined after hematoxylin–eosin (HE) and myelin basic protein (MBP) staining respectively at 10 weeks after HI.**

-A) Representative examples of brain area loss at ventricular and hippocampal levels of the brain after HE staining

-B) The ratio of ipsilateral/contralateral areas (ventricular level and hippocampal level) after HE staining

-C) Representative examples of white matter changes at ventricular and hippocampal levels of the brain after MBP staining

-D) The white matter ratio of ipsilateral/contralateral areas (ventricular level and hippocampal level)

** EPO5 vs vehicle P<0.01; n= 10 per group (SHAM group n=5)

Analysis of ipsilateral surface area

The effect of EPO on long term brain damage was determined at 10w. The morphological changes in the brain as visualized after HE staining are shown in figure 2. The ipsilateral ventricle was mildly dilated secondary to tissue loss in the caudate putamen, and there was still a 35% loss of tissue in the ipsilateral hippocampus. The lesion volumes were similar in male and female mice. In addition, we did not detect a significant effect of EPO treatment (5kU/kg, 20kU/kg) in the ratio of ipsilateral / contralateral area in males or females. (figure 3A, 3B).

In contrast to what was observed for total area loss as determined after HE staining, the data in figure 3C and 3D show that a significant reduction in the HI-induced loss of MBP staining was obtained only in females after treatment with 5kU/kg EPO compared with vehicle ($P<0.01$). However, there was no effect in either males or females of 20kU/kg EPO.

Discussion

In this study we found no neuroprotective effect of EPO in our histological parameters when determined 24 h after the last EPO treatment (72h after HI). In the long-term, we observed a positive effect of EPO treatment on sensorimotor function and white matter injury when using 5kU/kg of EPO. However, the beneficial effect of EPO was only observed in female animals.

Gender effects of post-hypoxic neuroprotective interventions have been described after hypothermia and after treatment with the drug 2-iminobiotin (Nijboer et al., 2007; Bona et al., 1998). Also in those studies only females benefitted from these neuroprotective interventions. Our present results on the gender-specific effects of EPO treatment after neonatal HI are in line with the study by Wen et al. (Wen et al., 2006). These authors described gender-specific long-term protective effects of EPO treatment in a neonatal stroke model (Wen et al., 2006). Although it has been shown convincingly that circulating estradiol can lower the sensitivity for HI injury in females (Hurn et al., 2005), it is not likely that female sex hormones play already a major role in P9 neonatal mice. Indeed, we did not observe any gender difference in the extent of long-term HI brain injury in either sensorimotor function or brain damage in the vehicle group. Mechanisms other than a hormonal influence have been considered to explain gender effects in studies on brain damage, including gender-dependent gene expression and properties of female neuronal cells (Hurn et al., 2005). In a clinical investigation using

peripheral blood cells from healthy individuals, the EPOR alleles, EPORA 1 and EPORA 10, were present in a significantly higher frequency in females than in males (Zeng et al., 2001). Although there is no report about the gender difference of EPOR in the human brain, we would like to propose that the higher frequency of EPOR in females may contribute to the gender dependent neuroprotection of EPO. In any case, our results indicate that gender has to be taken into account even early in life, when designing strategies to protect the neonatal brain against injury.

It has been reported that EPO does not cross the blood–brain barrier in a detectable amounts when given at doses appropriate for erythropoiesis (200–400 U/kg) (Juul et al., 1999). Although the dose of EPO used in our studies is clearly above the range used for anemia treatment, there is no consensus as to the optimal dose, dose frequency, or dosing interval when using EPO to treat brain damage (Bührer et al., 2007). Pharmacokinetics of EPO have demonstrated that i.p. administration produced a higher plasma concentration than subcutaneous (s.c.) administration. At the same time, i.p. injection also resulted in a more pronounced penetration into the brain than s.c. injection after HI (Statler et al., 2007). We administered EPO i.p. in our study and used a dose that is well above the dose used for treatment of anemia. Therefore, we expect that in our study EPO could reach the brain in sufficient amounts.

In our study, the beneficial effect of 5kU/kg EPO on sensorimotor function and white matter damage in females was stronger and lasted longer than the effect of a higher (20kU/kg) dose. Interestingly, an inverted U-shaped dose-response curve for EPO has also been desescribed in a neonatal rat HI model (Kellert et al., 2007); 5ku/kg injection resulted in more neuroprotection than 2.5kU/kg or 30kU/kg; three injections had a stronger beneficial effect than one injection, although increasing treatment to 7 injections did not further improve the effect of EPO. It is not completely clear which mechanisms underlie the loss of positive effects at very high (>20kU/kg) doses of EPO.

Toxicity of EPO at very high dosage has been reported in animal experiments. In a neonatal rat HI model, administration of EPO at 20kU/kg significantly increased the number of degenerating cerebral neurons (Weber et al., 2005). In our female animals, however, a mild improvement of sensorimotor function was shown at 5 w after EPO treatment with 20kU/kg, but the improvement did not last into the adult period (10 w). The toxicity of the very high dose of EPO may partially result from increased hematocrit-associated side effects, such as hypertension and thrombo-embolism, which may increase the infarction volume of the brain and neuronal cell death (Paschos et al., 2008).

Additionally, two types of EPO receptors have been identified in an in vitro study: a homodimeric (EpoR/EpoR) receptor and a heterodimeric (CD131/EpoR) receptor (Brines et al., 2004). The protective action of EPO occurs when EPO binds to the heterodimeric CD131/EpoR, but protective effects of EPO will be lost if all EPO is bound by homodimeric receptors (Brines et al., 2004). EPO itself is also involved in the degradation of EPOR (Walrafen et al., 2005). It is possible that extremely high doses of EPO will promote the degradation of EPOR leading subsequently to a loss of the protective effects of EPO.

The rodent model of neonatal HI injury has been widely used since it was developed in 1981 (Rice et al., 1981), with some variation in the hypoxic duration (Ditelberg et al., 1996; McAuliffe et al., 2006; van der Kooij et al., 2009b). Previous studies have indicated that treatment with EPO was only effective provided a certain amount of brain matrix tissue was left (Spandou et al., 2005). In our study, we used a mild model of HI brain damage by applying only 45min 10% O_2. We specifically used this mild model to increase the potential beneficial effects of EPO.

Endogenous regeneration of neuronal tissue after HI insult is obviously not enough. EPO has been reported to have neurogenerative properies (Iwai et al., 2007). There is a 17-mer peptide neurotrophic sequence called epopeptide AB which exists in the structure of EPO. This peptide has been considered to induce proliferation, differentiation and survival of neuronal cells (Campana et al., 1998). During in vitro and in vivo studies, administration of EPO enhanced differentiation of embryonic neural stem cells into neurons, upregulated the expression of neurotrophic factors in the SVZ and increased the generation of neurons in the injured striatum (Gonzalez et al., 2007; Shingo et al., 2001; Wang et al., 2004). Our study indicated that (progenitor) cell proliferation in the SVZ was increased by treatment with EPO at 5kU/Kg after neonatal HI in females. However, we did not observe a beneficial effect of EPO treatment on HE staining in the long-term. This being said, we cannot rule out the possibility that proliferation and differentiation of progenitor cells have contributed to the improved functional outcome in EPO-treated females. Notably, EPO treatment after HI did reduce the loss of MBP staining in females. We do not know at present, however, whether the effect of EPO on MBP staining in the brain is dependent on the protection of oligodendrocyte precursors, increased formation of these cells or on stimulation of maturation oligodendrocytes. However, it may be that EPO improved axonal remodeling in our model in view of the improved sensorimotor function.

In conclusion, EPO treatment for neonatal HI is only effective in female mice provided a moderate dose of EPO is administrated.

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Chapter 4

Neuroprotective effects of hypothermia after neonatal hypoxic-ischemic brain injury are gender dependent

Xiyong Fan[1,2,3], Frank van Bel[1], Cobi J. Heijnen[2], Michael A. van der Kooij[1,2], Floris Groenendaal[1*]

[1]Department of Neonatology and [2]Laboratory for Neuroimmunology and Developmental Origins of Disease (NIDOD), University Medical Center Utrecht, the Netherlands

[3]Department of Paediatrics, Peking University First Hospital, Beijing, China

*Correspondence

Dr. Floris Groenendaal, MD, PhD

Department of Neonatology

University Medical Center Utrecht

KE 04.123.1

PO Box 85090

3508 AB Utrecht, the Netherlands

telephone: +31-88-75 5 4545

fax: +31-88-75 5 5320

Email: f.groenendaal@umcutrecht.nl

Abstract

Hypothermia has neuroprotective effects after perinatal hypoxia-ischemia (HI) in infants. In the present study we investigated long-term effects of moderate hypothermia in rats on sensorimotor function in the 'Vannucci-Rice' model of neonatal HI. P7 rats were subjected to HI, and moderate hypothermia was applied for 3h immediately after HI, while rectal temperature was sustained between 32.5-33°C. Neurodevelopmental outcome was assessed with the cylinder rearing test (CRT) performed at 3w and 6w of age. The non-impaired paw preference was calculated as (right - left)/(right + left + both)×100. Histological investigation was performed with hematoxylin–eosin (HE) and luxol fast blue (LFB) staining. At 3w of age, the non-impaired paw preference of HI rats was found similar in female and male rats. Hypothermic treatment reduced the paw preference in females ($P<0.001$) but not in males. At 6w of age, the ipsilateral paw preference was 45 ± 4 % (female) and 41 ± 4 % (male) in the normothermia group, and hypothermia reduced the paw preference to 19 ± 4 % in females ($P<0.001$), and 34 ± 7 % in males ($P=0.37$). Histology showed a significant reduction (~20%) of brain tissue loss and white matter damage only in females after hypothermia. In conclusion, moderate hypothermia has neuroprotective effects on sensorimotor function and brain lesion volume in females, whereas no protection could be demonstrated in males.

Key words

hypothermia, neonate, hypoxia, ischemia, neuroprotection, gender

Introduction

Despite the advances in obstetric and neonatal care, hypoxia-ischemia (HI) is still an important cause of neonatal brain injury and is associated with long-term neurological sequelae such as cognitive dysfunction, developmental delay, seizures, and sensory or motor impairment (Glass et al., 2007). Although the details of the mechanisms leading to brain damage are still not very clear, calcium influx (Perlman, 2006), free radical formation (Kumar et al., 2008), excitotoxicity (Papazisis et al., 2008) and inflammation (Nijboer et al., 2009) likely contribute to HI brain injury. Evidence from studies involving neonatal HI indicate that reduction of brain temperature by 2°C to 5°C provides neuroprotection by preserving energy metabolism, reducing edema, reducing accumulation of free radicals, inhibiting necrosis and apoptosis, and improving behavioral outcome (Gunn et al., 1997; Wagner et al., 2002; Ohmura et al., 2005).

Selective head cooling and systemic cooling are two methods commonly used for hypothermia in clinical practice. Of the two possible methods, moderate total body hypothermia has been used most commonly (Edwards et al., 2010).

Recently, gender dependent neuroprotection has been shown in animals models treated with 2-iminobiotin after neonatal HI (Nijboer et al., 2007). Until now, studies have reported variable results concerning the gender effect on the long-term neurodevelopmental outcome after HI and hypothermia (Bona et al., 1998; Hosono et al., 2009; Loidl et al., 2000). In the present study, we used the well-known P7 rat HI model (Rice et al., 1981) with systemic hypothermia after HI. Aim of the present study was twofold. First, we investigated whether hypothermia has neuroprotective effects on sensorimotor function and histology. Second, we examined a possible contribution of gender to this intervention.

Methods

Animals

Experiments were performed in accordance with international guidelines and approved by the Experimental Animal Committee of the University Medical Center Utrecht. Wistar rats were bred at the animal facility and surgery was performed at seven days of age (P7). Rats were housed on a normal day night cycle, weaned at the age of 4 weeks and single-housed for the remainder of the experiment. Food and water were available ad libitum. All analyses were performed in a blinded set-up.

Experimental model

In P7 pups the right common carotid artery was ligated under anesthesia (isoflurane, 5% induction/1.5% maintenance in O_2:N_2; 1:1). After 1.5 h of recovery, rats were subjected to 8% O_2 in N_2 for 90 min at a temperature of 37°C (Rice et al., 1981). Sham-treated controls underwent anesthesia and incision but no artery occlusion or hypoxia (4 males, 4 females).

56 rat pups (28 males, 28 females) underwent unilateral hypoxic-ischemia. 4 rats (2 males, 2 females, 7.1%) died from hypoxia before randomization and treatment.

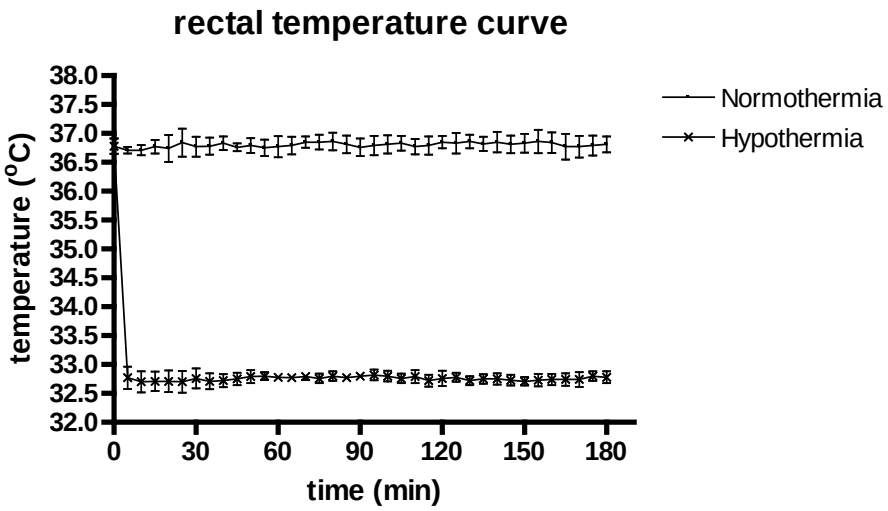

Figure 1.

The pattern of rectal temperature (mean±SD) of "sentinel" rats (n=10) in an infant incubator by 3-hour intervention of normothermia (37°C) or hypothermia (32.5-33 °C).

Hypothermia

Hypothermia started immediately after hypoxia. Animals were placed into an infant incubator for 3h which was temperature and humidity-controlled. Rectal temperature was continuously monitored in "sentinel" pups in the same incubator by using a calibrated (<0.1°C deviation) temperature probe (BIO-BRET-4, Bioseb, Vitrolles cedex, France) (Hobbs et al., 2008). The sentinel pups were not included in the final analysis and sacrificed immediately after hypothermia according to literature (Hobbs et al., 2008). The incubator temperature setting was adjusted to keep the rectal temperature between 32.5°C-33°C. Rectal temperature was recorded every 5 min. Animals for normothermia treatment were placed in another infant incubator, and the rectal temperature (kept

between 36.5°C-37°C) was measured in the same way to the hypothermia group (figure 1).

Cylinder rearing test

Cylinder rearing test (CRT) was used to assess sensormotor functions in neonatal rodent HI models (van der Kooij et al., 2009). At 3 w and 6 w of age between 9 and 10 am, animals were individually placed in a Plexiglas transparent cylinder (3 weeks: 11 cm ø× 30 cm height; 6 weeks: 25 cm ø×30 cm height) and observed for 3 min in the housing room. Initial non-impaired forepaw (left/right/both) preference of weight-bearing contacts during full rear was recorded. The relative proportion of right (ipsilateral) forepaw contacts was calculated as: (right - left)/(right + left + both)×100 (Chang et al., 2005).

Histology

Rats were sacrificed at 6w of age with pentobarbital (300 mg/kg i.p.) and perfused with 4% formaldehyde in phosphate-buffered saline. Brains were mounted in paraffin from which coronal sections (8µM) were cut (at -3.20 mm from bregma). Brain sections were stained with hematoxylin–eosin (HE) and luxol fast blue (LFB). Both hemispheres were outlined on full section images with a Nikon D1 digital camera (Nikon, Tokyo, Japan). Brain areas for HI were outlined manually using Photoshop CS4 software (Adobe Systems Inc., San Jose, CA, USA) and the ratio of ipsi- to contralateral areas was calculated (Nijboer et al., 2007). The area of LFB staining in both hemispheres was quantified using ImageJ software (http://rsb.info.nih.gov/ij/, 1997-2006) and the ratio of ipsi- to contralateral areas was also calculated (Nijboer et al., 2007).

Statistics

Statistical analysis was performed using SPSS and Graphpad Prism software. Data are presented as means ± SEM and a p-value<0.05 was accepted as statistically significant. One-way ANOVA with Bonferroni post tests was used to analyze group differences.

Results

Paw preference (cylinder rearing test)

At the age of 3w and 6w (2w and 5w after HI), we assessed sensorimotor function using the CRT. Sham control animals did not show a paw preference in the CRT (figure 2). At 3 and 6 weeks of age hypothermia reduced the non-impaired forepaw preference in HI-

females from 69 ± 6 % to 31 ± 7 % (3w, P<0.001) and 45 ± 4 % to 19 ± 4% (6w, P<0.001). In HI-males hypothermia did not affect non-impaired forepaw preference: 65± 8 % to 55 ± 8 % (3w, P=0.16), and 41 ± 4 % to 34 ± 7 % (6w, P=0.37) (figure 2).

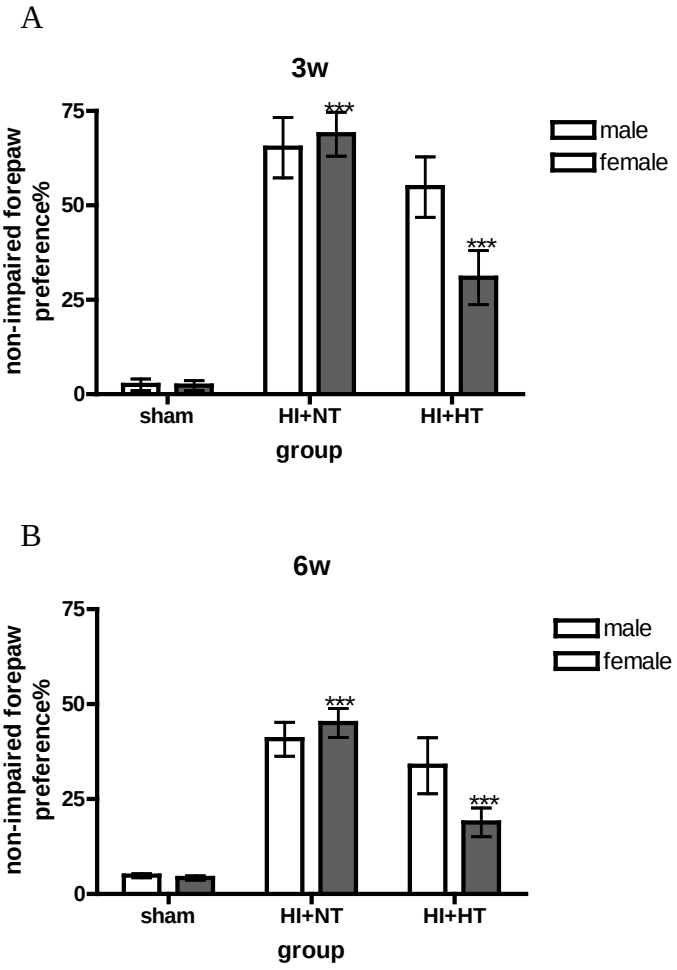

Figure 2. **Effect of hypothermia on preference to use the non-impaired (ipsilateral) paw in the cylinder rearing test.**

Male and female P7 rats were exposed to HI and treated with 3h hypothermia or normothermia. At 3w (A) and 6w (B) old, sensorimotor function was assessed in the cylinder rearing test.

HI=hypoxia-ischemia, HT=hypothermia, NT=normothermia

*** HI+NT vs HI+NT P<0.001; n=13 per group (sham group n=4)

Analysis of ipsilateral brain lesion volume

The effect of hypothermia on brain damage was determined at 6w. The morphological changes in the brain as visualized after HE staining are shown in figure 3A. There was a ~75% loss of tissue at the ipsilateral hippocampal level after HI. The lesion areas were similar in male and female rats. Hypothermia showed a 20% protection of brain tissue loss in females only (P<0.05) (figure 3B).

Similar to what was observed for total area loss as determined after HE staining, the data in figure 3C and 3D show that a significant reduction (~20%) of white matter damage in the LFB staining was present only in females after treatment with hypothermia (P<0.01). In addition, there was no statistically significant effect on brain lesion volume in males in either HE or LFB staining.

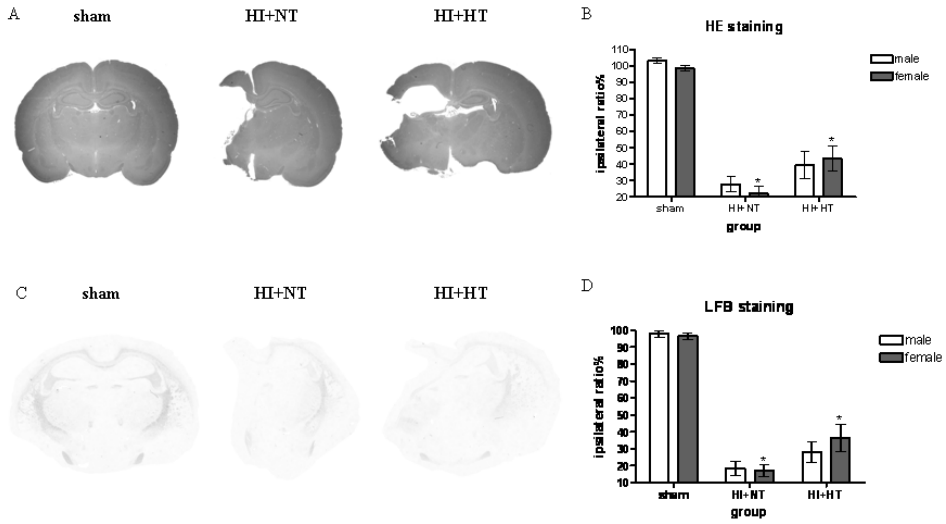

***Figure 3*. Effect of hypothermia treatment on brain area loss and white matter loss as determined after hematoxylin–eosin (HE) and luxol fast blue (LFB) staining respectively at 6 w of age (5w after HI).**

-A) Representative examples of brain area loss at hippocampal levels of the brain after HE staining

-B) The ratio of ipsilateral/contralateral areas (hippocampal level) after HE staining

-C) Representative examples of white matter changes at hippocampal levels of the brain after LFB staining

-D) The white matter ratio of ipsilateral/contralateral areas (hippocampal level)

HI=-hypoxia-ischemia, HT=hypothermia, NT=normothermia

* HI+NT vs HI+HT in females P<0.05; n= 13 per group (sham group n=4)

Discussion

In the present study, we examined the neuroprotective effects of moderate hypothermia in a commonly used rat model of neonatal HI (Rice et al., 1981). We demonstrated a pronounced neuroprotective effect of moderate hypothermia in females only.

Clinical trials have shown that the use of hypothermia as standard therapy for asphyxiated full term neonates improves long-term outcome (Edwards et al., 2010), with a risk difference on the combined rate of death and severe disability of -0.11 and a number to treat of 9. Gender differences were not mentioned in this meta-analysis of human clinical trials.

Several *in vitro* and *in vivo* studies have also demonstrated the neuroprotective properties of hypothermia on biochemical marker changes in a rat model (Hasegawa et al., 2009; Fukui et al., 2006). However, since the effect of moderate hypothermia has been assessed most of the time only at early timepoints, we cannot exclude that the therapy may only delay the expression of brain damage (Trescher et al., 1997). In our present study, we determined the effects of moderate hypothermia on long-term behavioral and histological outcome. We observed a good improvement of sensorimotor function and a reduction of brain damage in female rats but not in male rats. The results on the gender-specific effects of hypothermia in our study support the data of Hosona et al. and Hoeger et al. (Hosono et al., 2009; Hoeger et al., 2006) who demonstrated improvement of long-term behavior including motor function as determined by the rota-rod test in females only.

Gender effects have been demonstrated to play a role in neuroprotection of neonatal brain damage with treatment of 2-iminobiotin and erythropoietin (Nijboer et al., 2007; Wen et al., 2006). These effects could be detected on the level of reduction in lesion volume as well as behavioral tests. Female rodents apparently received more benefits from the neuroprotective interventions. Importantly, the gender dependent effects of treatment are not associated with gender differences in the severity of cerebral injury after the insult as such (Nijboer et al., 2007). Moreover, the gender-specific effects of treatment of brain injury are not simply a result of a difference in sex hormones, since there is hardly estrogen secretion at the time of the insult (P7) and the week thereafter. So it is more likely that gender differences in the response to therapy result from the use of different cellular pathways such as a gender specificity in the activation of different apoptotic signaling routes after HI (Nijboer et al., 2007; Hurn et al., 2005). Our experiment has shown that gender effects also contribute to the therapeutic effect of

hypothermia in long-term neurodevelopmental outcome. Previously, studies have alluded to gender differences in the neuroprotective effects of moderate hypothermia, but have not examined this in detail (Bona et al., 1998). However, taken all studies together, the data point to stronger protective effects in females compared to males (Hoeger et al., 2006).

Recent studies have also shown that the main benefit of hypothermia might be to increase the therapeutic time window, allowing to combine the treatment with other therapeutic strategies after HI (Dietrich et al., 1993; Guan et al., 2000; Hobbs et al., 2008). It is likely that hypothermia combined with other therapies will have more neuroprotective effects. A recent study in neonatal rats indicated that hypothermia combined with the gaseous anesthetic Xenon confers greater protection in both behavioral tests and histology after HI than either treatment alone (Hobbs et al., 2008). However, gender differences have not been mentioned in this study. Combination of hypothermia with either erythropoietin (Cariou et al., 2008) or melatonin (Hlutkin et al., 2008) also showed increased neuroprotection in comparison with hypothermia alone in adult animal models. However, we show here that hypothermia as such does not only delay the damage in female rats, but also confers protection of grey and white matter.

In conclusion, moderate systemic hypothermia after neonatal hypoxia-ischemia has a pronounced neuroprotective effect on sensorimotor function and brain volume loss in females but not in males. Further research has to be performed to investigate whether the treatment in females and males may be improved when combined with pharmacological neuroprotection (see Chapter 5).

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Chapter 5

The effect of combined treatment with hypothermia and EPO on brain injury and behavior in neonatal rats after hypoxia-ischemia

Xiyong Fan[1,2,3], Frank van Bel[1], Michael A. van der Kooij[1,2], Cobi J. Heijnen[2], Floris Groenendaal[1]*

[1]Department of Neonatology and [2]Laboratory for Neuroimmunology and Developmental Origins of Disease (NIDOD), University Medical Center Utrecht, the Netherlands

[3]Department of Paediatrics, Peking University First Hospital, Beijing, China

***Correspondence**

Dr. Floris Groenendaal, MD, PhD

Department of Neonatology

University Medical Center Utrecht

KE 04.123.1

PO Box 85090

3508 AB Utrecht, the Netherlands

telephone: +31-88-75 5 4545

fax: +31-88-75 5 5320

Email: f.groenendaal@umcutrecht.nl

Abstract

Both moderate hypothermia and erythropoietin (EPO) have been reported to have neuroprotective effects as a treatment for perinatal hypoxia-ischemia (HI). We investigated a possible synergic effect of the combination of hypothermia and EPO on the sensorimotor function and lesion area in a rat model of neonatal HI. P7 rats were subjected to HI, and hypothermia (rectal temperature 32.5-33°C) was applied for 3 hours starting immediately after HI. EPO was injected intraperitoneally in a dose of 5kU/kg at 0h, 24h and 48h after HI. Neurodevelopmental outcome was assessed by cylinder rearing test at 3w and 6w of age. Lesion volume was determined by hematoxylin-eosin (HE) staining. White matter loss was explored using luxol fast blue (LFB) staining. At 3w of age, hypothermia alone improved non-impaired forepaw preference exclusively in females during cylinder rearing test, and EPO alone did not have a significant effect at this time point. Combining hypothermia and EPO improved paw preference in both males ($P<0.01$) and females ($P<0.001$). At 6w of age, either hypothermia or EPO alone improved paw preference in females respectively, but not in males. Combining hypothermia with EPO did not augment improvement in females at 6w. In males the combined treatment resulted in a non-significant reduction of the paw preference. HE staining showed a significant reduction (~20%) of brain tissue loss only in females after hypothermia treatment alone ($P<0.05$). Staining with LFB indicated a significant reduction (~20%) of white matter damage after hypothermia treatment alone ($P<0.05$) and a trend (~15%) after hypothermia-EPO combination in females only ($P=0.08$). In conclusion, both hypothermia and EPO resulted in long-term benefits in sensorimotor function and brain injury only in females. The combination of hypothermia and EPO had no synergistic effect in females. In males only a short-lasting improvement of sensorimotor function could be shown with the EPO-hypothermia combination.

Key words

hypothermia, erythropoietin, neonate, hypoxia-ischemia, neuroprotection

Introduction

Perinatal hypoxia-ischemia (HI) is an important cause of neonatal brain injury and is associated with long-term neurological sequelae such as cognitive dysfunction, developmental delay, seizures, and sensory or motor impairment (Barnett et al., 2002; Glass et al., 2007). Several newborn animal studies indicate that reduction of brain temperature by 2°C to 5°C for 3h (if staring no later than 6h after HI) provides neuroprotection by preserving energy metabolism, reducing edema, reducing accumulation of free radicals, inhibiting necrosis and apoptosis, and improving behavioral outcome (Gunn et al., 1997; Groenendaal et al., 2009; Hosono et al., 2009; Wagner et al., 2002; Ohmura et al., 2005; Vannucci et al., 2004).

Since reduction of brain damage by hypothermia was shown to be limited (see Chapter 4) and only 1 in 6 children benefit from hypothermia in clinical practice (Gluckman et al., 2005), a combination with other strategies may improve long-term neurodevelopment (van Bel and Groenendaal, 2008).

Erythropoietin (EPO) has been proven to reduce free radical formation, inappropriate pro-inflammatory and apoptotic activity (Sun et al., 2005; Kellert et al., 2007). EPO also stimulates neurogeneration as a trophic factor. Both in vitro and in vivo studies have indicated that EPO stimulates neuronal differentiation from neural progenitor cells (Shingo et al., 2001; Wang et al., 2004). Most experimental studies in neonatal animals with EPO treatment after HI were indeed beneficial with respect to histological outcome (van der Kooij et al., 2008; Sola et al., 2005). Furthermore, EPO treatment of perinatally asphyxiated human term neonates showed improvement of neurological outcome (Zhu et al., 2009). In adult patients with cardiac arrest a combined treatment with hypothermia plus EPO has shown to be neuroprotective (Cariou et al., 2008).

In the present study, we examined whether a combined treatment of hypothermia and EPO reduced brain injury and improved long-term behavioral outcome in a P7 rat HI model. Behavioral tests were performed during the adolescent and adult periods to assess neurodevelopmental outcome.

Methods

Animals

Experiments were performed in accordance with international guidelines and approved by the Experimental Animal Committee of the University Medical Center Utrecht. Wistar

rats were bred at the animal facility of Utrecht University. Experiments were performed at seven days of age (P7). Rats were housed on a normal day night cycle, weaned at the age of 4 weeks and single-housed for the remainder of the experiment. Food and water were available ad libitum. All analyses were performed in a blinded set-up.

Experimental model

P7 pups were anesthetized (isoflurane, 5% induction/1.5% maintenance in O_2:N_2O; 1:1) and the right common carotid artery was ligated. After 1.5 h of recovery, rats were subjected to 8% O_2 in N_2 for 90 min (n = 26) at a temperature of 37°C (Rice et al., 1981). Sham-treated controls underwent anesthesia and incision but no artery occlusion or hypoxia (12 males, 12 females).

110 rat pups (55 males, 55 females) underwent unilateral hypoxic-ischemia. Six rats (3 males, 3 females) (5.5%) died during hypoxia before randomization and EPO treatment, but no deaths occurred after HI insult.

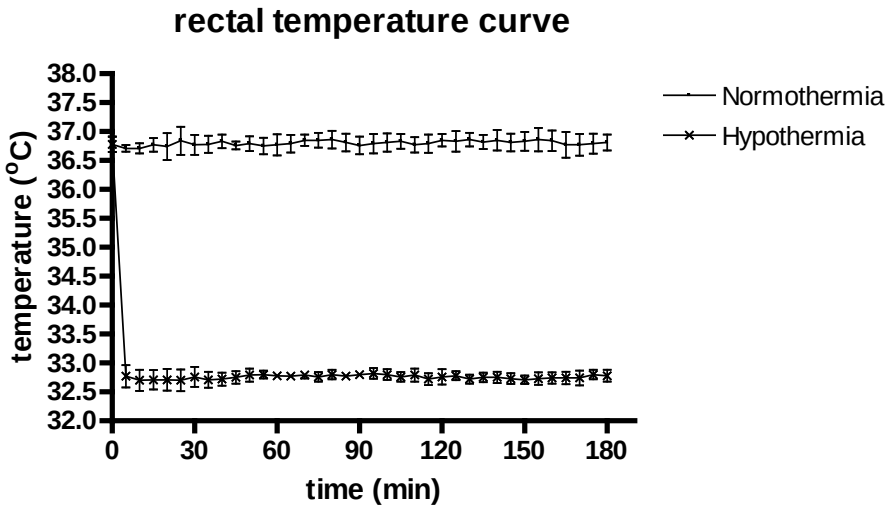

Figure 1.

The pattern of rectal temperature (mean ± SD) from so called "sentinel" rats (n=10) in an infant incubator by 3-hour intervention of normothermia (37°C) or hypothermia (32.5-33 °C)

Hypothermia

Hypothermia was started immediately after hypoxia. Animals (including 8 sham treated animals) were placed into an infant incubator for 3h (30 males, 30 females), and rectal

temperature was continuously measured in additional so called “sentinel” pups (gender randomized) in the same incubator by using a calibrated (<0.1°C deviation) temperature probe (BIO-BRET-4, Bioseb, Vitrolles cedex, France) (Hobbs et al., 2008). These sentinel pups were excluded from analysis and sacrificed immediately after hypothermia, as the stress of carrying a probe may affect outcome (Hobbs et al., 2008). The incubator temperature setting was adjusted to keep the rectal temperature between 32.5°C-33°C. Rectal temperature was recorded every 5 min. Animals (8 sham treated animals included) for normothermia treatment were placed in another infant incubator (30 males, 28 females), and the rectal temperature, which was sustained between 36.5°C-37°C, was measured in the same way (figure 1).

EPO administration

EPO (EPREX, Janssen-Cilag B.V., Netherland) was injected intraperitoneally (ip.) at 5kU/kg immediately after hypothermia, with two additional doses given at 24h and 48h after hypoxia respectively. Vehicle treated animals were given sterile saline injections in the same way.

Cylinder rearing test

The cylinder rearing test (CRT) was used to assess sensorimotor function (Schallert et al., 2000). At 3 weeks and 6 weeks of age between 9 and 10 am, animals were individually placed in a Plexiglas transparent cylinder (3 weeks: 11 cm ø× 30 cm height; 6 weeks: 25 cm ø×30 cm height) and observed for 3 min in the housing room. Initial non-impaired forepaw (left/right/both) preference of weight-bearing contacts during full rear was recorded. The relative proportion of right (ipsilateral) forepaw contacts was calculated as: (right - left)/(right + left + both)×100 (Chang et al., 2005).

Histology

Rats were sacrificed at 6w of age with pentobarbital (300 mg/kg i.p.) and perfused with 4% formaldehyde in phosphate-buffered saline. Brains were mounted in paraffin from which coronal sections (8μM) were cut at hippocampal level (-3.20 mm from bregma). Brain sections were stained with hematoxylin–eosin (HE) and luxol fast blue (LFB). Both hemispheres were outlined on full section images with a Nikon D1 digital camera (Nikon, Tokyo, Japan). Brain areas for HI were outlined manually using Photoshop CS4 software (Adobe Systems Inc., San Jose, CA, USA) and the ratio of ipsi- to contralateral areas was calculated (Nijboer et al., 2007). The area of LFB staining in both hemispheres was

quantified using ImageJ 1.42q software (http://rsb.info.nih.gov/ij/, 1997-2006) and the ratio of ipsi- to contralateral areas was also calculated (Nijboer et al., 2007).

Statistics

Statistical analysis was performed using SPSS and Graphpad Prism software. Data were presented as means ± SEM and a p-value<0.05 was accepted as statistically significant. One-way ANOVA with LSD post tests was used to analyze group differences in area loss of ipsilateral hemispheres, the result from cylinder rearing test.

Results

Paw preference (cylinder rearing test)

At 3w and 6 w of age, we assessed sensorimotor function using the CRT. Sham-treated rats did not show any paw preference during rearing in the CRT (paw preference <±5%, figure 2).

At 3 w (2 w after the HI insult) HI resulted in 65 ± 8 % (male) and 68 ± 6 % (female) paw preference of the unimpaired forepaw in the normothermia groups (figure 2A). Hypothermia alone improved paw preference at 31 ± 7 % in females only, whereas EPO did not result in significant changes. The combination of hypothermia and EPO improved paw preference significantly (37 ± 7 % in males, 28 ± 5 % in females) (figure 2A).

At 6w of age (i.e. 5 weeks after the HI insult), 41 ± 4 % (male) and 45 ± 4 % (female) paw preferences were seen after normothermia in both males and females. Hypothermia or EPO alone showed improvement of paw preference in females (19 ± 4 % and 34 ± 4 % respectively), but not in males. Combination of hypothermia with EPO did not augment neuroprotection in females during this period (16 ± 4%) (figure 2B). Combination of hypothermia with EPO in males resulted in a non-significant reduction of the paw preference (at 30 ± 5%).

Analysis of ipsilateral surface area

The effect of hypothermia and EPO on brain damage was determined at 6w. The morphological changes in the brain as visualized after HE staining are shown in figure 3A. There was a ~75% loss of tissue at the ipsilateral hippocampal level after HI. The lesion areas were similar in male and female rats. Hypothermia alone indicated a 20% protection of brain tissue loss only in females (P<0.05) (figure 3C). However, there was no effect in either males or females of EPO treatment alone. Combination of hypothermia

and EPO also resulted in a non-significant reduction (~10%) of brain tissue loss in both genders (figure 3C).

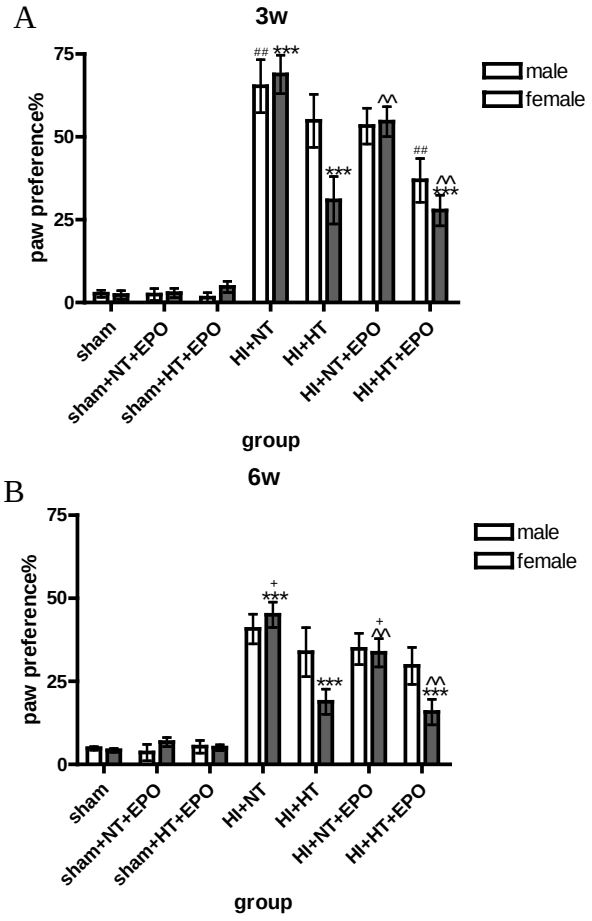

***Figure 2*. Effect of hypothermia and EPO on preference to use the non-impaired (ipsilateral) paw in the cylinder rearing test.**

Male and female P7 rats were exposed to HI and treated with 3h hypothermia or normothermia, combined with EPO (5kU/kg) or vehicle. At 3w (A) and 6w (B) of age, sensorimotor function was assessed in the cylinder rearing test.

EPO=erythropoietin, HI=hypoxia-ischemia, HT=hypothermia, NT=normothermia

*** HI+NT vs HI+HT, HI+NT vs HI+HT+EPO in females $P<0.001$

^^ HI+NT+EPO vs HI+HT+EPO in females $P<0.01$

HI+NT vs HI+HT+EPO in males $P<0.01$

+ HI+NT vs HI+NT+EPO in females $P<0.05$

n=13 per group (sham group n=4)

Similar to what was observed for total area loss as determined after HE staining, the data in figure 3B and 3D showed a significant reduction (~20%) of white matter damage in the LFB staining only in females after treatment with hypothermia alone (P<0.01). No effect was seen in either males or females with EPO treatment alone. However, there was only a trend of reduction (~15%) of white matter damage in females after hypothermia-EPO combination (P=0.08).

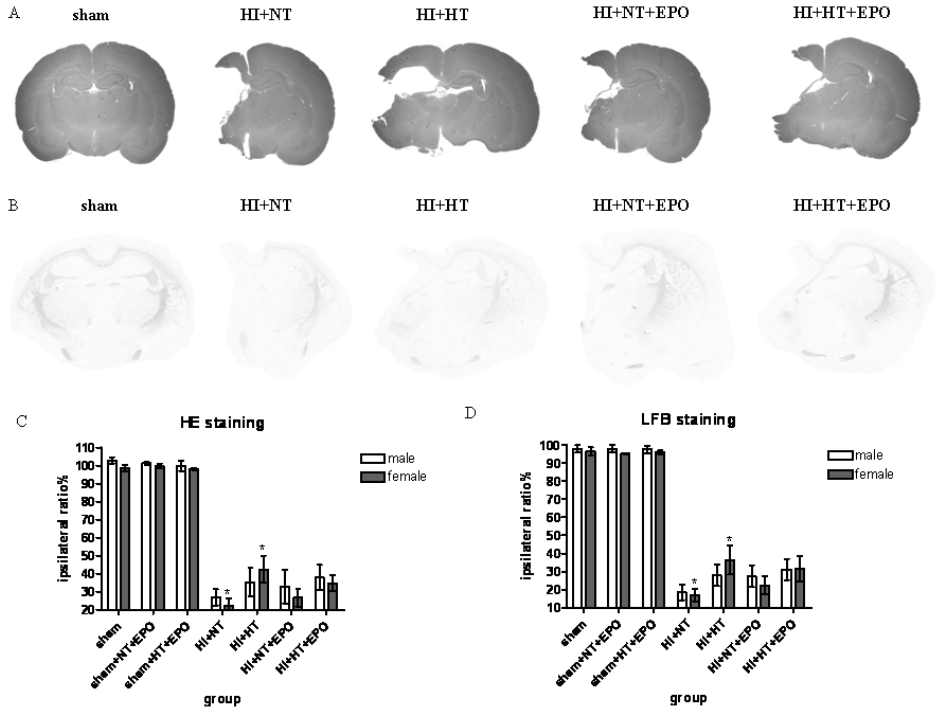

***Figure 3*. Effect of hypothermia and EPO treatment on brain area loss and white matter loss as determined after hematoxylin–eosin (HE) and luxol fast blue (LFB) staining respectively at 6 w of age (5w after HI).**

-A) Representative examples of brain area loss at hippocampal levels of the brain after HE staining (Examples of sham+NT+EPO and sham+HT+EPO groups are similar to sham group, and they are not shown here)

-B) Representative examples of white matter changes at hippocampal levels of the brain after LFB staining (Examples of sham+NT+EPO and sham+HT+EPO groups are similar to sham group, and they are not shown here)

-C) The ratio of ipsilateral/contralateral areas (hippocampal level) after HE staining

-D) The white matter ratio of ipsilateral/contralateral areas (hippocampal level)

HI=hypoxia-ischemia, HT=hypothermia, NT=normothermia

* HI+NT vs HI+HT in females P<0.05; n= 13 per group (sham group n=4)

Discussion

In the present study, we used a neonatal hypoxic-ischemic rat model to assess the protective effects of moderate hypothermia combined with EPO applied immediately after HI. We investigated the sensorimotor function outcome at adolescent and adult periods. Moreover we determined the effect of the combined treatment on lesion area and white matter loss. The hypothermia conferred long-lasting protection only in females, but EPO-hypothermia combination did not augment the protection by hypothermia alone. In males no major permanent neuroprotection could be obtained with either strategy.

EPO has been increasingly used in relatively high dosages (0.5-3kU/kg) in (preterm) infants to improve long-term outcome (Juul et al., 2008; Bierer et al., 2006; Fauchère et al., 2008). Moreover, EPO has been accepted as a neuroprotective pharmacological approach (van der Kooij et al., 2008; Sola et al., 2005). Moreover, it has been used, combined with hypothermia, in adult patients after out-of-hospital cardiac arrest (Cariou et al., 2008).

The mechanism of action of hypothermia as a therapy for neonatal hypoxic-ischemic brain injury is still under investigation. More and more studies considered that the main benefit of hypothermia might be the delay of the expression of brain damage thereby extending the therapeutic window, allowing additional therapeutic interventions after HI (Dietrich et al., 1993; Guan et al., 2000; Hobbs et al., 2008; Trescher et al., 1997). Hypothermia combined with Xenon has already demonstrated to have greater protection in neonatal rats in both behavioral tests and histology after HI than either treatment alone (Hobbs et al., 2008). Combination with melatonin (Hlutkin et al., 2008), magnesium (Zausinger et al., 2003), and neurotrophic factors (Berger et al., 2004) also shows synergic neuroprotection in animal models of adult stroke.

Our study is the first to examine the hypothermia-EPO combination in newborns (animal model) to assess neuroprotection. We found short-lasting but not long-lasting improvement of sensorimotor function with the combined treatment in males compared to single treatment, whereas addition of EPO did not further improve outcome in females compared with hypothermia alone. Since it is known that hypothermia might increase the therapeutic time window and delay the appearance of “secondary energy failure” (Dietrich et al., 1993; Guan et al., 2000), this combination may promote the protective effects of EPO during this therapeutic window and generate synergy in males. To explain the non-synergic effect of hypothermia combined with EPO in females, the neuroprotective mechanisms of these two interventions should be considered. Recently,

both the hypothermia and EPO have been recognized to reduce free radical formation, inhibit inflammation response and reduce apoptotic activity (Groenendaal et al., 2009; van der Kooij et al., 2008; Wagner et al., 2002; Sun et al., 2005; Kellert et al., 2007). In our study, single treatment with hypothermia has already shown a strong protection in females, and is probably powerful enough to block the secondary energy failure. Meanwhile, treatment with EPO under normothermia also showed the improvement of sensorimotor function in females, therefore no further effect appeared when hypothermia-EPO combining. It has already been reported that apoptotic effects in males are not the same as in females. Both in vitro and in vivo studies indicated that female neurons predominantly use the caspase-dependent pathway of apoptosis while male neurons prefer to use of the caspase-independent pathway (Du et al., 2004; Zhu et al., 2006). In animal models of neonatal HI, reduction of caspase activities has been shown after either EPO or hypothermia treatment (van der Kooij et al., 2009; Gressens et al., 2008). In males there was no effect of EPO under normothermic conditions, but probably the hypothermia works by allowing EPO to act under conditions of decreased metabolic demand. Under this condition, there may be some unknown pathways which contribute to the short-lasting protective in males.

Gender differences are important in neonatal HI. Studies have reported that the neuroprotective effect of hypothermia may show gender difference (Bona et al., 1998). Females are protected more than males as far as motor function, cognitive function and other behavior are concerned (Hoeger et al., 2006). Gender effects have also been demonstrated to play a role in other post-hypoxic neuroprotective strategies such as 2-iminobiotin and EPO (Nijboer et al., 2007; Wen et al., 2006). These effects on neonatal brain injury of are probably not simply a result of the hormonal influence but are properties of gender dependent gene expression and the properties of the female neuronal cell (Hurn et al., 2005). As we mentioned above, male and female neurons have different pathways of apoptosis. Translocation of apoptosis-inducing factor (AIF) is considered to contribute to the caspase-independent pathways in males (Zhu et al., 2006). Our previous data has also shown contribution of gender effect, which benefits females only, to the improvement of sensorimotor function when treated with EPO or hypothermia (see Chapter 3 and Chapter 4). In the present study, we found combined therapies benefited males only on the short-term (3w), but no long-lasting protection (6w) can be seen from either functional improvement or histology outcome. Results of the present study indicated that the combined therapy of hypothermia and EPO might be in

particular useful for males who did not show improvement with a single neuroprotective strategy.

In conclusion, in males the combination of EPO and hypothermia provides short-lasting, but not long-lasting improvement of sensorimotor function. In females, long-lasting benefit has already been seen using hypothermia, whereas hypothermia-EPO combination does not further improve outcome. Although we did not find strong benefits from the combination of hypothermia and EPO, we still suggest that single or combined therapy should be chosen according to different insults and different genders.

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Chapter 6

The p53 inhibitor pifithrin-μ protects against neonatal hypoxic-ischemic brain damage

Xiyong Fan[1,2,3], Cora H. Nijboer[2], Michael van der Kooij[1,2], Jitske Zijlstra[2], Annemieke Kavelaars[2], Frank van Bel[1], Cobi J. Heijnen[2*]

[1]Department of Neonatology and [2]Laboratory of Neuroimmunology and Developmental Origins of Disease (NIDOD), University Medical Center Utrecht, the Netherlands

[3]Department of Paediatrics, Peking University First Hospital, Beijing, China

[*]**Correspondence**

Cobi J. Heijnen, PhD

Lab. for Neuroimmunology and Developmental Origins of Disease (NIDOD)

University Medical Center Utrecht

KC03.068.0

Lundlaan 6

3584 CX Utrecht, the Netherlands

Telephone: +31-88-75 54359

Email: c.heijnen@umcutrecht.nl

Abstract

Background: Hypoxia-ischemia (HI) during the perinatal period remains a frequent cause of morbidity and mortality in the human neonate. The need for new therapeutic strategies is still high at present. Previously we have shown that inhibition of the transcription factor NF-κB using the TAT-NBD peptide has strong neuroprotective effects on HI-induced brain damage in neonatal rats. Interestingly, treatment with the TAT-NBD peptide prevented the early increase in mitochondrial association of the pro-apoptotic molecule p53 that occurred during the first hours after the insult. In the present study we investigated whether short-term treatment with pifithrin-μ (PFT-μ), an inhibitor of p53 mitochondrial association, protects against HI brain injury in neonatal rats. **Methods**: 7-Day-old (P7) Wistar rats were subjected to unilateral carotid artery occlusion followed by hypoxia (8% O_2, 120 min) and were treated intraperitoneally with PFT-μ (8 mg/kg) at 0 and 3 h post-HI. **Results**: PFT-μ treatment after HI completely prevented the HI-induced increase in mitochondrial p53 association. Moreover, PFT-μ treatment reduced MAP2 loss as a marker of neuronal damage by ~66% as determined at 2 days after HI. Neuroprotection by PFT-μ treatment was associated with strong inhibition of apoptotic pathways at 24 h after the insult; HI-induced cytosolic cytochrome *c* and subsequent activation of caspase 3 were almost completely prevented after PFT-μ treatment. PFT-μ treatment also significantly improved sensorimotor and cognitive function as determined at 6-10 weeks after the insult. Improved functional outcome was associated with long term reductions of infarct size by ~79 % and white matter loss by ~70 %. **Conclusion**: Prevention of mitochondrial association of p53 by PFT-μ treatment after HI markedly improves functional outcome and reduces lesion size caused by HI in neonatal rats. We propose that short-term inhibition of mitochondrial p53 association can develop into a novel and powerful neuroprotective strategy.

Key words

neonatal, hypoxia-ischemia, p53, pifithrin, neuroprotection

Introduction

Hypoxia-ischemia (HI) in the perinatal period in human neonates is associated with high mortality and morbity with life-long consequences such as cerebral palsy, mental retardation and other major neurodevelopmental disabilities (Volpe, 2001; Ferriero, 2004). Despite improvements in perinatal care and extensive experimental studies to design a successful therapy for these infants, clinical therapies are very limited. Although hypothermia, the most widely applied clinical intervention, has been shown to improve outcome of asphyxiated babies, the improvement is moderate and hypothermia appears to be only effective in mildly-affected children (Azzopardi et al., 2009; Gluckman et al., 2005; Shankaran et al., 2005; Edwards et al., 2010). Thus, there is a need for effective (add-on) pharmacological interventions, especially for severely-affected neonates (Perlman, 2006; Gonzalez and Ferriero, 2008; Cilio and Ferriero, 2010).

Apoptosis or programmed cell death is thought to be the major form of cell death in the developing nervous system and plays an important role in several neurodegenerative disorders including ischemic brain damage (Mattson, 2000; Mattson et al., 2001; Broughton et al., 2009). The tumor suppressor gene and transcription factor p53 is a central player in apoptotic cell death.

Various types of cellular stress, including DNA damage and hypoxia increase p53 protein levels, which are normally maintained at low steady-state levels (Culmsee and Mattson, 2005).

p53 acts as a transcription factor and enhances expression of pro-apoptotic target genes, including Bcl-2 family members PUMA, Noxa and Bax, which act together at the mitochondria to induce mitochondrial outer membrane permeablilization (MOMP). In addition, transcriptional repression of anti-apoptotic genes can contribute to the pro-apoptotic effect of p53 (Oren, 2003). Importantly, p53 can also have pro-apoptotic effects via a *transcription-independent* pathway that involves effects of p53 directly at the mitochondria (Speidel 2010; Vaseva and Moll, 2009; Green and Kroemer, 2009). In response to a death stimulus, p53 rapidly translocates to the mitochondria where it induces Bax pore formation leading to MOMP and release of pro-apoptotic factors like cytochrome *c* from the mitochondria into the cytosol. The increase cytosolic level of cytochrome c then activates caspase 3-dependent apoptotis (Saelens et al., 2004; Garrido et al., 2006; Galluzzi et al., 2009).

We have previously shown that exposure of neonatal rats to cerebral HI, induced mitochondrial association of p53 early after the insult (0.5-6 h) (Nijboer *et al.*, 2008).

Furthermore, we have shown that peripheral administration of the established NF-κB inhibitor TAT-NBD after HI results in strong neuroprotection (Nijboer *et al.*, 2008; van der Kooij et al., 2010). Remarkably, neuroprotection by TAT-NBD was associated with nearly complete inhibition of the mitochondrial translocation of p53 and prevention of the release of cytochrome *c* to the cytosol and activation of the executioner caspase 3. These findings led us to the hypothesis that inhibition of the HI-induced increase in p53 association with the mitochondria may have neuroprotective effects in vivo. To test this hypothesis we used pifithrin-μ (PFT-μ), a small molecule inhibitor of p53 association with the mitochondria (Strom et al. 2006). PFT-μ inhibits p53 binding to the mitochondria by reducing its affinity to Bcl-2 and Bcl-xL. It is already known that PFT-μ treatment rescues mouse thymocytes from radiation-induced p53-mediated apoptosis *in vitro.* Moreover, PFT-μ has been shown to provide radioprotection in mice *in vivo*. (Erster *et al.*, 2004; Strom *et al.*, 2006).

In the present study we investigated the protective effect of PFT-μ treatment after HI on HI-induced apoptosis, brain damage and sensorimotor as well as cognitive function.

Materials and Methods

Animals

The animal committee of Academic Biomedical Center Utrecht (DEC-ABC) approved all experiments. At postnatal-day 7 (P7), Wistar rats underwent occlusion of the right common carotid artery under isoflurane anesthesia followed by 120 min hypoxia (8% O_2). Sham controls underwent anaesthesia and incision only. All analyses were performed in a blinded set-up.

PFT-μ (Sigma-Aldrich, Steinheim, Germany) was dissolved in DMSO (20 mg/ml), diluted to 0.8 mg/ml in PBS and administered i.p. at a dose of 8 mg/kg. Vehicle-treated HI animals received an injection of 4% DMSO in PBS. Animals received injections of vehicle or PFT-μ directly (0 h) and 3 h after HI.

Histology

Rats were terminated by an overdose pentobarbital and perfused with 4% paraformaldehyde in PBS. Coronal paraffin sections (8 μm) (at -3.20 mm from bregma) were stained with hematoxylin-eosin (HE), luxol fast blue (LFB) or with mouse-anti-MAP2 (Sigma-Aldrich, Steinheim, Germany) followed by biotin-labeled horse-anti-mouse

antibody and revealed using Vectastain ABC kit (Vector-Labs, Burlingame, CA) and diaminobenzamidine.

Full section images were captured with a Nikon D1 digital camera (Nikon, Tokyo, Japan). Brain areas staining positively for MAP2 or HE were outlined manually using image processing tools in Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA, USA) and the ratio of ipsi- to contralateral areas was calculated (Nijboer *et al.*, 2007). The area of LFB staining in both hemispheres was quantified using ImageJ software (http://rsb.info.nih.gov/ij/, 1997-2006) and the ratio of ipsi- to contralateral areas was calculated (Nijboer *et al.*, 2007).

Western blotting

Rats were terminated by an overdose of pentobarbital, brains were rapidly collected and frozen in liquid nitrogen. Cytosolic and mitochondrial brain protein fractions of ipsi- and contralateral hemispheres were prepared as described (Nijboer *et al.*, 2007). Proteins were separated by SDS-PAGE, transferred to Hybond-C membranes (Amersham, Buckinghamshire, UK) and revealed using mouse-anti-p53, rabbit-anti-cleaved caspase 3 (both Cell Signaling, Danvers, MA), mouse-anti-cytochrome *c* (BD Biosciences Pharmingen, San Jose, CA) or goat-anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) followed by peroxidase-labeled secondary antibodies, revealed by enhanced chemiluminescence (Amersham) and analyzed with a GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA).

Behavioral tests

Cylinder Rearing Test (CRT)

At 6 wks after HI, rats were individually placed in a Plexiglas transparent cylinder and observed for 3 min. Initial forepaw preference (left/right/both) of weight-bearing contacts during full rear were recorded. The relative proportion of preference to use the right (unimpaired) forepaw was calculated as: (right-left)/ (right + left + both)×100 (Schallert *et al.*, 2000; Nijboer *et al.*, 2009; van der Kooij *et al.*, 2010).

Adhesive Removal Task (ART)

At 6 wks after HI, stickers (tough-spots, Diversified Biotech, Boston MA) were placed on the left and right forepaw and the latency to removal was recorded. The order for sticker placement on left or right forepaw was alternated between and within animals and we determined the mean time until complete removal of three stickers per forepaw after one

training sticker for each paw (Schallert *et al.*, 2000; Grow *et al.*, 2003, Nijboer *et al.*, 2009; van der Kooij *et al.*, 2010).

Novel object Recognition Task (NORT)

At 9 wks after HI, rats were trained for NORT by placing them individually in a cage of 45 x 35 x 20 cm without bedding under red light conditions according to the protocol described by Bevins and Besheer (2006). Two minutes after placement of the rats in the test environment, 2 identical objects were placed in the back left and right corners and animals could freely explore the 2 objects for 10 minutes. During the 5 minutes test session one of the objects was replaced by a novel object. The training-to-testing interval was 1 hour. Training and test sessions were video-taped and time of interaction with the novel and familiar object were analyzed using Observer software (Noldus, Wageningen, The Netherlands) by a trained observer blinded to treatment. The percentage of time of interaction with the novel object was calculated as follows: (time spent with novel object)/ (total time spent with both objects). Objects used were transparent glass balls (object A; diameter 7.5 cm) or green cone-shaped pottery cups (object B; diameter 4-10 cm, height 6 cm). The use of object A or B as familiar or novel object as well as the placement of the novel object in the left or right corner were randomized between all animals and treatment groups.

Statistical Analysis

Data were normally distributed, are presented as mean ± SEM, and were analyzed by one-way ANOVA with Bonferroni post-tests as indicated.

Results

Effect of PFT-μ treatment on mitochondrial p53 levels

Previously, we have shown that cerebral HI induced by unilateral carotid occlusion followed by systemic hypoxia in P7 rats increased mitochondrial p53 levels of the ipsilateral hemisphere from 0.5–6 h after HI. Maximal levels of mitochondrial p53 were observed at 3 h post-HI (Nijboer *et al.*, 2008). Here, we confirm that the HI insult induces a significant increase in the level of mitochondrial p53 at 3 h post-HI in the ipsilateral hemisphere (figure 1). Importantly, intraperitoneal treatment with PFT-μ (8 mg/kg) immediately after HI completely prevented this HI-induced increase in mitochondrial p53

(figure 1). This finding is in line with the proposed mechanism of action of PFT-μ by Strom *et al.* (2006).

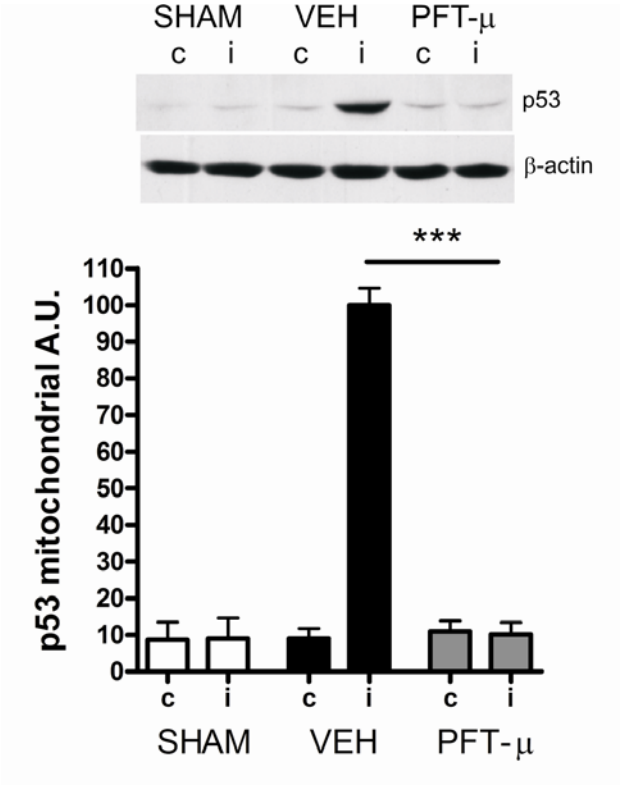

Figure 1. **Effect of PFT-μ treatment on mitochondrial p53 translocation**

P7 rats were subjected to HI, treated with vehicle (VEH) or PFT-μ directly after HI and p53 levels were quantified by Western Blot analysis in mitochondrial fractions of contra- (c) and ipsilateral (i) hemispheres at 3 h post-HI. ***p<0.001 Sham controls n=4, vehicle- and PFT-μ-treated n=6. Inset shows a representative Western blot.

Effect of PFT-μ treatment on short-term brain damage

Induction of HI in P7 rats resulted in severe unilateral brain damage in vehicle-treated animals with ~78% loss of microtubule associated protein 2 staining (MAP2) as a marker of neuronal damage at 48 h after HI (figure 2A,2B). Intraperitoneal administration of PFT-μ directly after HI and 3 h later (0/3 h), potently reduced ipsilateral MAP2 loss by ~66% ($p<0.001$; figure 2A,2B). No MAP2 loss was detected in the contralateral hemisphere or in sham-operated animals.

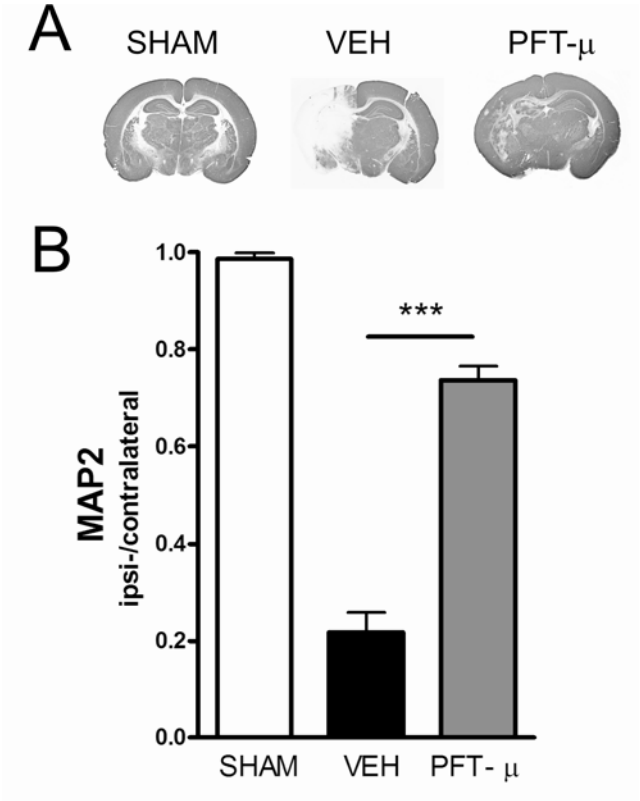

Figure 2. **Effect of PFT-μ treatment on early neuronal damage**

A: Representative photographs of MAP2 loss.

B: Ratio ipsi-/contralateral MAP2 positive area at 48 hours post-HI as a measure of neuronal damage. Vehicle or PFT-μ was administered i.p. at 0 and 3 h post-HI. No MAP2 loss was observed in the contralateral hemisphere. SHAM n=5, VEH n=13, PFT-μ treated n=11. ***p<0.001.

Effects of PFT-μ on cell death markers

Mitochondrial p53 translocation facilitates MOMP with the subsequent release of mitochondrial proteins like cytochrome *c*, leading to the activation of caspase 3 and apoptotic cell death. Previously, we have shown that cytochrome *c* release into the cytosol and activation of caspase 3 in this model of neonatal HI could first be detected at 6 h after the insult and was strongly increased at 24 h after the insult (Nijboer *et al.*, 2008). Therefore, we now determined the effect of PFT-μ treatment on cytochrome *c* release into the cytosol and on activation of caspase 3 at 24 h after the insult (figure 3A). The data in figure 3A demonstrate that PFT-μ treatment significantly decreased the HI-

induced release of cytochrome *c* into the cytosol (figure 3A). Moreover, PFT-μ treatment markedly reduced the HI-induced activation of caspase 3 in the ipsilateral hemisphere (figure 3B).

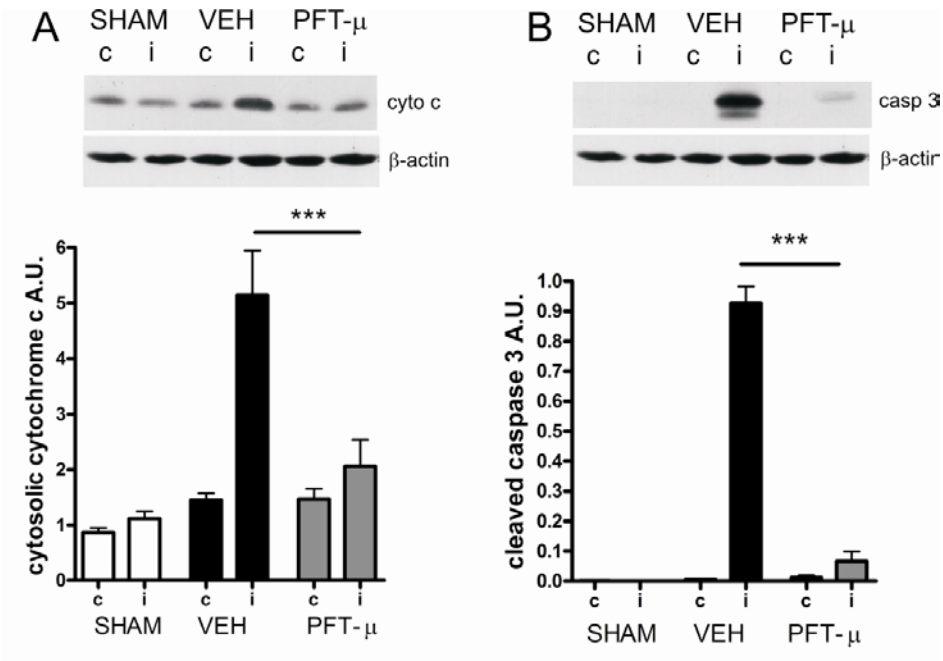

Figure 3. **Effect of PFT-μ treatment on apoptotic cell death after HI**

P7 rats were subjected to HI, treated with VEH or PFT-μ at 0/3h after HI and (A) cytosolic cytochrome c and (B) active (cleaved) caspase 3 were quantified by Western Blot analysis of cytosolic fractions of contra- (c) and ipsilateral (i) hemispheres at 24 h post-HI. ***p<0.001 Sham controls n=4, vehicle- and PFT-μ-treated n=8.

Sensorimotor function after PFT-μ treatment

To determine whether the early neuroprotective effects of PFT-μ treatment also translated into functional improvement, we determined sensorimotor function in two different tests at 6 wks after HI.

First, the effect of PFT-μ treatment on the HI-induced preferential use of the non-impaired forepaw was analyzed in the cylinder rearing test (CRT). At 6 wks after HI, vehicle-treated HI rats showed a clear preference for using the non-impaired forepaw in the CRT (figure 4A). Notably, treatment with PFT-μ significantly reduced paw preference

in the CRT by ~58% (figure 4A). Sham control rats did not show any paw preference during rearing in the CRT.

Next, the latency to remove an adhesive patch from either left (impaired) or right (non-impaired) forepaw was measured in the adhesive removal task (ART). In the ART, sham-operated animals did not show a difference in adhesive removal latency between left (L) and right (R) forepaw; latency was ~6 sec (figure 4B). In vehicle-treated HI rats, latency times to adhesive removal for the impaired (L) forepaw were significantly higher (~23 seconds) (figure 4B). Importantly, after PFT-μ treatment latency times for the impaired (L) forepaw were strongly reduced as compared to vehicle-treated HI rats and latency times did no longer differ from removal latencies for the non-impaired (R) forepaw or for both paws of sham-control littermates.

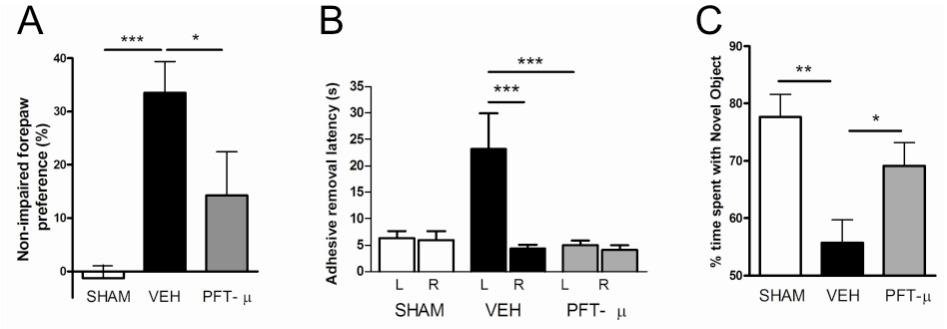

Figure 4. **Effects of PFT-μ treatment on sensorimotor and cognitive behavior**

Rats were subjected to HI at P7 and were tested for sensorimotor and cognitive function.

A: Rats were tested at 6 wks after HI in the cylinder rearing test (CRT) as an indication for laterizing sensorimotor defects. Preference to use the non-impaired (ipsilateral) paw during full rears was determined.

B: Rats were tested in the adhesive removal task (ART) at 6 weeks after HI. Adhesive removal latency from impaired left- (L) forepaw and unimpaired right- (R) forepaws was determined. The removal latency from the right forepaw did not differ between groups.

C: Rats were tested for cognitive function using the novel object recognition task (NORT) at 9 weeks after HI. After a training session, time spent with the familiar and the novel object were determined during a 5 min test period and the percentage time spent with the novel object was calculated. Total object interaction time did not differ between groups.

***p<0.001; **p<0.01 *p<0.05 Sham controls n=9, vehicle n=10, PFT-μ n=10.

Cognitive function after PFT-μ treatment

At 9 weeks after HI, rats were tested for cognition in the novel object recognition tasks. This test uses the natural preference of rats to explore a novel object in comparison to a familiar object (Bevins and Besheer, 2006). After training with two similar objects, we determined the preference for exploration of a novel object as a measure of memory.

Sham control rats clearly showed a preference for the novel object as they spent ~78% of the total object interaction time with the novel object (figure 4C). Exposure to the HI insult induced a marked loss in novel object recognition as vehicle-treated rats did not show a significant preference for interaction with either the familiar or the novel object; ~55% of total object interaction time was spent with the novel object (figure 4C). After PFT-μ treatment, novel object recognition was restored; PFT-μ-treated HI rats spent ~70% of total interaction time with the novel object (figure 4C).

Total time spent with the objects did not differ between the different groups; 11.4%, 12.9% and 13.8% of total NORT time (300 sec) was spent with the objects by sham-controls, vehicle-treated and PFT-μ-treated HI rats respectively.

Long-term effects of PFT-μ treatment on cerebral gray and white matter damage

To assess whether the improved sensorimotor and cognitive function after PFT-μ treatment was associated with long-lasting reduction in brain damage, lesion volume and white matter loss were determined at 10 weeks after the insult. PFT-μ treatment at 0 and 3 h post-HI, effectively decreased lesion size measured as a reduction in the area of the ipsilateral hemisphere with 79% compared to vehicle-treated littermates (figure 5A, figure 5C).

To determine white matter damage, loss of luxol fast blue (LFB) staining in the ipsilataral hemisphere was measured at 10 weeks after HI. Loss of ipsilateral LFB staining was significantly decreased after PFT-μ treatment (70%) (figure 5B, figure 5C). We did not observe damage in the contralateral hemisphere of HI or HI+ PFT-μ animals.

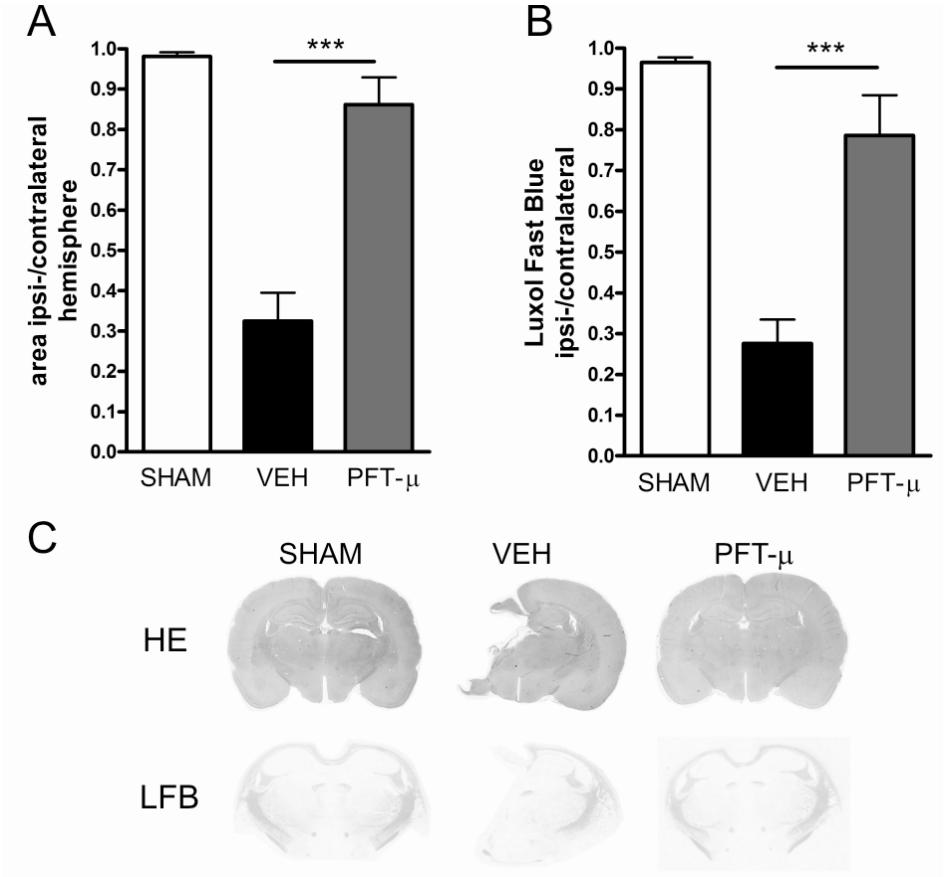

Figure 5. **Long-term effects of PFT-μ on cerebral gray and white matter damage**

A: HI-induced ipsilateral cerebral area loss expressed as area of the ipsi-/contralateral hemisphere at ~-3.20 mm from bregma at 10 wks post-HI.

B: HI-induced white matter damage expressed as the area of ipsi-/contralateral Luxol Fast Blue (LFB) staining at ~-3.20 mm from bregma at 10 wks post-HI as a measure of white matter loss.

C: representative examples of HE and LFB staining. Sham controls n=9, vehicle n=10, PFT-μ n=10; ***p<0.001.

Discussion

In the present study we describe for the first time the strong neuroprotective potential of PFT-μ in vivo. In our model of hypoxic-ischemic brain injury in neonatal rats, PFT-μ prevented the HI-induced increase in mitochondrial association of p53 and reduced apoptotic cell death measured by cytochrome *c* release into the cytosol and caspase 3

activation. Importantly, neuroprotection by PFT-μ was long-lasting and PFT-μ treatment potently improved sensorimotor and cognitive behavioral skills after HI.

Strom *et al.* (2006) selected PFT-μ from a chemical library of compounds, which were tested for their ability to inhibit p53-dependent apoptosis without affecting the transactivation properties of p53 in primary irradiated mouse thymocytes. When thymocytes were treated *in vitro* with PFT-μ these mice were protected against radiation-induced apoptosis. *In vivo,* mice could be partially rescued from bone marrow failure after lethal dose of irradiation by i.p. administration of PFT-μ. Further studies demonstrated that the anti-apoptotic effect of PFT-μ could be assigned to its ability of directly inhibiting p53 binding to the mitochondria.

Within the brain, p53 plays an important role during development as well as in neurodegenerative diseases such as ischemia (reviewed by Culmsee and Mattson, 2005). Neuronal p53 is activated in response to a range of stimuli including oxidative stress, DNA damage and calcium overload. Studies using p53-deficient mice or cells or p53 inhibiting strategies using pharmacological inhibitors of p53 or p53 antisense oligonucleotides, have implicated a pivotal role of p53 during neuronal apoptotic cell death (reviewed by Culmsee and Mattson, 2005). However, these studies have mainly focussed on the role of p53 as a transcriptional regulator and neuroprotective pharmacological studies have mainly used the compound pifithrin-μ (PFT-μ) which either inhibits nuclear translocation of p53 or inhibits DNA binding of p53, leading to inhibition of target-gene transcription and subsequent neuroprotection (Culmsee *et al.*, 2001, 2003; Plesnila *et al.*,2007; Leker *et al.*, 2004; Endo *et al.*, 2006 a,b; Cahill *et al.*, 2006, 2007; Luo *et al.*, 2009). In the present study, we are the first to show the key role of p53 translocation to the mitochondria in a neonatal cerebral hypoxic-ischemic damage. Moreover, we have shown that the drug PFT-μ is a very potent neuroprotective compound, which inhibits p53 association at the mitochondria after intraperitoneal administration.

Endo *et al.* (2006a, b) have described early mitochondrial accumulation of p53 in CA1 hippocampal neurons which correlated with apoptotic cell death after cerebral injury in an adult rat model. Additionally, p53 mitochondrial translocation was also observed in an ischemia-reperfusion model in the kidney and a genotoxic stress model in the liver. PFT-μ treatment in the latter model selectively inhibited the function of p53 at the mitochondria and reduced hepatocyte apoptotic cell death (Leu and George, 2007).

Since the discovery of p53 in 1979, the molecule has been widely acknowledged for its role as a nuclear transcription factor that transactivates numerous target genes

involved in apoptosis, DNA repair, cell cycle arrest and senescence after a broad range of stress stimuli. More recently, several *in vitro* studies using transcription-inactive p53 mutants, inhibitors of nuclear import, inhibitors of transcription/translation or a-nucleated cells provided evidence for an additional transcription-independent pro-apoptotic role of p53 (Caelles *et al.*, 1994; Haupt *et al.*, 1995; Chipuk and Green, 2003; Moll *et al.*, 2005). Marchenko *et al.* (2000) were the first to show that after cell stress, p53 rapidly translocates to the mitochondrial outer membrane and is involved in MOMP, release of mitochondrial proteins and caspase activation. Studies with mutated p53 which is targeted directly to the mitochondria and which has no residual transcriptional function, confirmed that mitochondrial p53 induces apoptosis (reviewed in Green and Kroemer, 2009; Vaseva and Moll, 2009; Speidel 2010). The pro-apoptotic function of p53 at the mitochondria relies on its interaction with and modulation of several Bcl-2 family members leading to activation of the Bax/Bak pore and release of cytochrome c from the mitochondria but the exact mechanism of action is still subject of dispute. A model proposed by Moll et al. claims that at the mitochondria p53 displaces the anti-apoptotic factors Bcl-xL and Bcl-2, from Bax leading to Bax pore formation and cytochrome c release (Mihara *et al.*, 2003). Interestingly, Strom *et al.* (2006) have shown that PFT-μ strongly reduced binding of p53 to both Bcl-xL and Bcl-2 in 293T cells. Consistent with this in vitro activity of PFT-μ, we describe here that in vivo administration of PFT-μ inhibits the HI-induced increase in p53 mitochondrial association in the ipsilateral hemisphere. This reduction in mitochondrial association of p53 prevented the subsequent release of cytochrome c from the mitochondria and the activation of caspase 3.

p53 is crucially important in preventing tumor formation and approximately 50% of all human cancers show mutations in p53 which inactivate the protein. Moreover, almost all other human cancers express mutations in proteins that deactivate the p53 pathway of apoptosis. Importantly, in tumors with mutated p53, sustained inactivation of the p53 apoptotic pathway is needed to maintain the tumor. Recently, this has been shown in *in vivo* animal models where induction of apoptosis was restored and tumor regression was accomplished when p53 function was re-established (reviewed in Vaseva and Moll, 2009). Therefore, we do not anticipate that the short term inhibition of one of the functions of p53, i.e. mitochondrial translocation, will promote oncogenesis.

Moreover, we did not observe any signs of abnormalities in the contralateral hemispheres at 10 weeks after HI.

The data presented here clearly show that PFT-μ treatment reduces HI-induced apoptosis and prevents early neuronal damage as MAP-2 loss at 48 h after the insult was markedly reduced in HI rats treated with PFT-μ. Perhaps more importantly, we also show that the early neuroprotective effect was associated with marked improvements at the level of sensorimotor function as well as cognitive function as determined at 6-9 weeks after the insult. This finding is important as we have shown previously in mice that the early functional improvement that was observed at 4 weeks after treatment with a very high dose of erythropoietin (20kU/kg) gradually disappeared over time (Chapter 3). The effect of erythropoietin on lesion volume as determined at 9 weeks after the insult was also much smaller than the effect of PFT-μ described here.

The long term effects on cognitive function of neuroprotective treatment after HI in neonatal rats have not been studied extensively. In an earlier study, our group used a food finding task (the modified hole board or mHB) to analyze cognitive function (Nijboer et al., 2009; van der Kooij et al., 2010). During this task, rats learn to find a food reward in cued cylinders. Interestingly, even our severe model of HI brain damage did not lead to significant impairments of memory function as assessed in this task. We now used the novel object recognition task as a measure of cognitive function. This task is based on the voluntary interaction of rats with familiar and novel objects (Bevins and Besheer, 2006). As rats are known to have a preference for novel objects, reduced interaction with a novel object is indicative of memory dysfunction. Our data clearly showed that exposure to HI at p7 severely impaired memory function as assessed in this task. As the novel object recognition task relies on voluntary behavior and does not involve food rewards, we suggest that the difference in outcome when comparing the effect of HI in these two tasks may be attributed to the strong effect of the food reward on performance in the modified hole board. The reduced preference for the novel object of HI animals cannot be explained by possible sensorimotor deficits as there were no group differences in total time of interaction with the objects. Interestingly, using this novel object recognition task, we could show that short term PFT-μ treatment early after the insult not only improves sensorimotor function, but also cognitive performance as determined during early adulthood.

In this study we used a severe model of HI brain damage because clinical studies indicate that the currently used intervention for asphyxiated babies only has positive effects in children with moderate brain damage. We have preliminary data indicating that the therapeutic window for the neuroprotective effect of PFT-μ on HI brain damage is approximately 6 hours (data not shown). Similarly, we have shown before that the

therapeutic window for neuroprotective treatment with NF-κB inhibitor TAT-NBD or the JNK-inhibitor TAT-JBD is also 6 hours. Moreover, the neuroprotective effect of TAT-NBD treatment was clearly associated with prevention of mitochondrial accumulation (Nijboer et al., 2008). Notably, hypothermia also has to be applied within 6 h after the insult to have neuroprotective effects (Azzopardi et al., 2009; Gluckman et al., 2005; Shankaran et al., 2005; Edwards et al., 2010). One of the questions that needs to be answered before further development of PFT-μ for clinical application is whether p53 translocation to the mitochondria is also inhibited by hypothermia. It is possible that hypothermia delays such active processes involved in neuronal death (Cilio and Ferriero, 2010). If so, PFT-μ treatment could be considered as an add-on therapy that could be started upon termination of hypothermia to improve neuroprotection in severely affected cases. However, in view of the strong protective effects of PFT-μ, one may wonder whether hypothermia is still needed when the drug can be administered to the baby at the appropriate time. Moreover, p53 may prove to be of great value for prematurely born babies with asphyxia, since no therapy is currently available for this group of patients.

In conclusion, our results show that mitochondrial association of p53 is an important factor in initiating neuronal damage after neonatal cerebral HI. Moreover, our present data demonstrate that PFT-μ administered intraperitoneally has strong neuroprotective effects even in our model of severe HI brain damage. Further studies are required to investigate the possibility of developing a novel intervention strategy for babies to protect the neonatal brain after a hypoxic-ischemic insult.

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Chapter 7

Summary and Discussion

Summary

In **Chapter 1** the possible mechanisms and the current promising neuroprotective strategies after neonatal hypoxia-ischemic (HI) brain injury have been summarized. Based on the mechanisms, therapies should be concentrated on inhibition of the production of reactive oxygen species or free radicals, anti-inflammation and anti-apoptosis in the early stage (first 6h after birth) while on stimulation of neurotrophic properties in a later stage. Combination of moderate hypothermia and pharmacological interventions is probably the next step of consideration.

One important factor playing a role in brain injury is the hypoxia-inducible factor-1α (HIF-1α). During neonatal HI, HIF-1α protein expression is stabilized so that it can regulate the expression of several target genes, such as erythropoietin (EPO), which plays a role in neuronal cell survival and death. In **Chapter 2**, the role and regulation of HIF-1α in neonatal HI is summarized, and several possible pathways are described that are involved in promoting the neuroprotective effect of HIF-1α via inducing expression of the neurotrophic target genes while inhibiting its neurotoxic effects which increase the level of the pro-apoptotic protein p53.

The neuroprotective properties of EPO have been reported, but contradicting evidence with respect to its efficacy exists (van der Kooij et al., 2008). In **Chapter 3**, the effect of EPO treatment on short-and long-term outcome after HI was investigated in a p9 mice model. EPO showed to improve sensorimotor function and white matter damage, but did not reduce grey matter lesion volume. Furthermore, EPO also enhanced proliferation of progenitor cells in the brain. However, these modest neuroprotective effects were shown to be both dose-dependent and gender-dependent. In the present study, only female mice benefited from EPO after HI when treated with 5kU/kg (EPO).

Moderate hypothermia is the only method available today for asphyxiated term newborns with moderate encephalopathy that has shown to have a modest neuroprotective effect provided the therapy is started within 6h after birth (Edwards et al., 2010). Only 1 out of 9 children benefits from this intervention (Edwards et al., 2010). In **Chapter 4 and 5**, the potential neuroprotective effects of 3h hypothermia was tested in a p7 rat HI model. With hypothermia alone (**Chapter 4**), the improvement of sensorimotor function and reduction in brain lesion were found only in females.

We checked whether the combination of hypothermia and EPO improved the efficacy of the treatment instead of applying EPO alone in **Chapter 5**. However, no synergic or

additive effect of the hypothermia-EPO combination was found in females. In males there was only a short-lasting improvement of sensorimotor function after the combined therapy of hypothermia and EPO in males, which disappeared after 3 weeks.

Apoptosis is thought to play an important role in neonatal HI brain damage (Gill et al., 2002). Neuronal p53 acts as a transcription factor for various pro-apoptotic molecules. Moreover, it has a direct effect on mitochondria. In **Chapter 6**, pifithrin-μ, a p53 inhibitor, was administrated to the p7 rat after HI. Pifithrin-μ completely prevented the HI-induced transport of p53 to the mitochondria resulting in improved sensorimotor and cognitive function and in a profound reduction in brain lesion.

Discussion

Neonatal brain injury and therapeutic interventions

In the rodent models of HI, the severity of HI injury seems to direct the selection of the intervention to reduce post HI brain damage. The relation of severity of HI to the changes in brain structure and behavior has been investigated in animal models (McAuliffe et al., 2006; van der Kooij et al., 2009a). Severe HI leads in more than 70% to extensive loss of brain tissue as well as to abnormalities in sensorimotor function and cognitive performance (McAuliffe et al., 2006; van der Kooij et al., 2009a). Also in clinical practice, the severity of HI dictates the efficacy of hypothermia treatment in newborns with an HI-induced encephalopathy: moderate but not severe post-HI encephalopathy can be reduced with hypothermia (Azzopardi et al., 2007; Gluckman et al., 2005). In the present study, the benefits of EPO in the reduction of white matter damage were shown significantly in a mouse model with 45 min hypoxia but not in a rat model with 90 min hypoxia as was shown by van der Kooij et al. (2009b). However, in a study of Spandou et al. (2005), effective regeneration of brain tissue was shown after treatment with EPO in a similar rat model with 60 min hypoxia. The differences may be partially explained by the severity of HI. We propose that the neuroregenerative effect of EPO requires a certain amount of remaining brain tissue after the insult, loss of 75% or more of a hemisphere after 90 min hypoxia in rats may therefore greatly reduce the possibility of EPO to regenerate the brain. Considering the apparently contradictive effects of EPO after moderate vs severe hypoxia, in particular with respect to neurotrophic effects, this drug is probably more suitable to be used in asphyxiated newborns with mild to moderate but not in neonates with severe encephalopathy.

However, pifithrin-μ, a p53 inhibitor, may be an optimal choice after severe brain damage. In the present study, the inhibition of p53 resulted in a significant improvement of behavioral function and nearly complete protection of the brain, even after 120 min hypoxia (severe brain damage). Furthermore, p53 only increased significantly under sustained or severe hypoxic conditions (Halterman et al., 1999). Therefore, inhibition of p53 may be more meaningful to use if severe HI-induced brain injury has occurred.

Dosing and the administration of EPO after perinatal hypoxia-ischemia

Nowadays, the optimal dosage of EPO is still under investigation when administrated as a neuroprotective drug. In our experiments EPO was injected in two different doses (5kU/kg and 20kU/kg). The effects were either neuroprotective (5kU/kg) or neurotoxic

(20kU/kg). It has been reported that EPO does not cross the blood–brain barrier (BBB) in detectable amounts when given to the HI-injured babies at doses appropriate for erythropoiesis (200–400 U/kg) (Juul et al., 1999). Injection of EPO at 5kU/kg has been demonstrated to cross the BBB in a mouse model of brain trauma (Brines et al., 2000). In the present study, the benefits of EPO at 5kU/kg were demonstrated by improvement of behavioral function and by a reduction in white matter lesion volume. When EPO was administrated at a very high dose (>20kU/kg), the toxic effect was shown to increase the number of degenerating cerebral neurons (Weber et al., 2005). Also in our study, the protective effect disappeared with respect to behavioral function and histological outcome after administration of 20kU/kg EPO. In clinical practice, EPO has been indicated to induce the vasoconstriction and hypertension after the initiation of treating anemia in uremic patients (Paschos et al., 2008; Rancourt et al., 2010). It can not be excluded that loss of neuroprotective capacity of EPO at very high dose may result from the side effects of hypertension, increasing the infarction volume of the brain and neuronal cell death.

The frequency and therapeutic time window of the EPO seem also an important factor. In a neonatal rat model after HI, three injections of EPO at 5kU/kg had a better neuroprotective effect than a single injection, but increasing treatment to 7 injections did not further improve the effect of EPO (Kellert et al., 2007). Evidence from animal experiments indicates that EPO should be given at the beginning or within a short (up to 6 hours), critical time period after the actual HI insult to achieve the neuroprotective effect (Dame et al., 2001). In a study with a neonatal rat model after HI, the expression of EPOR increases immediately after HI and reaches its peak at 24h, but endogenous expression of EPO is downregulated early by HI and that it appears later after 4 days after HI (Spandou et al., 2004). In that case, the rationale for using exogenous EPO as a neuroprotective agent early after HI may compensate for the delayed endogenous EPO expression and might work via optimal EPOR expression after 24 h. With the considerations discussed above, it seems reasonable to administrate EPO at three doses and to start immediately after HI as in the present study.

Inhibition of p53 and the anti-apoptotic strategies

In this thesis, inhibition of p53 translocation to the mitochondria with pifithrin-μ led to nearly complete recovery of HI-induced brain damage in neonatal rats. In the previous studies, administration of the NF-κB inhibitor TAT-NBD, which is also associated with nearly complete inhibition of the mitochondrial translocation of p53, showed similar

complete recovery of brain damage after HI (Nijboer et al., 2008; van der Kooij et al., 2010). Since these studies were all performed in a neonatal rat model after severe HI (120 min hypoxia), it is at least indicated that the promising anti-apoptotic therapies associated with inhibition of p53 may be considered for severe asphyxiated babies though the effect of p53 inhibitor has not been tested after mild HI.

Neuronal p53 is recognized to be activated in response to oxidative stress, DNA damage and calcium overload which all occur in the reperfusion/reoxygenation period after HI (Culmsee et al., 2005). p53 has been reported to translocate to the mitochondrial outer membrane after cell stress and leading to leakage of e.g. cytochrome-c and activation of caspases which subsequently leads to cell apoptosis (Marchenko et al., 2000). Although the mechanisms of this nearly complete protection against brain damage are not known, the inhibition of p53 translocation to the mitochondria may play an important role after severe HI.

We do not yet know if p53 translocation to the nucleus also happens after mild HI and consequently whether pifithrin-μ might work after mild injury. This should be investigated in future studies.

The importance of the time window of treatment and duration of hypothermia

Based on studies in animal models and human neonatal magnetic resonance spectroscopy, the time window during which therapeutic intervention is still possible has been estimated to be no longer than 6 hours after HI (Vannucci et al., 2004). Neuronal death after HI occurs mainly between 6-72 h or sometimes later (Sirimanne et al., 1996). Therefore, 72 hours of moderate hypothermia started within 6 hours of birth is widely accepted nowadays as a useful time window in clinical practice (Edwards et al., 2010). In animal experiments, intermittent feeding is required if more than 8h hypothermia is performed (Ohmura et al., 2005). In that case, the body temperature might not be well controlled which will hamper the efficacy of the treatment. One hour of hypothermia has been reported to be not neuroprotective in a neonatal swine model after ischemia (Laptook et al., 1999). Furthermore, delayed hypothermia starting at 3 hours after HI had no longer neuroprotective effect but exacerbated injury of organs in newborn pigs (Karlsson et al., 2008). In the present study, 3 hours hypothermia, which is most commonly used in rodent models (Hobbs et al., 2008), was performed immediately after HI, and we observed neuroprotection such as improvement of behavioral function and a decrease in lesion volume. There are also several controversial studies about the duration of hypothermia for 3-6h (Bona et al., 1998; Covey et al., 2007). Trescher et al.

reported delayed brain injury and no neuroprotection was detected when hypothermia was performed 3h after HI in neonatal rats (Trescher et al., 1997). Taken together, it may be clear that the optimal duration of hypothermia still needs further investigation.

The appropriate combination based on hypothermia

Based on the theory of "secondary energy failure", hypothermia may provide the possibility to "buy time", in order to successfully use other (pharmacologic) interventions, by preserving energy metabolism elongating the therapeutic time window (Gunn et al., 1997). Although hypothermia alone has already shown improvement of brain lesion volume and behavioral function in female rats only, a (short-term) neuroprotective effect in males seems only to exist when hypothermia was combined with EPO. Theoretically, combination of hypothermia and other neuroprotective interventions should provide a synergic or additive effect. It has also been reported that the gaseous anesthetic Xenon combined with hypothermia generated better neuroprotection than hypothermia alone in neonatal rats of both genders after HI (Hobbs et al., 2008). In our study, discussed in this thesis, no further benefits could be detected in females when given the hypothermia-EPO combination.

The intervention of pifithrin-μ has not yet been performed in combination with hypothermia, and there is no report about the relationship between hypothermia and expression of p53 (Cilio et al., 2010). Since strong neuroprotection has been seen with pifithrin-μ alone, it is not known if more benefits can be expected under an elongated therapeutic window induced by hypothermia.

Gender effect in the interventions of EPO, hypothermia and pifithrin-μ

In this thesis, the effect of gender has been carefully studied. It appeared that only females benefited from treatment with either EPO or hypothermia. However, we did not find any gender difference of treatment with pifithrin-μ. The gender-dependent effect of EPO may be caused by a difference in subtype of EPO receptor (EPOR) as was shown in a clinical investigation with healthy individuals (Zeng et al., 2001). There are also reports about a different role for sex hormones as related to the activity of EPO: testosterone promotes the activity of EPO while estradiol inhibits the activity of EPO during hypoxia (Rochira et al., 2009; Mukundan et al., 2004). In a study with adult mice, the local level of estrogen in the male brain is most likely to be higher than in females (Ivanova et al., 2000). Therefore on the basis of these data, the high level of estrogen may be involved in the inhibition of the neuroprotective activities of EPO in male brain.

However, in the neonatal rodent models, it is not likely that sex hormones play a major role as endogenous estrogen production is not initiated yet.

Hypothermia may not only act via down-regulation of cellular metabolism, but also via increasing anti-inflammation and anti-apoptosis, finally leading to a decrease in the apoptotic executioner molecule caspase-3 (Ohmura et al., 2005; Gressens et al., 2008; Hasegawa et al., 2009). It is known that females predominantly use the caspase-dependent pathway of apoptosis while males merely use the caspase-independent pathway (Nijboer et al., 2007; Zhu et al., 2006). In that case, it is not surprising that especially females benefit from hypothermia treatment. Activation of p53 pathway occurs in both genders equally (Wang et al., 2001), therefore, both genders should be able to benefit from the treatment with pifithrin-μ.

In summary, the neuroprotection of perinatal HI brain injury should be a tailored with respect to gender. For females after mild to moderate HI, EPO or hypothermia should be given as early as possible. For males or severe asphyxiated babies, inhibition of p53 may become a very promising drug, especially in the prevention of severe disability later in life.

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Chapter 8

Nederlandse samenvatting (Summary in Dutch)

Samenvatting

In hoofdstuk 1 worden mechanismen beschreven die ten grondslag liggen aan hersenschade die pasgeborenen oplopen bij zuurstofnood rond het geboorteproces. Vervolgens worden hersenenbeschermende strategieën besproken om hersenschade door zuurstofnood te beperken.

Hoofdstuk 2 beschrijft een eiwit, “Hypoxia-inducible factor 1α”, dat ervoor zorgt dat na zuurstofnood neurotrofe stoffen worden gevormd en minder hersenceldood (apoptosis) optreedt.

Hoofdstuk 3 beschrijft resultaten van behandeling met een ander eiwit, het erythropoietine of EPO, gegeven aan pasgeboren muizen na zuurstofnood. Het bleek dat EPO, in voldoende hoge dosering toegediend, met name bij vrouwtjesmuizen bescherming gaf tegen hersenschade na zuurstofnood.

In hoofdstukken 4 en 5 werden pasgeboren ratten na zuurstofnood met matige lichaamskoeling behandeld. Alleen vrouwtjesratten hadden minder schade. Als EPO werd toegevoegd tijdens lichaamskoeling werden de resultaten niet beter in vrouwtjes maar werd wel kortdurende bescherming gezien in mannetjesratten.

In hoofdstuk 6 werd de werking onderzocht van pifithrin-μ, een stof die door zuurstofnood geïnduceerde hersenceldood of apoptosis kan voorkomen. Het bleek dat deze stof, zelfs na ernstig tekort aan zuurstof in pasgeboren muizen, zowel in mannetjes- als in vrouwtjesmuizen een krachtige bescherming gaf van hersencellen en dat motorisch en cognitief functioneren op latere leeftijd veel beter waren in vergelijking met niet behandelde muizen.

Chapter 9

中文摘要 (Summary in Chinese)

中文摘要 (Summary in Chinese)

论文题目：新生儿缺氧缺血后脑损伤的神经保护策略：药物介入和亚低温研究

研究生：樊曦涌
单位：荷兰乌特勒支大学医学中心新生儿科
导师：F.van Bel 教授，C.J.Heijnen 教授
副导师：F.Groenendaal 博士

本文主要利用动物模型，研究了几种新兴药物以及亚低温在新生儿缺氧缺血后脑损伤中的治疗作用，对今后临床的相关治疗提供的新的信息。

第一章总结了目前在新生儿缺氧缺血后脑损伤方面的可能治疗策略，并重点强调基于脑损伤机理的治疗。

近年来发现，缺氧诱导因子(hypoxia inducible factor, HIF)-1α 在脑损伤中占有重要地位。在新生儿缺氧缺血过程中，HIF-1α 的表达被稳定下来，并对几种靶基因（如促红细胞生成素）的表达起到调节作用，从而调节神经细胞的存活和死亡。**第二章**总结了 HIF-1α 在新生儿缺氧缺血过程中的作用及其表达的调控，并描述了几种可能的途径来发挥它的神经保护作用，同时抑制其神经毒作用。

促红细胞生成素(erythropoietin, EPO)的神经保护特性已经有报道，但仍存在争议。**第三章**应用生后 9 天的小鼠模型，研究了 EPO 治疗对缺氧缺血性脑损伤后长期和短期预后的影响，发现 EPO 可以改善感觉运动功能和脑白质损伤，但没有减轻脑灰质的损伤。此外，EPO 也可促进前体细胞的增生。但所有这些作用均具有剂量依赖性和性别依赖性。在本研究中，这些作用只出现在给予 5kU/kg EPO 治疗的雌性个体中。

中度亚低温是目前对窒息后中度脑病足月儿的唯一可行的治疗方法。如果在生后 6 小时内开始应用该方法，可以获得一定程度的神经保护效果。然而，目前只有 1/9 的患儿显示明显的治疗效果。**第四章**和**第五章**对生后 7 天的大鼠缺氧缺血模型应用了 3 小时的亚低温治疗。研究显示，应用单一的亚低温治疗（**第四章**），只能改善雌性大鼠的感觉运动功能以及降低脑损伤程度，**第五章**应用相同的动物模型进一步研究了亚低温和 EPO 联合治疗的作用。在雌性大鼠中，EPO 和亚低温的联合治疗并未显示进一步的协同效应，而雄性大鼠在联合治疗后只显示了短期的感觉运动功能改善。

细胞凋亡在新生儿缺氧缺血性脑损伤后有重要作用。p53 是一个具有促凋亡作用的转录因子。**第六章**对生后 7 天的大鼠缺氧缺血模型应用了 p53 抑制剂 pifithrin-μ。研究显示，pifithrin-μ 完全抑制了缺氧缺血诱导的线粒体内 p53 的增加，改善了感觉运动功能和认知功能，并且极大的减轻了脑损伤。

List of abbreviations

AIF	apoptosis-inducing factor
ARD1	arrest-defective-1
ARNT	aryl hydrocarbon nuclear translocator
ART	adhesive Removal Task
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BNIP3	BCL2/adenovirus E1B interacting protein 3
CA	cornu ammonis
CAM	cell adhesion molecules
CRT	cylinder rearing test
C-TAD	C-terminal domain
DFO	deferoxamine
3,4-DHB	3,4-dihydroxybenzoate
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthases
EPO	erythropoietin
EPOR	EPO receptor
ER	endoplasmic reticulum
FGF	fibroblast growth factors
GSK3	glycogen synthase kinase-3
GS9L	gliosarcoma 9 line
HBO	hyperbaric oxygen
HE	hematoxylin–eosin
HEK293	human embryonic kidney 293
HGF	hepatocyte growth factor
HI	hypoxia-ischemia
HIF	hypoxia inducible factor
HIF-1α	hypoxia-inducible factor-1α
HIF-1α CKO	HIF-1α conditional knock-out
HIFdn	dominant-negative form of HIF-1α

H/IPC	hypoxic/ischemic preconditioning
HRE	hypoxia response elements
HT	hypothermia
2-IB	2-iminobiotin
IFN-γ	interferon-γ
IGF-1	insuline-like growth factor 1
IL-1β	interleukin-1β
iNOS	inducible nitric oxide synthases
JBD	JNK binding domain
JNK	c-Jun N-terminal kinase
LFB	luxol fast blue
MAP-2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
mHB	modified hole board
mNPCs	midbrain-derived neural precursor cells
MOMP	mitochondrial outer membrane permeablilization
MSCs	mesenchymal stem cells
NBD	NFκB essential modulator Binding Domain
NF	nuclear factor
NFκB	nuclear factor kappa B
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NORT	novel object recognition task
NOS	nitric oxide synthases
nNOS	neuronal nitric oxide synthases
NPBI	non-protein bound iron
NSC	neural stem cells
NT	normothermia
N-TAD	N-terminal domain
ODDD	oxygen-dependent degradation domain
P	phosphorylation
P7	postnatal-day 7
P9	postnatal day 9

PBS	phosphate buffered solution
PC12	pheochromocytoma 12
PFT-μ	pifithrin-μ
PHD	prolyl hydroxylases
PHI	prolyl-4-hydroxylase inhibitor
PMNL	polymorphonuclear leukocytes
pVHL	von Hippel Lindau protein
rhEPO	recombinant human erythropoietin
ROS	reactive-oxygen species
RCC4	renal cell carcinoma 4
SOD	superoxide dismutase
SVZ	subventricular zone
TGF	transforming growth factors
TNF	tumor necrosis factor
VEGF	vascular endothelial cell growth factor

Acknowledgements

Now that this thesis has been completed, I am proud of getting the chance to share all my research results with you. Being able to succeed in finishing all my research in 2 years, I really need to give great thanks to all the people who helped me during this period.

I would like to give great appreciation to my supervisor Prof. Frank van Bel. It is his much care, contribution and encouraging helping me overcome the difficulties one by one during my research. I also thank his contribution to my clinical skills and care to my everyday life. His support during my study made me understand how to convert experimental research to clinical practice and how to be both an excellent doctor and good scientist.

I would like to thank my co-supervisor Prof. Cobi J. Heijnen a lot. It is her great contribution to my experimental experience and writing of scientific papers which helped me much to efficiently organize my research and finish my 4 projects in such a short period. It is her assistance helping me to solve all the problems during my study. From her I learned the most important attitude to scientific research and the problem solving ability.

I would like to thank my co-supervisor Dr. Floris Groenendaal very much. His help during my research, especially during the project of hypothermia, was an important reason that I got this promising and conceivable data. I also thank him for enriching my clinical knowledge in neonatology and MRI.

I also would like to thank Dr. Annemieke Kavelaars a lot for her important suggestions and all the advice concerning my experiments. It was always her help and nice ideas making me easily go through some key points.

I would like to give many thanks to my colleague Michael A. van der Kooij. Thanks for his patient teaching of the basic experimental skills related to my research. With his help, I can manage my subsequent projects easily.

I would like to thank Dr. Manon Benders in the Department of Neonatology. Thanks for her organizing the MRI and brain segmentation study. Her teaching enriched me a lot in the knowledge of neonatal image analysis.

I also would like to thank the colleagues of the “Hypoxia-ischemia” group, Cora Nijboer and Cindy T.J. van Velthoven. Thanks for sharing their experience with me and giving me helpful advice. Especially, I would like to thank the technician Selana: thanks for her great help to my histology study when the deadline of my thesis submission was coming soon.

I need to thank Anibal, Niels, Hanneke, Ilona, Jitske, Jesseca, Pieter, Huijing, Elke, Vanessa and all the other colleagues in our NIDOD lab, with their help during my study and my life in the Netherlands, I always feel happy.

I also thank all the secretaries, Daniëlle Nijssen, Ineke Krijgsman, Bertina Hol, Moniek Donker, Demelza Kielstra and Noëline de Zilwa. Thanks for all the work they have done before and after I came to Utrecht.

I would like to thank Helma, Sabine and other staffs of the GDL. Thanks for their care of my large number of animals and their help performing my animal science studies.

I would like to thank Maarten Rijpert, Michelle Westerhuis, Marieke Claas and other colleagues in our office. Whenever I had questions, they always tried their best to help me.

I really would like to thank the Defense Committee. Thanks a lot for their willing to take time reading my thesis carefully in a very tight schedule.

I would like to thank Huijing and Anibal again for willing to be the “paranimfs” of my thesis defense. Thanks for their assistant.

Finally, I would like to thank Prof. Congle Zhou, Prof. Jie Ding, and Prof. Yang Ke. Thanks a lot for all their great help to provide me such a good chance to study abroad. I also appreciate the financial support from Peking University for my living expense.

In the end, I would like to thank my parents, my girlfriend Yuwen Su and other relatives. Thanks for their care about me everyday. They always encourage me and tried their best to help me whenever I met difficulties.

Xiyong Fan
May 2010

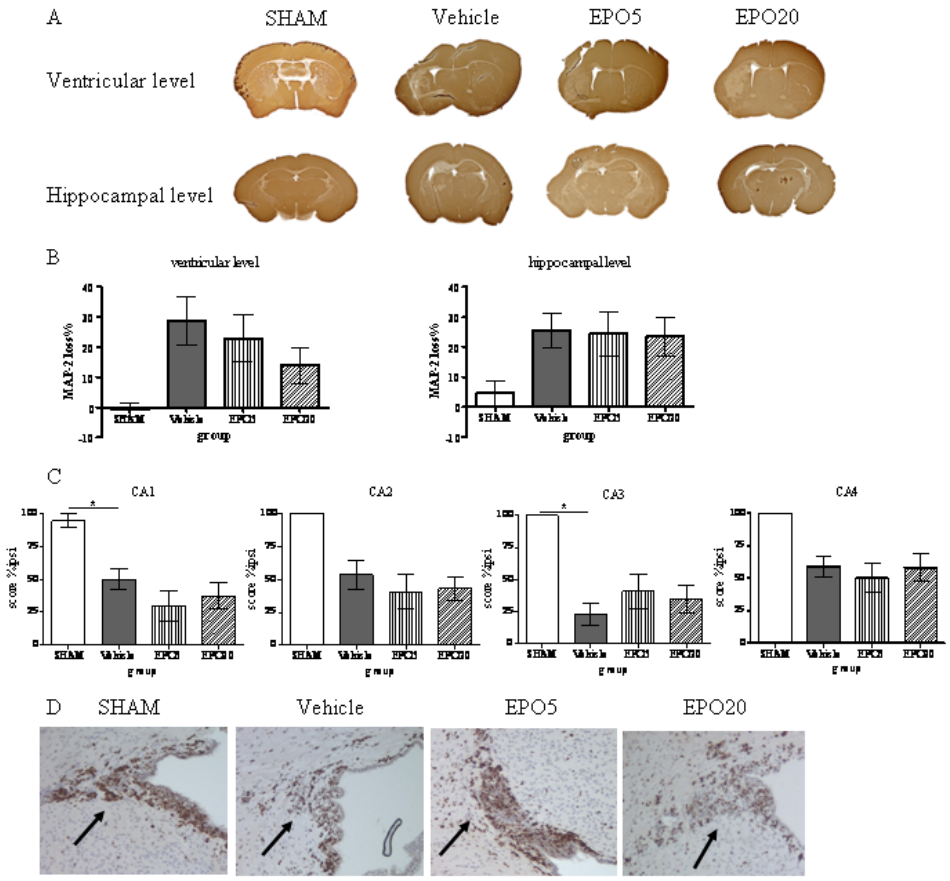
Curriculum Vitae

Xiyong Fan was born on 19 November, 1980, Hohhot of China. He graduated from Peking University in 2004 and was honored Bachelor's Degree of Clinical Medicine. Then he received 3 years further resident doctor training as well as postgraduate study in Pediatric Department of Peking University First Hospital and was honored the Master's Degree in Pediatrics in 2007. In 2006, he got the medical licence and was registered as a pediatrician in Peking University First Hospital. Between 2007 and 2008, he served as the chief resident doctor in Peking University First Hospital and continued his postgraduate study following Prof. Congle Zhou. During this period, he got a lot of experience in pediatrics and learned the skills in diagnosis of perinatal brain damage by using cranial ultrasound and MRI. He also published eight relevant research papers.

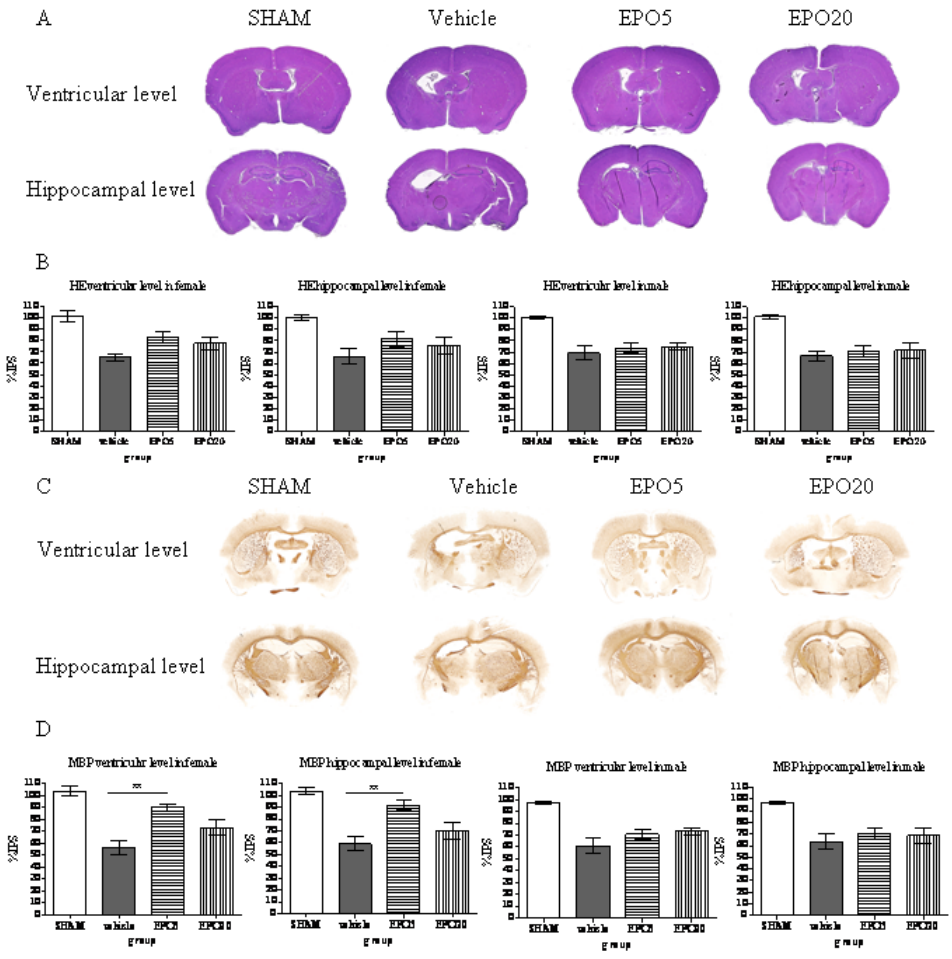
In August 2008, he came to University Medical Centre Utrecht (the Netherlands) and started his PhD research program supervised by Prof. Frank van Bel and Prof. Cobi J.Heijnen in Department of Neonatology and Laboratory for Neuroimmunology and Developmental Origins of Disease (NIDOD). He received training of neurobiological technologies and laboratory animal science. He conducted the experiment of "Effect of erythropoietin (EPO) during neonatal hypoxic-ischemic brain damage" and "Therapeutic hypothermia in neonatal rat model". In October 2009, he got the chance to attend the conference of Neuroscience 2009 in the USA to present his data about neuroprotective effect of EPO. In May 2010, his research was also accepted by the 2010 Pediatric Academic Societies' Annual Meeting in Canada. During the 2 years study, he also participated in some clinical training under the supervision of Prof. Frank van Bel in the Neonatal Intensive Care Unit (NICU) as well as in the clinical project of "brain segmentation of preterm infant in MRI".

Color figures

Chapter 3: *Figure 1*



Chapter 3: *Figure 3*



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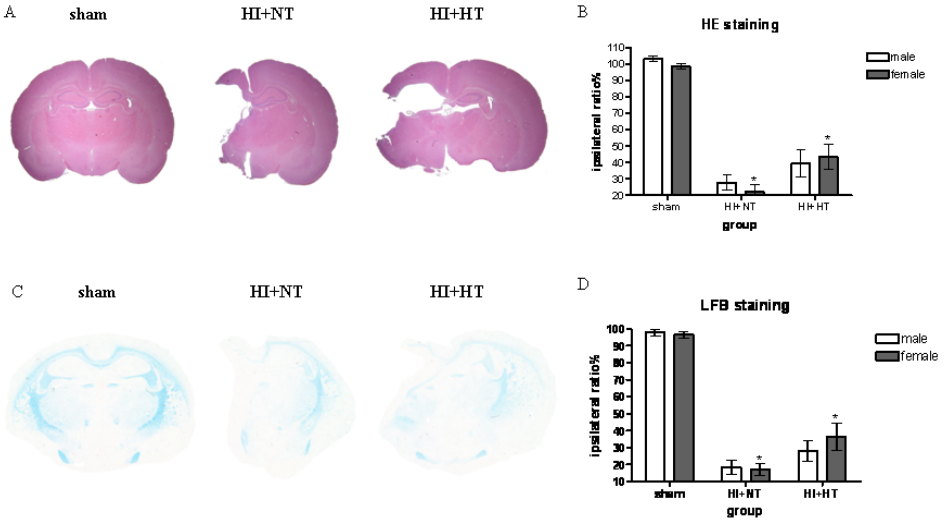

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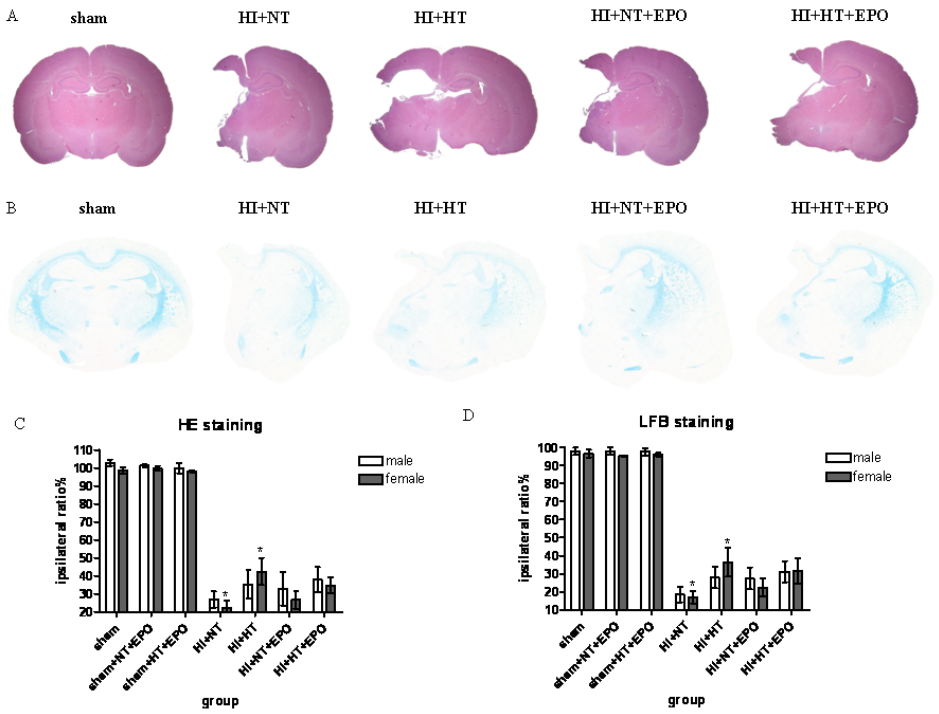
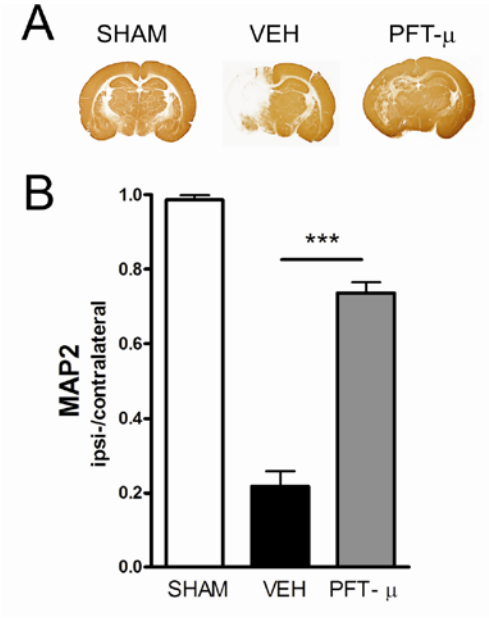


Chapter 6: *Figure 2*



Chapter 6: *Figure 5*