Neuroprotection of the Newborn brain:
Therapeutic Targets & Behavioral Outcome

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Chapter 1

General Introduction

GENERAL INTRODUCTION

1. HYPOXIA-ISCHEMIA

Perinatal hypoxia-ischemia (HI) or birth asphyxia is a clinical condition in which a critical combination of low cerebral oxygenation and hypoperfusion results in brain injury, hypoxic-ischemic encephalopathy (HIE) (Scher, 2001; Volpe 2001). These conditions are collectively referred to as hypoxia-ischemia (HI) in this thesis. HI affects 3-5 per 1,000 live births and is often accompanied with death or severe functional deficits (Cowan et al. 2003; de Haan et al. 2006). It is estimated that 30-40% of infants die and 20-40% develop a broad range of neurofunctional deficits (Yu et al. 2003; Bracewell et al. 2002). The resultant neurological impairments seen in survivors after neonatal HI includes epilepsy, cerebral palsy (CP), learning disabilities (memory & executive function), visual and hearing impairments and the occurrence of behavioral disorders including ADHD (Krägeloh-Mann et al. 1999; Lindström et al. 2006; Rennie et al. 2007). Unfortunately, a standard therapy that prevents the detrimental sequelae induced by HI is not available yet. However, current developments in search for novel therapeutics against HI injury are promising.

The most frequently applied animal model for neonatal HI has been developed by Rice and colleagues about three decades ago as a modification of the model described earlier by Levine S. using adult rats (Levine et al. 1960; Rice et al. 1981). The 'Vannucci-Rice' model involves unilateral ligation of the common carotid artery in seven-day-old (P7) rat pups followed by a hypoxic period which results in ipsilateral cerebral injury (Rice et al. 1981). Many factors may influence the resulting brain damage including (but not exclusively) the animal species, the strain used, ambient temperature, hypoxic duration and the percentage of oxygen inhalation (Vannucci & Vannucci, 2005; Sheldon et al. 1998). In our experiments P7 rat pups and P9 mice pups have been used. Based on histological similarities it was suggested that animals at this age may model the human neonate at a gestational age of 30-34 weeks (Vannucci & Vannucci, 2005).

In section 2 an overview is provided regarding the neuropathological processes and mechanisms underlying HI-induced brain injury. The neuroprotective strategies that aim to inhibit the underlying pathways responsible for HI-brain injury vary and some of those strategies are highlighted in section 3. In other studies the neurofunctional consequences of neonatal HI-injury has also been addressed as detailed in section 4.

2. HI NEUROPATHOLOGY

Prolonged periods of HI interferes with oxidative phosphorylation and results in serious energy failure (ATP-depletion). Due to a lack of ATP cellular pumps fail to function and cytosolic calcium accumulates in neurons. The neuronal calcium overload in turn activates calcineurin, μ-calpain and phospholipidases and induces excessive neurotransmitter release (notably glutamate) (White et al. 2000).

Cytochrome oxidase within mitochondria requires oxygen to function. Under hypoxic conditions free radicals are released by cytochrome oxidase within mitochondria and leak out into the cytoplasm after the internal buffering capacity within mitochondria is reached. In addition, iron (not bound to protein) and nitric oxide are accumulating upon HI and both make important contributions to the pool of oxygen-free radicals. Oxygen free radicals can damage cell membranes through peroxidation and damage DNA as well as proteins. Breakdown of ATP and free fatty acids in turn stimulates the synthesis of xanthine oxidase and prostaglandins respectively. Importantly, in the production process of both xanthine oxidase and prostaglandins free radicals are formed.

Several important molecular pathways including the NF-κB and JNK-pathway are activated upon neonatal HI. Both pathways are involved in neuroinflammation (cytokine transcription) and neuronal cell death after neonatal HI and may act complementary (Nijboer et al. 2009a). All of the abovementioned mechanisms and pathways promote cell death and occur in the early phase after HI.

In addition, a variety of chemokines are secreted upon HI attracting inflammatory cells to the lesion site. The chemokines MIP-1α (macrophage inflammatory protein-1 alpha), MIP-1ß and MCP-1 (monocytes chemoattractant protein 1) activate microglial cells. Upon activation microglia migrate to the site of neuronal injury and start removing the local cellular debris. Activated astrocytes accompany the microglial cells towards the lesion site where these cells start the formation of a 'glial scar' (Silver and Miller, 2004). Although the glial scar may enclose the lesion thereby preventing further deterioration of the healthy tissue surrounding the lesion, it also inhibits neuroplasticity while the injury perpetuates through further release of inflammatory cytokines (o.a. interleukin (IL)-1 and tumor necrosis factor (TNF)α) and chemokines (Bona et al. 1999). These in turn recruit more microglia to the lesion as well as macrophages (Alvarez-Diaz et al. 2007) while also leukocyte infiltration takes place. The events related to the inflammatory reaction constitute a delayed ischemic response which corresponds to a second wave of cell death.

Cell death is classically manifested as either apoptosis or necrosis and both types of cell death can be found after neonatal HI. Early neurodegeneration after HI was reported to be necrotic while delayed cell death was apoptosis (Northington et al. 2001). In a more recent study it appeared that the necrotic-apoptotic phenotype is

not as black-and-white as was previously suggested. In fact, many dying cells after neonatal HI display a mixed phenotype exhibiting both apoptotic- and necrotic-like features (Northington et al. 2007). In contrast to necrosis, apoptosis is an energy-demanding process, it was therefore suggested that energy availability ultimately determines whether the apoptotic cell death program is completed.

In response to HI, some molecular routes are induced that, depending on the type and severity of the insult as well as the metabolic state of the cells affected, may be either beneficial or detrimental such as the involvement of the hypoxic inducible factor (HIF1). HIF1 plays a key role during neonatal HI. Upon HI, default HIF-1α subunit degradation is prevented which allows its binding to the ubiquitously available HIF-1ß to form HIF1. HIF1 mediates gene transcription thereby stimulating erythropoiesis, angiogenesis as well as anti- and pro-apoptotic pathways and necrosis. Mild HI was found to promote cell survival through formation of HIF thereby inducing EPO and VEGF (vascular endothelial growth factor) while under severe HI cell death was induced through transcription of BNIP and P53 (inducing mainly apoptosis) or other factors including calpains (inducing mainly necrosis) (Fan et al. 2009).

3. TREATMENT STRATEGIES

The proposed neuroprotective therapeutic approaches have been shown to inhibit, counteract or restore the neuronal damage after neonatal HI and are classified below based on the proposed mechanism of action through which neuroprotection is achieved.

3.1 Influencing the energy balance

As discussed in section 2, energy failure is a hallmark of HI pathology. Therefore it is not surprising that the amount of initial energy availability will influence the final outcome after neonatal HI. While the results from experiments using hypoglycemia are mixed (Perlman 2006), hyperglycemia was found to prevent HI-brain injury in immature animals (Vannucci & Mujsce 1992). Post-HI glucose injections to neonatal rats, however, did not reduce cerebral injury and tended even to exacerbate cerebral injury (Sheldon et al. 1992). Since clinical interventions for neonatal HI are to be initiated only *after insult* hyperglycemia is therefore not a suitable approach to combat neonatal HI.

3.2 Glutamate

Early after HI extracellular levels of the excitatory neurotransmitter glutamate are increasing. The surplus of extracellular glutamate in turn will result in excessive

neuronal depolarization of glutamate receptors NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isox-azole proprionate) and kainate receptors thereby initiating cell death (Jensen et al. 2005).

Treatment strategies that aimed to stop the detrimental effects of glutamate overload are often targeted at the receptors. For example, the NMDA-antagonists ketamine, memantine and MK-801 reduced white matter injury in rats after neonatal HI (Hagberg et al. 1994; Spandou et al. 1999; Manning et al. 2008). Furthermore, topiramate and NBQX act as AMPA-antagonists and reduced brain injury after neonatal HI in rats (Hagberg et al. 1994; Follet et al. 2004). Unfortunately glutamate antagonists have been shown to induce neurotoxicity thereby limiting its usefulness as a potential therapeutic (Ikonomidou et al. 1999).

3.3 Preventing free radical formation

Excessive production of free radicals and the incapacity of the neonatal brain to deal with this threat early after HI stimulated the search for anti-oxidative strategies. A large number of compounds have been tested that inhibit free radicals to exert their detrimental effects and may be divided upon their mode of action.

Xanthine oxidase contributes to oxygen free radical production. Compounds including allopurinol and oxypurinol inhibit xanthine oxidase and limit free radical production. Allopurinol has been shown to limit brain damage after neonatal HI in rats (Palmer 1993) while oxypurinol was reported not to be neuroprotective in rats after neonatal HI (Feng et al. 2003). In three clinical trials involving neonates after HI the effects of allopurinol were assessed (van Bel et al. 1998; Benders et al. 2006; Gunes et al. 2007). A recent meta-analysis concluded that the three available clinical trials lacked power to demonstrate whether allopurinol is beneficial after neonatal HI (Chaudhari & McGuire 2008).

Non-protein bound iron plays an important role in the perpetuation of free radical formation (van Bel et al. 1998). Deferoxamine (DFO) shows modest neuroprotective effects in animal models for neonatal HI (Palmer et al. 1994; Bergeron et al. 2000; Sarco et al. 2000; Peeters-Scholte et al. 2003; Papazisis et al. 2008). The afforded neuroprotection after DFO-treatment was thought to result from its properties as iron scavenger (Ferriero, 2001). However, studies have shown DFO causes stabilization of HIF1 (Mu et al. 2005). Therefore, the beneficial effects seen after DFO administration may be similar to HI-preconditioning (Jones et al. 2008). Nicotine may be used as iron scavenger as well; nicotine infusion in a piglet model for neonatal HI was shown to decrease both non-protein bound iron and extracellular glutamate (Andresen and Saugstad, 2008). The putative neuroprotective potential for nicotine still needs to be established.

Other free radical inhibitors that have shown neuroprotective properties in animal models for neonatal HI include Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) (Yasuoka et al. 2004; Noor et al. 2005), melatonin (Carloni et al. 2008; Signorini et al. 2009) and cobalt chloride (Jones et al. 2008).

3.4 Inhibition of the NF-κB and/or JNK-pathway

The NF-κB and JNK-pathway are important molecular routes induced upon neonatal HI.

Neonatal HI induces two NF-κB activation peaks in the rat (Nijboer et al. 2008a). Inhibition of the NF-κB pathway may lead to strong neuroprotection but timing is crucial. Early inhibition of NF-κB activation was found to strongly protect the brain from neonatal HI (Nijboer et al. 2008a, b). However, inhibition of both NF-κB activation peaks or inhibition of the second peak alone exacerbates neonatal HI-injury in rats (van den Tweel et al. 2006; Nijboer et al. 2008b). In addition inhibition of the JNK-pathway after neonatal HI was also found to moderately reduce cerebral injury in rats (Nijboer et al. 2009a). Surprisingly, HI-induced production of both pro- and anti-inflammatory was not affected after inhibition of either the NF-κB or JNK-pathway. After inhibition of both the NF-κB and JNK-pathway a reduction in HI-induced TNF-α production was observed (Nijboer et al. 2009a).

However, inhibition of both NF-κB and JNK-pathway reduced the neuroprotective effect compared to NF-κB inhibition alone. Moreover, inhibition of TNF-α with etanercept also decreased the neuroprotective effect of NF-κB inhibition. In line, it was found that early inhibition of NF-κB was associated with a decreased expression of the cell-death inducing TNF-R1 and an increased expression of the pro-survival TNF-R2 (Nijboer et al. 2009a).

Therefore, preservation of intact TNF-α production may in fact be vital for the underlying neuroprotection seen after early inhibition of NF-κB after neonatal HI (Nijboer et al. 2009a).

The putative neurofunctional improvement that may have been associated with inhibition of the NF-κB or JNK-pathway remained to be determined and is dealt with in Chapter 5 and Chapter 6 respectively.

3.5 Inhibition of cerebral inflammation

The developing inflammatory response after neonatal HI often persists long after the initial injury leaving a time-window through which intervention may be possible.

A number of anti-inflammatory strategies have been proposed to antagonize the cytokines involved after neonatal HI. In this respect antagonists that target the IL-1 receptor have been shown to reduce excitotoxicity and the corresponding infarct size after neonatal HI in rats (Hagan et al. 1996; Quiniou et al. 2008). Furthermore, TNFα

inhibition using etanercept reduced the amount of cerebral injury after neonatal HI in rats (Nijboer et al. 2009a). It must be noted, however, that inhibition of TNFα does not always result in neuroprotection since binding of TNFα to the TNF-R1 induces cell death but TNFα binding to the TNF-R2 may promote cell survival (Fontaine et al. 2002; Nijboer et al. 2009a).

Other anti-inflammatory strategies are aimed at reducing the activity of the inflammatory cells itself. For instance, minocycline is a compound that inhibits microglial activity. Minocycline administration was associated with reduced neuroinflammation and resulted in neuroprotection in rats after neonatal HI (Arvin et al. 2002; Fan et al. 2006; Carty et al. 2008). In contrast, in mice after neonatal HI minocycline was found to worsen the brain outcome (Tsuji et al. 2004). An explanation for this apparent species-dependent difference remains to be found. A number of issues need to be resolved before clinical approval of minocycline for neonatal HI is justified. These include the therapeutic window and the long-term histological and functional outcome (Buller et al. 2009; Nijboer et al. 2009b). It may be possible that minocycline only has short lasting effects with no effect on long-term outcome (Nijboer et al.2009b).

In addition, antibodies that target and deplete neutrophils also reduces neonatal HI injury in rodents but only when administrated prior to insult (Hudome et al. 1997; Palmer et al. 2004; Nijboer et al. 2008c).

3.6 Preventing apoptosis

The cascade of events that ultimately leads to apoptotic cell death is completed upon cleavage of caspase 3. In a number of studies the effects of direct inhibition of caspase activity and related proteins downstream of the pathway that leads to cell death have been explored.

To illustrate, the non-specific caspase inhibitors quinoline-Val-Asp(Ome)-CH2-O-phenoxy (Q-VD-OPh) and boc-aspartyl-(OMe)-fluoromethyl-ketone (BAF) showed neuroprotection after neonatal HI in rats (Cheng et al. 1998; Renolleau et al. 2007; Yin et al. 2007).

Administration of 2-imminobiotin protected females –but not males- after neonatal HI through inhibition of cytochrome *c* release and a reduction of caspase 3 (Nijboer et al. 2007). The calpain inhibitor MDL 28170 also proved to effectively inhibit caspase 3 as well thereby reducing both necrotic and apoptotic cell death in a model after neonatal HI in rats (Kawamura et al. 2005).

Bcl-xL is an anti-apoptotic protein that counteracts the release of apoptotic proteins, administration of Bcl-xL was found to protect rats after neonatal HI through inhibition of caspase 3, caspase 9 and AIF (Yin et al. 2006).

3.7 Brain Cooling

Hypothermia is gaining interest as a treatment for HI-injury. In animal models for HI it was found that cooling the animal resulted in neuroprotection while being well tolerated (Shankaran, 2009).

The combination of hypothermia and a pharmacological compound is often suggested as a promising novel approach to combat neonatal HI in newborns (van Bel and Groenendaal, 2008). In animal models for neonatal HI the combination of hypothermia to xenon, the NMDA antagonist MK-801 and to a pan-caspase inhibitor (BAF) made an improvement over therapy with hypothermia or the compound alone (Adachi et al. 2001; Alkan et al. 2001; Ma et al. 2005; Martin et al. 2007).

The precise mechanism of action for hypothermia is not known but likely involves the preservation of cerebral energy, a normalization of protein synthesis and a reduction of free radical production.

Clinical studies have confirmed the neuroprotective effect of hypothermia (Jacobs et al. 2007) although death or severe disability was not reduced (Azzopardi et al. 2009). The Cool Cap® proved beneficial for children with mild to moderate HIE showing less severe changes on the amplitude-integrated EEG (Glückman et al. 2005). In another trial whole body cooling after HI tended to exert beneficial effects in both moderate and severe HIE (Shankaran et al. 2005). Additional clinical trials are scheduled as well as follow up of the first major trials aimed to evaluate long-term outcome and the effect of hypothermia.

3.8 Regeneration of the brain

A recent development is the use of stem cells as potential treatment for neonatal HI. Consistent with findings in adult models for stroke; neurogenesis is also induced upon neonatal HI and sustained for months (Yang et al. 2007; Yang & Levison 2007). Only a small percentage of initial immature neurons survived at long-term and of those surviving cells only minute numbers could be identified neurons (Ikeda et al. 2005; Yang and Levison, 2007; van Velthoven et al. 2009a).

Therefore, stem cell transplantation may work by stimulating the endogenous neuroregeneration seen after neonatal HI. Recent studies performed in neonatal rats and mice showed that post-HI mesenchymal stem cell and multipotent progenitor cell injection induced neuroregeneration (Yasuhara et al. 2006; Yasuhara et al. 2008; van Velthoven et al. 2009a).

It was argued that upregulation of growth factors by stem cell treatment after neonatal HI may be even more important than the number of initially surviving cells per se (van Velthoven et al. 2009b). In this respect, insulin growth factor 1 (IGF-1) (Lin et al. 2009), brain derived neurotrophic factor (BDNF) (Almli et al. 2000; Han et al. 2000), neuronal growth factor (NGF) (Holtzman et al. 1996) and VEGF (Feng et al.

2008) were shown to have neuroprotective properties when administrated after HI-insult in neonatal rodents. Treatments using modified stem cells that secrete enhanced levels of specific growth factors may hold a great promise for future therapies.

4. FUNCTIONAL PROPERTIES AFTER NEONATAL HYPOXIA-ISCHEMIA

Importantly, despite the fact that what ultimately determines the usefulness of a therapeutic strategy is the functional outcome, most studies have assessed neuroprotection based on a histological evaluation but often neglected the neurofunctional aspects of brain recovery.

The functional outcome based on behavior after an HI-insult in animal models may be classified into motor-behavior, cognitive functionality and other behavioral measurements (e.g. impulsivity, attention deficits, aggression). Here we provide an overview of possible behavioral tests performed in animals after neonatal HI based on this classification.

4.1 Motor outcome after neonatal hypoxia-ischemia

Motor deficits are often seen in neonates that suffered from perinatal HI (Rennie et al. 2007; Rutherford et al. 2010). To understand the consequences of neonatal HI in animal models on neurodevelopmental motor behavior tests are used such as the righting-, gait-, geotaxis- reflexes as well as the cliff aversion, wire hanging maneuver and the elevated body swing tests (Lubics et al. 2005; Ten et al. 2003; Fan et al. 2006; Yasuhara et al. 2008).

Frequently used behavioral paradigms to study long-term outcome in motor behavior in animal models for neonatal HI include the adhesive removal test, apomorphine-induced circling behavior, cylinder rearing test, rota-rod and staircase test (Jansen and Low, 1996; Tomimatsu et al. 2002; Grow et al. 2003; Ten et al. 2004; Chang et al. 2005). The motor tests used to assess long-term motor outcome as mentioned above -except the rota-rod- are based on the consequences of unilateral lesion development in animal models for neonatal HI.

In the adhesive removal task the latency upon sticker removal from the forepaw is measured as primary outcome. It took neonatal HI-treated adult animals longer to remove the adhesive from the contralateral (affected) forepaw as compared to the ipsilateral (non-affected) forepaw (Grow et al. 2003) and will be further discussed in Chapter 5-6.

Lateralized motor deficits may also be visualized after administration of the direct dopamine (D1- and D2-like) receptor agonist apomorphine. Systemic apomorphine ad-

ministration to HI-treated animals was found to induce ipsiversive rotational asymmetry which was not seen in control animals (Jansen and Low, 1996; Demers et al. 2005).

In the cylinder rearing test (also discussed in Chapters 4-6) animals are placed in a cylinder after which the animals will start rearing. The number of weight-bearing forepaw contacts with the wall of the cylinder are noted (left, right or both) when the animal rears. Sham-controls show no forepaw preference but HI-treated animals showed ipsilateral (non-affected) forepaw preference when rearing (Chang et al. 2005).

The rota-rod (see also Chapter 6) is an elevated treadmill which requires intact motor skills and sensorimotor integration to remain on the rod. In comparison to sham-controls, HI-treated animals showed reduced durations to remain on the rod (Ten et al.2004).

In the staircase test animals are allowed to retrieve food pellets which require skilled reaching and grasping. Effective food retrieval is measured for each separate forepaw; HI-treated animals retrieved less food rewards with the impaired forepaw and made more errors (Tomimatsu et al. 2002).

4.2 Cognitive outcome after neonatal hypoxia-ischemia

The majority of published studies that assessed animal behavior after neonatal HI have focussed solely on motor outcome. It was found, however, that childhood survivors of neonatal HI are at risk for cognitive defects even in the absence of motor disabilities (Rennie et al. 2007). This finding underlines the importance of careful follow up studies that take into account the cognitive performance of animals tested.

Cognitive performance in animal models for neonatal HI is tested in a limited number of behavioral paradigms; the Morris water navigation task, the 8-arm radial maze and the novel object recognition task.

In animals models for neonatal HI the Morris water navigation task is the cognitive test used par excellence (Balduini et al, 2000; Almli et al. 2000; Ten et al. 2003; McAuliffe etr al. 2006; McAuliffe et al. 2007; Gonzalez et al. 2009). Animals are placed in a pool of water in which an escape platform is located. Initially the location of the platform is visible but the platform is submerged during testing. The animals are required to remember the location of the platform using spatial information (extra-maze visual cues). The latency to reach the platform and the duration of time spent in the target quadrant are taken as primary outcome indicative for cognition. HI-treated animals are typically impaired on these cognitive parameters.

Albeit the most widely used cognitive test for neonatal HI, a number of disadvantages especially in murine studies using the Morris water navigation task are reported. For example, animals with higher swimming speeds are more likely to reach the platform by chance. Furthermore, mice are often reluctant to perform the task

and instead show floating behavior or wall hugging behavior (Wahlsten et al. 2003). Recently it was found that the Morris water navigation task induced an increased stress response in mice compared to the land-based Barnes maze (Harrison et al. 2009). Finally, in the water maze mice are unable to perform their full behavioral arsenal which makes it difficult to determine whether confounding factors could have contributed the poor performance of a particular animal.

The 8-radial arm maze consists of a central arena and eight arms some baited with a food reward. HI-treated animals were slightly impaired based on decreased numbers of retrieved food rewards and increased number of revisits to arms from which food was already eaten (Ikeda et al. 2002).

During the novel object recognition task animals are first presented with two identical objects. During a subsequent trial one of the objects is replaced by a novel object. Rats and mice have a natural tendency to explore the novel object more intense. Therefore, the time spent in the vicinity of the novel object as compared to the familiar object is taken as an indication for intact cognition. HI-treated animals show a reduced interest in the novel object as compared to sham controls (McAuliffe et al. 2007; Pereira et al. 2008). Possible confounding factors which can partly be controlled for may include neophobia for an object or the possibility that animals may have an innate preference for a particular object or material (Dere et al. 2007).

To conclude, there is a need for more refined behavioral tests that allow a cognitive assessment in animal models for neonatal HI. A novel cognitive behavioral test for neonatal HI (the modified hole board) is proposed in Chapters 4-6.

As mentioned in section 1, apart from motor- and cognitive dysfunctions HI in neonates may also elicit emotional disturbances which are recognized later in life. Few studies have investigated the neuropsychological status in animal models for neonatal HI. Pioneering studies as described above have just started unravelling the plethora of emotional disturbances that may have accompanied the HI-insult. Once a neuroprotective strategy is found to be safe, beneficial and effective at short-term it will be pivotal to evaluate the motor-, cognitive- and emotional outcome at long-term.

THESIS OUTLINE:

The research described in this thesis focuses on the evaluation of neuroprotective strategies in animal models for neonatal HI. Neuroprotection was assessed by not only by histology but was focused on the behavioral outcome seen in animal models for neonatal HI.

First we studied the role of erythropoietin after hypoxic-ischemic (HI) brain injury in Chapter 2-3. In Chapter 2 we provide an overview of the studies in which erythro-

poietin (EPO) has been used as a neuroprotective agent in *in vitro* and *in vivo* models for HI-injury. Chapter 3 explores the potential beneficial consequences of combining two individually neuroprotective compounds; EPO and DFO.

In Chapter 4 we studied whether severe but also mild cerebral injury could lead to distinct long-term motor impairments. Moreover we explored whether the modified hole board was sensitive enough as a test to visualize impairments in cognition after severe and mild HI.

In Chapter 5 the effects on histological and functional (motor- and cognitive) outcome of inhibition of the NF-κB pathway after neonatal HI was explored using a specific NEMO-binding domain peptide.

Chapter 6 describes the histological changes of JNK-inhibition and the molecular basis that may underlie the neuroprotection afforded by the specific JNK-binding domain (JBD)-peptide. In addition we employed behavioral tests to assess motor functions and cognition after JBD-treatment.

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Chapter 2

Neuroprotective properties and mechanisms of erythropoietin in *in vitro* and *in vivo* experimental models for hypoxia/ischemia

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ABSTRACT

Besides its established function in erythropoiesis, erythropoietin (EPO) is currently also appreciated for its neuroprotective effects. The detrimental sequelae of prolonged cerebral hypoxia and ischemia have been shown to attenuate by EPO treatment. After binding to the EPO receptor, EPO is capable of initiating a cascade of events which -via different pathways- may lead to neuroprotection. The circumstances that determine which specific signalling route(s) are activated by EPO are largely unknown. We aim to provide the reader with a timely overview on the use of EPO in models of stroke and hypoxia-ischemia and to discuss the molecular events that underlie its neuroprotection.

1. INTRODUCTION

Erythropoietin (EPO) was originally recognized as a humoral mediator involved in the maturation and proliferation of erythroid progenitor cells (Carnot and Deflandre, 1906) but is now appreciated for its neuroprotective effects on the central nervous system as well.

In vitro and *in vivo* studies in adult and neonatal animal models revealed a neuroprotective role of exogenous EPO administration (Noguchi et al., 2007; Sola et al., 2005b). Clinical relevance for the use of EPO as a neuroprotective agent was enhanced when the 34kD glycoprotein was found to cross the blood-brain barrier (BBB) after peripheral administration (Brines et al., 2000). EPO was tested in clinical trials as a possible treatment for adult stroke and found to be both safe and beneficial (Ehrenreich et al., 2002).

Neuroprotection by EPO has been shown to associate with anti-apoptosis, neuroregeneration and anti-inflammation (Sola et al., 2005b). The biological effects of EPO mediated by the EPOR are accomplished via at least three different pathways; after binding to the EPO-receptor (EPOR), EPO induces the phosphorylation of Janus kinase (JAK) 2 thereby activating 1) the phosphoinositide 3-kinase (PI3K)- serine-threonine kinase AKT, and/or 2) signal transducer and activator of transcription (STAT) 5 and/or 3) nuclear factor (NF)-κB pathway (Sola et al., 2005b). How the selection of the specific pathways is determined by the cell, is not known, although cell type, metabolic status of the cell and receptor availability will be implicated in this phenomenon.

On the other hand, results obtained from studies employing EPO variants, demonstrated that although these alternatives were less or even uncapable of binding the EPOR, neuroprotective properties were retained (Belayev et al., 2005; Coleman et al., 2006; Erbayraktar et al., 2003; Leist et al., 2004; Villa et al., 2007; Wang et al., 2004c; Wang et al., 2007). Consequently, mechanisms for neuroprotection by EPO may not be as straightforward as thought and hint towards a binding-site other than the EPOR-homodimer. A receptor constellation involving the EPOR and the common beta receptor (CβR) subunit (the CβR- is a known component of the IL-3, IL-5 and granulocyte/macrophage colony stimulating factor (GM-CSF) receptor) has been proposed as an alternative binding site for EPO (Brines et al., 2004).

In this review we will first discuss the data on the neuroprotective effects of EPO *in vitro*. Then we will evaluate the role of EPO as a neuroprotective agent against brain injury in adult and neonatal animal models for stroke and hypoxia-ischemia (HI). Next we will discuss the anti-apoptotic, neuroregenerative and anti-inflammatory routes for neuroprotection by EPO. Finally we will present recommendations for future research.

2. ENDOGENOUS PRODUCTION OF EPO

As a consequence of hypoxia, degradation of hypoxia-inducible factor (HIF)-1α is prevented which allows the molecule to heterodimerize with HIF-1β to form HIF-1. HIF-1 induces a.o. transcription of endogenous EPO (Jones and Bergeron, 2001; Sharp et al., 2004a) (Fig. 1, top left). Especially astrocytes, but also oligodendrocytes, endothelial cells, neurons and microglia were found to produce EPO (Bernaudin et al., 2000; Bernaudin et al., 1999; Chin et al., 2000; Masuda et al., 1994; Meloni et al., 2006; Sugawa et al., 2002). The homodimeric EPOR has been demonstrated on neurons, astrocytes, endothelial cells and microglia (Bernaudin et al., 1999; Brines et al., 2000; Nagai et al., 2001; Yamaji et al., 1996).

In mice, both EPO and the EPOR were found to be upregulated in blood vessels upon cerebral ischemia (Bernaudin et al., 1999). Hypoxic preconditioning was found to increase HIF-1, VEGF and EPO production (Jones and Bergeron, 2001; Sharp et al., 2004b), thereby protecting the rodent brain from subsequent ischemic injury (Bernaudin et al., 2002; Malhotra et al., 2006).

Prolonged hypoxia, however, induces cell death (Felderhoff-Mueser et al., 2000). The detrimental consequences as a result from prolonged hypoxia were found to be partly counteracted by an increase in endogenous EPO production from astrocytes (Digicaylioglu et al., 1995; Marti et al., 1996; Masuda et al., 1994).

3. NEUROPROTECTION BY EPO *IN VITRO*

EPO was shown to provide protection from hypoxic and toxic insults in ex-vivo and cultured neuronal cells as well as in cultures of endothelial cells (see table 1). For example, cellular damage induced by prolonged hypoxia in hippocampal neurons or endothelial cells was effectively counteracted by EPO (Chong et al., 2002; Lewczuk et al., 2000). Cell death as a result of serum depletion in PC12 cells (rat pheochromocytoma derived cells) was reversed by pre-treatment with EPO (Koshimura et al., 1999).

1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) owes its neurotoxicity to its conversion by mono-amine B expressing glial cells into 1-Methyl-4-phenylpyridinium (MPP^+) after passage of the BBB. EPO treatment given simultaneously with the MPP^+ challenge, however, was capable of saving PC12 cells from cell death (Wu et al., 2007b).

Cell death due to glutamate challenge in cortical and hippocampal neurons was abolished by pretreatment with EPO (Morishita et al., 1996). N-methyl-D-aspartate acid (NMDA) mimics the action of glutamate on the NMDA-receptor and the accompa-

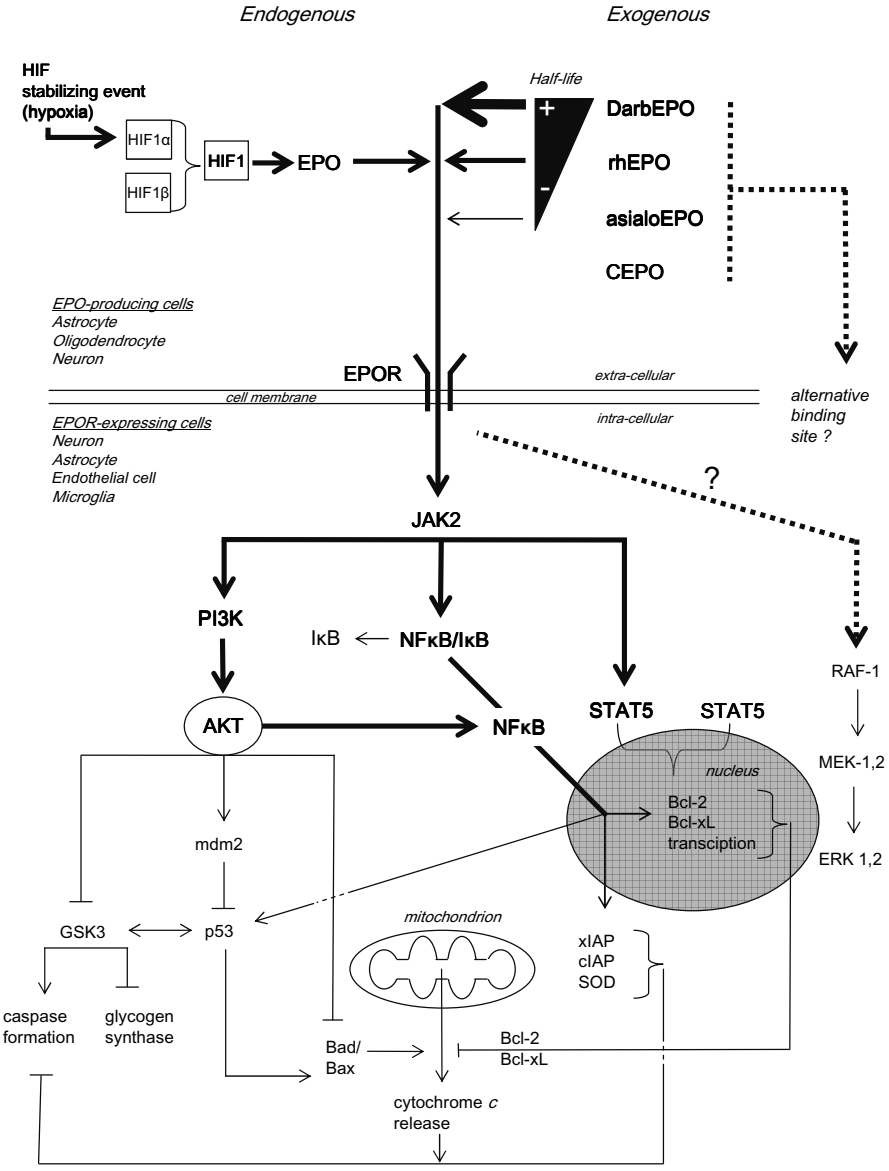

Figure 1. Neuroprotective pathways employed by EPO.
During hypoxia, HIF-1 is formed which induces the transcription of endogenous EPO. Endogenous and exogenous EPO are capable to bind and stimulate the extracellular EPOR as depicted. CEPO does not bind the EPOR but may exert neuroprotective actions via alternative binding sites. After binding of EPO to the EPOR, JAK2 is phosphorylated and induces PI3K thereby activating AKT. AKT may inhibit GSK3 and subsequent caspase formation while increasing glycogen synthase activity. Moreover, AKT activates mdm2 through which p53 may be inhibited. The protein p53 acts pro-apoptotic by stimulating cytochrome *c* translocation from mitochondia via Bax. Finally, AKT inhibits Bad/Bax functioning leading to diminished cytochrome *c* release. Stimulated by AKT or

via JAK2 phosphorylation, NFκB is unbound from its inactive NFκB-IκB complex in the cytosol and translocates to the nucleus. NFκB induces transcription of p53, x-linked and cellular inhibitors of apoptosis (xIAP and cIAP respectively), superoxide dismutase (SOD) and the anti-apoptotic genes Bcl-2 and Bcl-x_L. Nuclear transcription of Bcl-2 and Bcl-x_L genes is also possible via the STAT5 homodimer induced by JAK2 phosphorylation. JAK2 phosphorylation also induces Raf-1, in turn activating MEK-1,2 and leading to ERK-1 and 2 phosphorylation; whether the ERK-pathway activation takes place through the EPOR directly is unknown.

nied cell death after exposure. Pre-treatment with EPO has been shown to attenuate the toxicity of NMDA exposure (Digicaylioglu et al., 1995).

Staurosporine induces cell death via inhibition of protein kinases through prevention of ATP binding. Pre-treatment as well as concurrent treatment with EPO could limit cell death in SH-SY5Y cells by about 50% from cell death induced by staurosporine (Um et al., 2007).

Oxygen and glucose deficiency (OGD) is considered as a model for hypoxia-ischemia *in vitro*. Neuroprotection of cortical neurons from OGD-induced damage has shown to be possible by adding EPO to the hippocampal slices *in vitro* (Ruscher et al., 2002). Transfer of the culture supernatant from OGD-treated astrocytes to OGD- challenged neurons protected the neurons *in vitro* via endogenously produced EPO in the supernatant of cultured astrocytes; administration of soluble EPOR abolished or a specific EPOR antibody reduced EPO's protective effect. Therefore, EPO released from astrocytes may act as a paracrine protective mediator for neurons (Ruscher et al., 2002).

4. NEUROPROTECTION BY EPO *IN VIVO*

The promising neuroprotective properties by EPO as shown by initial *in vitro* studies were ensued by *in vivo* studies in which neuroprotection was demonstrated after exogenous EPO administration. Exogenous EPO includes recombinant human (rh) EPO or other EPO variants such as DarbEPO, asialoEPO and carbamylated EPO (CEPO). EPO variants differ from rhEPO by their binding capacity to the EPOR. DarbEPO contains additional oligosaccharide chains thereby extending the circulation duration compared to recombinant human EPO. In contrast, the circulation duration of asialoEPO is dramatically reduced. Moreover, CEPO has been reported not to bind the EPOR at all (Fig. 1, top right).

The experimental characteristics (experimental model, route of administration, EPO dosage, EPO type, time of administration etc.) and neuroprotective effects of EPO for individual *in vivo* studies have been outlined in table 2 for adult- and in table 3 for neonatal animals. Included in the tables are studies that assessed the neuroprotective role of EPO by measuring the amount of brain injury via histology.

Table 1. Summary of neuroprotective effects of EPO in *in vitro* studies

Reference	Model	Cell culture	Dose (pM)	Administration	Assessment (h)	Improvement (%)
Koshimura '99	Serum deprivation	PC12	100	-6, -3, & -1 d	0	**100**
			10			54
			1			53
			0.1			23
Wu '07	MPP^+	PC12	8.8	0	24	**100**
			29.4			100
			88			100
			2.9			53
			294			35
Morishita '97	Glutamate	Cortical neurons	30	-24 h	0	**100**
			300			100
			3			27
	Glutamate	Hippocampal neurons	30			**100**
			300			100
			3			36
Lewczuk '00	Anoxia	Hippocampal neurons	100	0	15	**100**
Digicaylioglu '01	NMDA	Cortical neurons	147	-3 h	0	**82**
Chong '02	Anoxia	Endothelial cells	29.4	-1 h	24	**81**
			2.94			70
			0.3			61
			0.03			NS
			294			NS
			2.9 E3			NS
Ruscher '02	OGD	Cortical neurons	2.9	-48 h	24	**65**
Um '07	Staurosporine	SH-SY5Y	25	-24 h	24	**59**
			250			56
			2.5 E3			40
			25	0	24	55
			250			51
			2.5 E3			40

The model for brain injury is noted next to the references. The EPO concentration used is indicated in pM. The column Administration describes the time of EPO administration relative to the onset of the neurotoxic threat (the minus-prefix indicates pretreatment. Column Assessment indicated time points at which neuronal damage was evaluated, the amount of relative protection provided after EPO may be found in the column Improvement. Optimum improvement scores for each study are in bold. E3, to the third power; NS, denotes no significant change.

Table 2. Summary of neuroprotective effects of EPO in adult *in vivo* studies

Reference	Animal	Route	Dose (kU/kg*)	Administration	Assessment (d)	Improvement (%)
MCAO plus reperfusion						
Yu '05	Rat ♂	i.p.	5	10 min, 1 h	1	**79**
Sirén '01	Rat ♂	i.p.	5	0	1	**75**
Brines '00	Rat ♂	i.p.	5	-24 h	-	**63**
				0	-	63
				3 h	-	60
				6 h	-	31
				9 h	-	NS
Leist '04[1]	Rat	i.v.	5 µg/kg	1 h	1	**55**
			50 µg/kg	1 h	1	50
			50 µg/kg	4 h	1	35
			5 µg/kg	4 h	1	NS
Belayev '05[2]	Rat ♂	i.p	10 µg/kg	0	14	**49**
					3	NS
Erbayraktar '03[3]	Rat ♂	i.v.	44 µg/kg	0	1	**47**
Wang '04[1]	Rat ♂	i.v.	5	6 h	28	**27**
			50 µg/kg	6 h		**27**
			0.05			NS
			0.5			17
			1.15			NS
Villa '07[1]	Rat ♂	i.v.	50 µg/kg	1 h	1	**25**
Wang '04	Rat ♂	i.p.	7 x 5 or 10/d	24 h	28	**NS**
Permanent MCAO						
Li '07	Mice ♂	i.p.	5	-30 min, 1, 2 d	3	**58**
Bernaudin '99	Mice ♂	i.c.v.	0.4 µg/kg	-24 h	1	**47**
Sadamoto '98	Rat	i.c.v.	28 x 1 U/d	0	28	**29**
			28 x 5 U/d			29
			28 x 0.2 U/d			21
Transient BCCAO						
Calapai '00	Gerbil ♂	i.c.v.	25 U	0	7	**61**
			5 U			43
			0.5 or 2.5 U			NS
			100 U			NS
			50 U			29
		i.p.	12.5 or 25 U			NS
Sakanaka '98	Gerbil ♂	i.c.v.	5 U/d	0 and 1-7 d	8	**59**
			25 U/d			59
			2.5 U/d			51
			0.5 U/d			NS

The model for brain injury is noted above the references. The species of animal (rat/mice/gerbil) and route of administration are indicated. EPO dosage is in kU/kg unless indicated otherwise. The column Administration describes the time of EPO administration relative to the onset of insult (the minus-prefix indicates pretreatment). The column Assessment indicated time

points at which neuronal damage was evaluated, the amount of relative protection provided after EPO may be found in the column Improvement. Optimum improvement scores for each study are in bold.
Abbreviations: NS, not significant; _, information unknown. Superscript numbers next to references indicate that alternative EPO variants were used in the respective study: 1, CEPO; 2, darbepoetin; 3, AsialoEPO.

Temporary middle cerebral artery occlusion (MCAO) is used in rodents as a model for transient focal cerebral ischemia and reperfusion (stroke). Temporary bilateral common carotid artery occlusion (BCCAO) in gerbils results in a milder form of focal cerebral ischemia in which the CA1 area of the hippocampus is selectively affected.

Initially the brain was considered impenetrable for EPO due to the BBB and therefore in early studies EPO was administered intracerebroventricularly (i.c.v.). Brain damage after focal cerebral ischemia in mice, rats and gerbils was found to be attenuated and cognitive aspects were improved by EPO (Bernaudin et al., 1999; Sadamoto et al., 1998; Sakanaka et al., 1998). The dogma of the BBB as an unconditional barrier for large proteins was challenged when recombinant human (rh)EPO was capable of protecting rodents from a number of brain injuries (including MCAO) after *systemic* administration while EPO could be detected in the spinal fluid indicating actual BBB passage (Brines et al., 2000). It remained to be tested, however, whether EPO was only capable of penetrating the brain because the insult had actually damaged the BBB (Calapai et al, 2000). A follow-up study resolved the issue by demonstrating that human and murine EPO and the even larger molecule darbepoetin were capable of penetrating the non-injured brain as well, albeit at low levels (Banks et al., 2004).

Systemic intraperitoneal (i.p.) and i.c.v. EPO administration immediately after BCCAO were compared in adult male gerbils and administration of EPO via both routes were found to inhibit the insult-induced increase in brain water content and both treatment routes led to improved brain histology (Calapai et al., 2000). In rodents after temporary MCAO, systemic EPO was also neuroprotective as indicated by reduced infarct volumes (Belayev et al., 2005; Prass et al., 2002; Siren et al., 2001; Wang et al., 2004a; Yu et al., 2005). Associated with the improved histological scores, motoric and cognitive performances as measured by scoring the postural reflex, forelimb placing, the corner and foot-fault test and behaviour in the Morris water maze were enhanced (Belayev et al., 2005; Sadamoto et al., 1998; Wang et al., 2004a; Wang et al., 2007; Yu et al., 2005).

The neuroprotective effects by EPO and EPO derivatives as shown in *in vitro* and in *in vivo* adult studies were followed by studies involving animal models for neonatal cerebral injury; the most widely studied animal models include the Vannucci-Rice (Rice, III et al., 1981) model for neonatal HI and MCAO for neonatal stroke.

Table 3. Summary of neuroprotective effects of EPO in neonatal *in vivo* studies.

Reference	Animal (PND)	Route	Dose (5kU/kg*)	Administration	Assessment (d)	Improvement (%)
Hypoxia-Ischemia						
Kellert '07	Rat 7	s.c.	5 x 3	0, 1, 2 d	2	**79**
			30	0	2	65
			5	0	2	44
			2.5 x 3	0, 1, 2 d	2	NS
			2.5	0	2	NS
			30	0	7	**86**
			5 x 3	0, 1, 2 d	7	64
			2.5 x 3	0, 1, 2 d	7	49
			5 x 7	0, 1 ... 6 d	7	NS
Spandou '04	Rat 7	i.p.	2	-10 min	4	**75**
Kumral '04b	Rat 7	i.p.	1	0	7	**62**
Wang '04[1]	Rat 7	i.p.	10	-4 h	5	**55**
						52
Aydin et al. '03	Rat 7	i.c.v.	20 U	0	7	**53**
Matshushita '03	Mice 7	i.p.	5	-1 h	7	**50**
						NS
Kumral '03	Rat 7	i.p.	1	0	3	**37**
Kumral '06	Rat 7	i.p.	1	0	2	**34**
Demers '05	Rat 7	s.c.	2.5 x 3	0, 1, 2 d	23	**NS**
MCAO plus reperfusion						
Gonzalez '07	Rat 10	i.p.	5	0	42	**70**
Wei '06	Rat 7	i.p.	10 x 3	-15 min, 1, 2 d	3	**59**
Chang '05	Rat 10	i.p.	5	0	14	**45**
Permanent MCAO						
Wen '06	Rat 7	i.p.	1 x 3	-15 min, 1, 2 d	77	**64**
					35	55
Sola '05	Rat 7	i.p.	1	15 min	7	**62**
			0.1			54
			5			47

The model for brain injury are noted above (in vivo) the references. The animal species (rat or mouse) and route of administration are indicated. EPO dosage is in kU/kg unless indicated otherwise. The column Administration describes the time of EPO administration relative to the onset of the neurotoxic threat (the minus-prefix indicates pretreatment. Column Assessment indicated time points at which neuronal damage was evaluated, the amount of relative protection provided after EPO may be found in the column Improvement. Optimum improvement scores for each study are in bold.

Abbreviations: NS, denotes no significant change. Superscript 1 next to the reference indicates that asialoEPO was used in the respective study.

EPO given prior to or after a neonatal insult in mice and rats reduced the infarct volume after HI (Kumral et al., 2003; Matsushita et al., 2003; McClure et al., 2006; Spandou et al., 2004; Sun et al., 2005; Wang et al., 2004c) and from permanent and temporary MCAO (Chang et al., 2005; Sola et al., 2005a; Wei et al., 2006) or brain damage as a result from ibotenate (Keller et al., 2006). Intracerebral ibotenate injection causes NMDA receptor mediated excitotoxic brain injury. The neuroprotective effects of EPO persisted when brain infarcts were assessed at 4, 6 and 10 weeks after MCAO (Gonzalez et al., 2007; Wen et al., 2006).

There is ample evidence that EPO improves behavioural and intellectual performances after neonatal insult. Righting and postural reflexes as well as grip traction performance were enhanced, asymmetries of forelimb use and rotation were attenuated, sensory neglect was reduced and when tested in the Morris water maze, the mean latency to find the target platform was reduced both at two and at 19 weeks after insult (Chang et al., 2005; Demers et al., 2005; Kumral et al., 2004; Spandou et al., 2004; Spandou et al., 2005; Wen et al., 2006).

Most effective treatments for both adult and neonatal animal models include EPO administration immediately after the insult while the extent of neuroprotection seems smaller after delayed administration (table 1 and 2). In the majority of studies, assessment of brain damage was performed only a few days (1-7) after the insult. Further research involving longer follow-up of the neuroprotective effects after EPO administration is warranted because brain injuries due to a hypoxic-ischemic insult are known to evolve over a period of 6-12 weeks and possible longer (Wen et al., 2006).

The positive long-term outcome of EPO was found to be gender dependent in rats subjected to focal cerebral ischemia (Wen et al., 2006). At 6 weeks after injury, both sexes displayed reduced cerebral infarct volume but protection was more prominent in females. At 12 weeks, the infarct in EPO-treated males was enlarged compared to 6 weeks earlier but remained stable in EPO-treated females. In contrast, in a short-term experiment using neonatal mice subjected to ibotenate injection, the protective actions by EPO were shown not to be gender-specific (Keller et al., 2006). Other neuroprotective strategies, like the use of 2-iminobiotin have shown to act gender specific (Nijboer et al., 2007) but it is too premature to conclude whether the same holds true for EPO. Gender specificity may, for an important part, be determined by the fact on what level of the signalling cascade of the EPOR the drug will interfere with the response of the target cell.

5. ANTI-APOPTOSIS, NEUROREGENERATION AND ANTI-INFLAMMATION AFTER EPO

Protective effects by EPO have been thought to result from a decrease in apoptosis, an increase in neuroregeneration and contributions to anti-inflammation. The anti-apoptotic effect of EPO after the insult is likely to be responsible for a proportion of neuronal survival as demonstrated by decreased numbers of apoptotic cells after EPO administration (Chong et al., 2002; Keller et al., 2006; Kellert et al., 2007; Lee et al., 2006; Matsushita et al., 2003; Sun et al., 2004; Wang et al., 2004c; Wei et al., 2006). In PC12 cells for example, EPO was found to reduce caspase 3 activities after MPP^+ challenge which was abrogated by the specific PI3K-blocker LY294002 (PI3K is downstream of the EPOR) (Wu et al., 2007a).

Apart from neuroprotection, EPO may also reduce neuronal injury by stimulating neuroregeneration as a trophic factor; indeed, *in vitro* studies have shown that neuronal progenitor cell production from pluripotent progenitor cells was stimulated by EPO (Shingo et al., 2001). Moreover, EPO stimulated *in vitro* neuronal differentiation from adult SVZ-derived neural progenitor cells (Wang et al., 2004a). *In vivo*, EPO was shown to act as a neurotrophic factor to cholinergic neurons after fimbria-fornix transsections in the adult rat (Konishi et al., 1993).

EPOR deletion in astrocytes has been shown to decrease the number of immature neurons migrating towards the area of infarct after focal cerebral ischemia (Tsai et al., 2006).

Inflammatory control by EPO is probably not directly controlled by EPOR-homodimer activation. Although *in vivo* EPO administration reduced TNFα, IL-6 and monocyte chemo-attractant -1 production in adult rats and reduced microglial activation and cerebral leukocyte influx in neonatal rats subjected to MCAO (Sun et al., 2005; Villa et al., 2003), EPO *in vitro* did not inhibit the cytokine response in astrocytes after exposure to neuronal homogenates nor did it modulate the response of human peripheral mononuclear cells or rat glial cells to lipopolysaccharide (Villa et al., 2007). Furthermore, carbamylated EPO (CEPO) does not bind to the EPOR but reduced microglial activation as well as the polymorphonuclear infiltrate in the brain (Villa et al., 2007; Wang et al., 2007) suggesting that direct activation of the classical EPOR is not paramount for inflammatory control.

Anti-inflammation by EPO is likely mediated via reduced neuronal cell death thereby attenuating the cerebral attraction of inflammatory cells that would have produced the cytokines in unprotected animals. A possible mechanism of EPO to control inflammation may be via BBB stabilization (see section 8). EPO has been reported to stabilize the BBB which in turn may have reduced cerebral invasion of inflammatory cells and inhibited persistent neuronal cell death.

6. SIGNALLING PATHWAYS AS A RESULT OF EPO RECEPTOR STIMULATION

A schematic drawing of endogenous EPO formation and the capabilities of endogenous and exogenous EPO to stimulate the EPOR and activate downstream molecular pathways enabling neuroprotection is depicted in figure 1.

Either endogenously or exogenously applied EPO may stimulate the EPOR to induce phosphorylation of JAK2 (Kawakami et al., 2000; Kawakami et al., 2001; Sola et al., 2005a). JAK2-phosphorylation in turn activates PI3K, induces the translocation and subsequent activation of NFκB and/or stimulates STAT5 homodimerization thereby initiating a number of downstream molecular cascades (Sola et al., 2005b).

The JAK2-PI3K pathway is crucial for the neuroprotective abilities of EPO. *In vivo* studies showed that inhibition of JAK2 or PI3K abolished the neuroprotective effects of EPO (Zhang et al., 2006; Zhang et al., 2007). PI3K activation subsequently induced AKT (Siren et al., 2001). AKT modulates multiple intracellular signalling routes influencing (for example) apoptosis, cell survival, synaptic signalling and the synthesis of glycogen and proteins. Target molecules of AKT include GSK-3, p53, cytochrome *c* and NFκB (Culmsee and Mattson, 2005; Datta et al., 1999).

GSK-3 mediates important processes such as glycogen synthesis, cell cycle and cell death. EPO was found to phosphorylate GSK-3β via AKT activation (Shang et al., 2007). Phosphorylation of GSK-3β inhibits the activity of GSK-3 and leads to both prevention of caspase 3 activation and an increase in glycogen synthesis. MCAO in mice increased GSK-3 activity (Bhat et al., 2000) and EPO reduced GSK-3 activity in this adult rat model for transient focal cerebral ischemia (Zhang et al., 2006). In contrast, in a rat model for neonatal HI, phosphorylated GSK-3β levels were unaltered after pretreatment with asialoEPO (Wang et al., 2004c). The reduced potential of asialoEPO to stimulate the EPOR may underlie the different results on GSK-3 activity compared to EPO. Abnormal (increased) GSK-3 activities are thought to play a major role in neurological disorders such as Alzheimer's disease and stroke (Bhat et al., 2004). Efforts are currently underway in search of GSK-3 inhibitors.

GSK-3 is known to induce p53 and *vice versa*. p53 may be suppressed via activation of mdm2 via AKT. In response to cell stress p53 is upregulated and the protein has been shown to initiate apoptosis (Culmsee and Mattson, 2005). *In* vitro, EPO was shown to reduce p53-mediated apoptosis (Lin and Benchimol, 1995) and in *in vivo* EPO administration and neuroprotection was associated with decreased ipsilateral hippocampal p53-protein expression after HI-insult (Juul et al., 2008). Therefore, p53 inhibition may be a promising target for neuroprotection.

Furthermore, EPO has shown to be capable of inhibiting cytochrome *c* release from mitochondria via AKT (Chong et al., 2003), either directly or by inhibition of the apoptotic proteins Bad/Bax. AKT facilitates NFκB activation by enhancing IκB degradation;

AKT was found to activate Ikkα. Ikkα, Ikkß and NEMO (NFκB Essential Modifier) form a complex capable of phosphorylating the inactive cytosolic NFκB-IκB formation. After phosphorylation, NFκB is released and induces nuclear transcription of NFκB specific genes regulating inflammation and apoptosis. AKT was also demonstrated to cooperate with other factors that induce NFκB activation (Kane et al., 1999). Stimulation of neurogenesis and astrocytic differentiation from neuronal stem cells by EPO *in vitro* was dependent of nuclear translocation of NFκB (Shingo et al., 2001). Moreover, the JAK2-NFκB pathway stimulated by EPO was pivotal for the neuroprotection from NMDA- or cytokine-induced nitric oxide (NO) production (Digicaylioglu and Lipton, 2001).

The JAK2-STAT pathway is likely to be important for the anti-apoptotic properties of EPO functioning. STAT5-KO mice embryos show severe anaemia in combination with increased levels of apoptosis in the erythroid progenitor cell population (Socolovsky et al., 1999; Socolovsky et al., 2001). STAT5 -a STAT member demonstrating pro-survival signals only (Debierre-Grockiego, 2004) - may be phosphorylated upon EPO/EPOR binding via JAK2 (Sola et al., 2005a). Phosphorylated STAT5 homodimerizes and enters the cell nucleus where the anti-apoptotic Bcl-2 and Bcl-x_L genes are transcribed. Bcl-2 and Bcl-x_L in turn are capable of preventing cytochrome *c* release from mitochondria. EPO has been found to increase STAT5 and the concentrations of Bcl-2 and Bcl-x_L *in vitro* and *in vivo* (Siren et al., 2001; Sola et al., 2005a; Wei et al., 2006; Wen et al., 2002). In addition, STAT5 may play a role in the neuronal differentiation of neural progenitor cells by STAT5-dependent upregulation of suppressor of cytokine signalling 2 as shown by an *in vitro* study (Wang et al., 2004b). Recently, *in vitro* neuroprotection by EPO to glutamate-induced cell death in murine hippocampal neurons was found not dependent on STAT5 (Byts et al., 2008). The precise role of the STAT5 pathway stimulated by EPO after brain injury remains to be determined, but will probably involve anti-apoptosis, differentiation and neurogenesis.

Finally, the ERK-pathway is also involved in the response after HI and can possibly be controlled by exogenous EPO. Neuronal ERK phosphorylation appeared early after HI-insult in damaged brain areas and was followed by ERK phosphorylation in astrocytes, microglia and oligodendrocytes (Wang et al., 2003). After cerebral ischemia, the importance of the ERK-pathway in the neuroprotective capacity of EPO ranged from either unimportant (Ruscher et al., 2002) or minor (Zhang et al., 2006) to crucial (Kilic et al., 2005; Siren et al., 2001).

7. ALTERNATIVE EPO SIGNALLING WITH EPO VARIANTS

EPO variants that do not bind the EPOR were shown to have neuroprotective properties. For example, the toxic effects of NMDA exposure of hippocampal slice cultures and NMDA-induced apoptosis in P19 (murine teratocarcinoma) cells were equally reduced by both EPO and CEPO treatment (Leist et al., 2004; Montero et al., 2007). AsialoEPO was also found to limit infarct volume after neonatal HI as potently as EPO itself (Wang et al., 2004c).

Since these non-erythropoietic variants do not bind to the homodimeric EPOR, it was claimed that the "classical" EPOR alone could not account for all the neuroprotective effects exerted by EPO. Therefore it was postulated that another binding site may exert neuroprotective effects by EPO.

In an early study using PC12 cells, accessory proteins were suggested to combine with the EPOR (Masuda et al., 1993). CD131, which we will refer to as CβR, matched the profile as candidate-component for an alternative EPO binding subunit. The CβR subunit was found to associate and functionally interact with the EPOR (Blake et al., 2002; Jubinsky et al., 1997) but was shown not to be required for normal hematopoiesis as observed in CβR-KO mice (Scott et al., 2000). Moreover, CβR-KO mice were not protected by EPO after compressional spinal cord injury and *in vitro*; staurosporine-induced toxicity was not attenuated by EPO in CβR-KO cardiomyocytes either (Brines et al., 2004). Therefore, EPOR-CβR heteromeres have been proposed as candidates for alternative binding sites for EPO, CEPO and other EPO variants. A confounding factor was the role the CβR subunit plays as part of the receptor for IL-3, IL-5 and GM-CSF (D'Andrea and Gonda, 2000). For example, neuroprotective properties were demonstrated using GM-CSF as well (Nakagawa et al., 2006; Schabitz et al., 2008).

Accordingly, the CβR as part of a heterocomplex with the EPOR was found to be fundamental for cytoprotection (Brines et al., 2004). Later on, the heteromeric EPOR-CβR was proposed to consist of one EPOR combined to two CβR subunits (Brines and Cerami, 2005). Concerning the apparent important role of CβR in neuroprotection, it is intriguing to consider whether 1) the EPOR upregulation seen after HI or EPO stimulation implicates the heteromeric EPOR-CβR exclusively and 2) whether the EPOR-CβR complex is primarily responsible for mediating neuroprotection.

In contrast, recently it was found in an *in vitro* study using PC12 and differentiated neuroblastoma SH-SY5Y cells that anti-apoptotic actions by EPO are critically dependent on the "classical" EPOR activating the STAT5, AKT and ERK signalling pathways while the CβR was undetectable in both SH-SY5Y and PC12 cells (Um et al., 2007). It would be interesting to investigate to what extent the "classical" EPOR and the EPOR-CβR differ in terms of their downstream molecular pathways and the implications with regard to the (type of) neuroprotection offered.

8. VASCULAR EFFECTS OF EPO

In adult and neonatal animals, revascularization was enhanced by EPO treatment after stroke and HI insult respectively (Iwai et al., 2007; Wang et al., 2004a). Consequently, in adult mice after focal cerebral ischemia, EPO was found to improve cerebral blood flow (Li et al., 2007a).

EPO treatment after permanent focal cerebral ischemia in mice increased the number of cells positive for the vessel marker glucose transporter 1 and bromo-deoxy-uridine (Li et al., 2007b). Protein levels of the angiogenic factors Tie-2, Angiopoietin-2 and VEGF were also increased by EPO treatment (Li et al., 2007a). EPO may stimulate angiogenic factor release either via an enhanced proliferation of endothelial progenitor cells or via an increased expression of endogenous growth factors or cytokines stimulating angiogenesis.

EPO possibly controls the inflammation after cerebral injury by stabilizing the BBB. When EPO was applied to mice 30 min prior to and at 3 consecutive days after focal cerebral ischemia, the BBB integrity markers occludin, α- and ß-catenin were preserved indicating BBB stabilization by EPO (Li et al., 2007b).

Taken together, the mechanisms by which EPO could improve the disturbed cerebral blood flow, stimulate angiogenesis and normalize the disrupted BBB integrity during/after stroke and HI are not understood in detail. A better understanding of the pathophysiology of the vascular system during cerebral insults will stimulate the search for novel therapeutics to improve outcome.

9. RECOMMENDATIONS AND CONCLUSIONS

The protective effects of EPO have been demonstrated in *in vitro* studies and in experimental animal models for cerebral injury, however, many questions still remain to be answered. It remains to be clarified as to whether EPO treatment is required at multiple time-points after brain damage with respect to its anti-apoptotic and anti-inflammatory effects, its effects on the vascular system and its effects on the regeneration of neuronal progenitor cells.

The detailed mechanisms responsible for EPO's neuroprotective effects are not understood. It is still not clear for example which of the molecular pathways are responsible for the neuroprotection by EPO. One possibility is that different routes are involved for different type of insults, e.g. the JAK2-NFκB pathway was found crucial for protection against NMDA and NO (Digicaylioglu and Lipton, 2001). Cells may also respond with a balanced activation of EPO-dependent specific molecular pathways based on the type of neurotoxic threat. Disentangling the molecular pathways em-

ployed by EPO has been difficult due to overlap between the pathways involved and putative compensatory mechanisms. Inhibition of specific key-proteins unique to the molecular cascades downstream of the EPOR after EPO-induced phosphorylation may elucidate our insight into EPO signalling. These novel insights into the mechanism employed by EPO will undoubtedly add potential neuroprotective targets to the pool which now includes GSK-3, NFκB and p53.

Control by EPO on the recruitment of inflammatory cells may result from EPOs effect on BBB stabilization or could be a beneficial "side-effect" of reduced neuronal cell death through binding sites other than the "classical" EPOR. The neuroprotective effects by EPO through BBB stabilization or alternative binding sites are intriguing and warrant further research which will add to our knowledge and is likely to have a major impact on the development of future therapies.

EPO has been shown to exert neuroprotective properties in a number of experimental models. For stroke, EPO has been reported to have beneficial effects (Ehrenreich et al, 2002). Frequently, the risk of increasing the number of circulating erythrocytes is stressed although detrimental consequences of extended EPO administrations have not been reported in experimental animal models for stroke or HI up to date (McPherson et al., 2007).

In contrast, chronic EPO usage has been associated with red cell aplasia (Casadevall et al., 2002). Hypertension as a consequence from increased blood pressure and an increased risk of thrombosis are other possible side effects of EPO treatment. EPO administered at a high dose for an extended period -required for the treatment of (neonatal) stroke and neonatal HI- may increase the risk for the abovementioned side effects.

Since it is not clear whether EPO can be safely applied under all circumstances, ongoing research involving the development and safety-profile of non-erythropoietic EPO alternatives should be encouraged (Ehrenreich, 2004). CEPO (and other non-erythropoietic variants) may hold great promise for future treatment of focal and global cerebral injury.

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Chapter 3

Combination of deferoxamine and erythropoietin: Therapy for hypoxia-ischemia induced brain injury in the neonatal rat?

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ABSTRACT

Deferoxamine (DFO) and erythropoietin (EPO) have each been shown to provide neuroprotection in neonatal rodent models of brain injury. In view of the described anti-oxidative actions of DFO and the anti-apoptotic and anti-inflammatory effects of EPO, we hypothesized that the combination of DFO and EPO would increase neuroprotection after neonatal hypoxic-ischemic brain injury as compared to single DFO or EPO treatment. At postnatal day 7 rats underwent right common carotid artery occlusion followed by a 90-min exposure to 8% oxygen. Rats were treated intraperitoneally with DFO (200 mg/kg), recombinant human EPO (1 kU/kg), a combination of DFO-EPO or vehicle at 0, 24 and 48 h after hypoxia-ischemia (HI) and were sacrificed at 72 h. DFO-EPO administration reduced the number of cleaved caspase 3-positive cells in the ipsilateral cerebral cortex. Early neuronal damage was assessed by staining for microtubuli-associated protein (MAP)-2. In our model 63 ± 9% loss of MAP-2 was observed after HI, indicating extensive brain injury. DFO, EPO or DFO-EPO treatment did not improve neuronal integrity as defined by MAP-2. Cerebral white matter tracts were stained for myelin basic protein (MBP), a constituent of myelin. Hypoxia-Ischemia strongly reduced MBP staining which suggests white matter damage. However, DFO, EPO and DFO-EPO treatment had no effect on the loss of MBP staining. Finally, HI-induced loss of striatal tyrosine hydroxylase-staining was not attenuated by DFO, EPO or DFO-EPO.

Although DFO-EPO treatment reduced the number of cleaved caspase 3^+ cells, treatment with DFO, EPO, or with the combination of DFO and EPO did not protect against gray or white matter damage in the experimental setting applied.

1. INTRODUCTION

Reoxygenation injury of the brain after perinatal hypoxia-ischemia (HI) is one of the most common risk factors for adverse neurodevelopmental outcome (Perlman, 2006). During HI and subsequent reoxygenation, several pathways are activated which lead to neuronal cell death. HI-induced cell death is associated with excess release of excitatory neurotransmitters, production of reactive oxygen species and pro-inflammatory mediators, and modulation of the expression of neurotrophic mediators (Blomgren et al. 2006; Bona et al. 1999; Nijboer et al. 2008; Perlman, 2006).

Deferoxamine (DFO) chelates free iron thereby preventing hydroxyl formation via the hydrogen peroxide-driven Fenton reaction (Gutteridge et al. 1979). Moreover, DFO can prevent accumulation of nontransferrin bound iron in endothelial cells and its subsequent activation attracting neutrophils and monocytes (Kartikasari et al. 2006). DFO was found to have neuroprotective properties in both postnatal day (p)7 rats and p7 mice when administered after HI (Palmer et al. 1994; Sarco et al. 2000).

Erythropoietin (EPO) has been shown to act as an anti-apoptotic, anti-inflammatory and neurotrophic mediator both *in vitro* and *in vivo* (van der Kooij et al. 2008). In adult rodents after middle cerebral artery occlusion (MCAO), it has been shown that administration of EPO was neuroprotective; the infarct volume was reduced and behavioral functions were improved (Belayev et al. 2005; Sirén et al. 2001). In rat pups, infarct size as a result from neonatal stroke induced by MCAO, either permanent (Sola et al. 2005; Wen et al. 2006) or with reperfusion (Chang et al. 2005; Gonzalez et al. 2007; Wei et al. 2006) was also reduced by EPO administration. In neonatal mice and rats EPO pre-treatment or treatment early after insult was also neuroprotective in animals subjected to the Vannucci-Rice model of HI-induced brain injury when assessed at 2-7 days after insult (Kellert et al. 2007; Kumral et al. 2003; Matsushita et al. 2003; Spandou et al 2005; Sun et al. 2004; Wang et al. 2004). Finally, the neuroprotective effect of EPO treatment after neonatal HI was reported to include a preservation of tyrosine hydroxylase (TH) positive fibers in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) (Demers et al. 2005; McClure et al. 2006; Sun et al. 2004).

The aim of our present study was to evaluate the neuroprotective potential of a combination of DFO and EPO in after HI in the newborn rat. Based on the described effects of DFO on free radical formation and the anti-apoptotic and anti-inflammatory capacities of EPO we hypothesized that combination of these two strategies may enhance neuroprotection as compared to the use of DFO or EPO alone.

2. MATERIALS AND METHODS

2.1 Animals

Experiments were performed in accordance with international guidelines and approved by the University Medical Center Utrecht experimental animal committee. Pregnant Wistar rats (Harlan, Horst, The Netherlands) were allowed normal delivery at the Central Laboratory Animal Institute (Utrecht, the Netherlands); the day of birth was considered day 0. Animals were kept on a 12:12 h light: dark cycle. Pups were subjected to surgical procedure as described earlier (Nijboer et al. 2007). In short, pups were anaesthetized (5 to 10 min) with isoflurane (5.0% induction, 1.5% maintenance) in O_2:N_2O (1:1). Blood flow in the right common carotid artery was permanently interrupted by thermocauterization, the incision was sutured and xylocaine spray (100 mg/mL) (AstraZeneca, Zoetermeer, The Netherlands) was applied. Animals were kept warm on a heated water mattress during surgery. After 1-2 h of recovery with the dam, pups were exposed to 8% O_2 in N_2 for 90 min. The gas mixture was humidified and preheated. During hypoxia, the animals were kept on a heated water mattress in an incubator. After hypoxia, the animals returned to their dams and were kept at room temperature. SHAM-treated controls underwent anesthesia and incision but received neither further surgical procedures nor hypoxia. Brains from completely untreated pups did not differ from SHAM-operated pups in any of the parameters tested (data not shown).

2.2 Treatments

DFO (Desferal®, Novartis Pharma BV, Arnhem, The Netherlands) was reconstituted in water and diluted in 0.9% NaCl solution as suggested by the supplier. Recombinant human EPO (epoetin beta, NeoRecormon®, Roche Diagnostics, Mannheim, Germany) was diluted in 0.9% NaCl solution. Pups received EPO (1 kU/kg), DFO (200 mg/kg), EPO-DFO or vehicle intraperitoneally (i.p.) immediately after and at 24 and 48 h after hypoxia.

2.3 Immunohistochemistry

At 72 h after HI animals received an overdose of pentobarbital (300 mg/kg) and were perfused with 4% paraformaldehyde in phosphate-buffered saline. Brains were embedded in paraffin. Coronal sections were cut (8 μM) at striatal, hippocampal and VTA/SNc level. Sections were incubated for 1 h at 37 °C with mouse-anti-MAP-2 (1:1,000, Sigma-Aldrich, Steinheim, Germany) or overnight at 4 °C with rabbit-anti-cleaved caspase 3 (1:800, Cell Signaling, Danvers, MA, USA), mouse-anti-myelin basic protein (MBP) (1:1,600, Sternberger monoclonals, Inc Lutherville, MD) or rabbit-anti-tyrosine hydroxylase (1:1,000, Pel-Freez Biologicals, Rogers, AR). Sections were stained with appropriate biotin-labeled secondary antibodies and Vectastain ABC (Vector Labo-

ratories, Peterborough, UK) after which staining was visualized using 3,3'- DiAminoBenzidine (Sigma-Aldrich, Steinheim, Germany) and ammonium nickel(II) sulfate hexahydrate.

As an indication for apoptotic like cell death we stained sections for caspase 3. For each animal, five stained sections were taken into account. The sections were examined using a semi-quantitative approach. The amount of caspase 3+ cells in the total ipsilateral hemisphere was estimated and scored accordingly. Sections that contained virtually no caspase 3+ cells were scored 0, sections with few cells (approx. 10-25) were scored 1, sections with several dozens of caspase 3+ cells were scored 2 and sections with an estimated total amount of caspase 3+ cells of over 100 were scored 3.

As a measure for neuronal integrity, we stained sections for MAP-2. Both hemispheres were outlined on full section images stained for MAP-2 and the surface for stained ipsi- and contralateral was calculated. The ratio between ipsi- and contralateral area was quantified and these values were compared between treatment groups (Nijboer et al. 2007). TH-staining in the striatal area was used as an indication for the intensity TH-immunoreactive (dopaminergic and noradrenergic) nerve terminals. We measured the density of TH-staining using ImageJ software (http://rsb.info.nih.gov/ij/). The ratio of ipsi vs. contralateral TH-staining intensity was calculated. All measurements were performed by one investigator blinded to experimental group.

2.4 Statistical Analysis

Data are expressed as mean ± SEM. Differences between SHAM, vehicle, DFO, EPO and DFO-EPO treated animals were compared using ANOVA and were regarded statistically significant if $p<0.05$. Post-test was performed using Bonferroni correction.

3. RESULTS

3.1 HI-induced apoptotic cell death (cleaved caspase 3)

Three days after HI, many cleaved caspase 3^+ cells were detected in the ipsilateral hemisphere (fig. 1A) whereas in the contralateral hemisphere we detected only a few caspase 3^+ cells. Staining was specific as omission of primary antibody did not result in staining (fig. 1B). We found that the semi-quantative apoptosis-score was significantly decreased after DFO-EPO treatment ($p<0.03$) while a trend towards decrease was observed after EPO treatment ($p=0.06$) (fig. 1C).

3.2 Gray matter damage

Gray matter damage was determined by quantification of loss of ipsilateral MAP-2 staining. HI induced significant loss of ipsilateral MAP-2 staining in vehicle treated

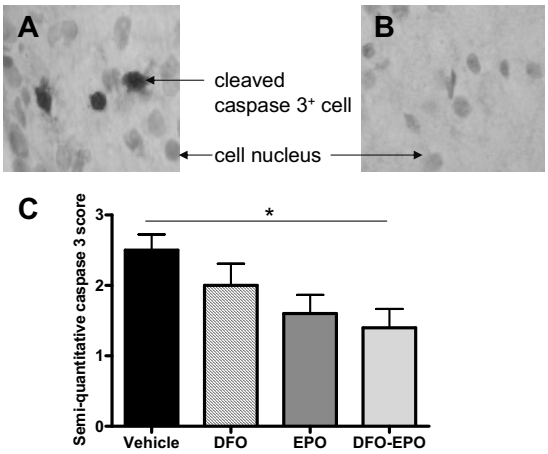

Figure 1.
At 3 days after HI, (A) cleaved caspase 3^+ cells were predominantly detected at the ipsilateral side of the cortex of the rat brain (shown is a vehicle treated animal). (B) No staining was observed when the primary antibody was omitted. (C) Semi-quantitative scores are depicted for cleaved caspase 3^+ cells as determined in five section of the ipsilateral hemisphere for each animal (n=7-10 animals per group)(*; $p<0.05$).

animals at hippocampal (63 ± 9%) and VTA/SNc level (64 ± 7%) (fig. 2A, B). Loss of ipsilateral MAP-2 staining was not influenced by treatment with DFO, EPO or the combination of DFO and EPO when assessed at either hippocampal or VTA/SNc level (fig. 2C and 2D).

3.3 White matter damage

To determine whether treatment with DFO, EPO or DFO-EPO influenced HI-induced white matter damage, sections obtained at 3 days after HI were stained for MBP. The insult reduced ipsilateral MBP staining considerably. DFO, EPO and DFO-EPO treatment did not attenuate the ipsilateral loss of MBP (data not shown).

3.4 Tyrosine hydroxylase staining

We evaluated TH-staining as a measure of integrity of dopaminergic neurons in striatal sections and sections at the VTA/SNc level. HI reduced the ipsilateral TH^+ intensity at striatal level (24 ± 6% TH^+ loss, $p<0.01$ fig. 3A) compared to SHAM-treated animals. DFO, EPO or EPO-DFO treatment did not attenuate loss of striatal TH intensity (fig. 3B). No significant loss of TH-staining intensity was found in HI-treated animals examined in VTA and SNc brain regions (data not shown).

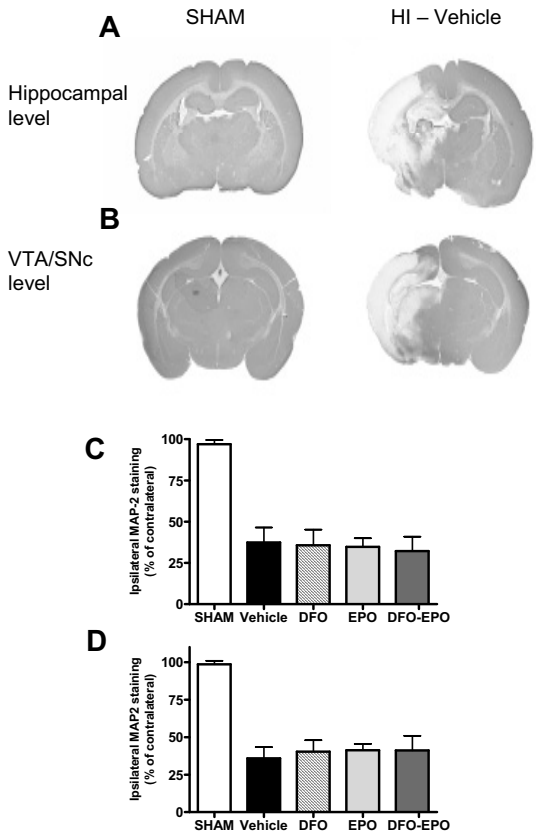

Figure 2.
MAP-2 staining as an indication for neuronal integrity was performed on rat brain sections of SHAM and HI treated animals at (A) hippocampal and (B) VTA/SNc level. Data represent mean ratio of ipsi/contralateral MAP-2-positive area for each treatment group at (C) hippocampal and (D) VTA/SNc level (SHAM: n=4, other groups: n=9-10 animals per group).

4. DISCUSSION

In the current study we show that the amount of cleaved caspase 3^+ cells, an indicator for apoptotic cell death, was reduced by about 50% by EPO or DFO-EPO treatment after HI, although the effect was statistically significant only in the DFO-EPO group. This finding is in line with the reported anti-apoptotic properties of EPO. DFO treatment alone did not affect the number of cleaved caspase 3^+ cells after HI. Furthermore, administration of DFO, EPO or DFO-EPO at 0, 24 and 48 h after HI did not reduce the loss of MAP-2, MBP or TH staining as determined at three days after the insult. Three days was chosen as experimental endpoint in this study because this coincides with the day after the last injection and because we hypothesized that we would be able to

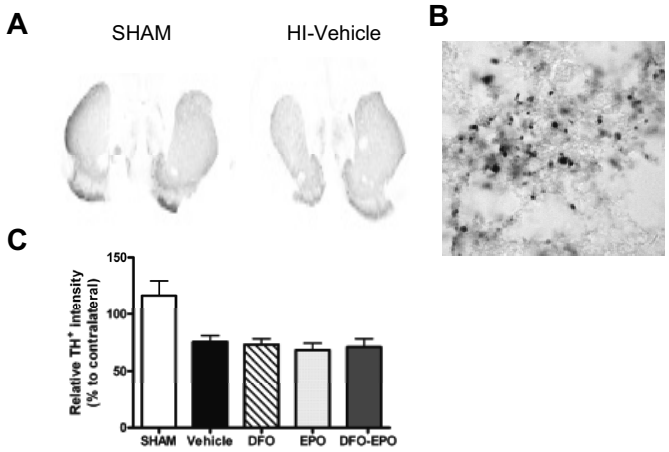

Figure 3.
(A) To measure loss of TH+ immunoreactive fibers in the striatum, TH-staining was performed on brain sections of SHAM and HI treated animals. (B) Loss of TH staining intensity was calculated for each treatment group (SHAM: n=3, other groups: n=7-10 animals per group).

see the anti-inflammatory and anti-apoptotic properties of EPO and DFO treatment. In addition, most studies involving neuroprotection after EPO have used similar end-points (van der Kooij et al. 2008).

The DFO dose of 200 mg/kg i.p. is similar to a study describing neuroprotective effects of DFO in a model of neonatal stroke using p10 rats (Mu et al. 2005). In mice, DFO (100 mg/kg s.c.) administered immediately after HI (30 min of hypoxia) was described to protect the animals as scored on a histological level (Sarco et al. 2000). Similarly, in a recent study the effects of DFO administration after a relatively mild insult (HI with 60 min of hypoxia) in p7 rats were assessed (Papazisis et al. 2008). Conventional histological examination at 7 days after the insult revealed that DFO (150 mg/kg) injected subcutaneously (s.c.) immediately after and at 24 h after insult reduced damage in the CA1 region of the hippocampus. In our experiments, the area of damage was much more extensive and therefore it was not possible to specifically assess DFO effects on CA1 integrity. In another study, DFO (100 mg/kg s.c.) administration in the p7 rat immediately after HI (2.25 h of hypoxia) reduced acute edema as determined at 42 h after HI and hemispheric atrophy analysed at 30 d after HI (Palmer et al. 1994). Duration of hypoxia in these experiments was longer as compared to our experimental setup. In addition, the readout for damage (edema early on vs. MAP-2 loss measured in our study) and timing of assessment (30 vs. 3 days after HI in our study) hampers a direct comparison. Nevertheless, we cannot exclude that difference in the route of administration of DFO (s.c versus i.p in our study) may have contributed to the lack of effect of DFO in our experiments.

The EPO dose (1 EPO injection for 3 consecutive days at 1 kU/kg i.p.) used in our experiments has been used earlier and shown to act neuroprotective (Sola et al. 2005). We have decided to use i.p. injections in our study since recently it was demonstrated that both a higher EPO plasma yield as well as detection of EPO after i.p. injection as compared to s.c. injection (Statler et al. 2007). Moreover, most studies that describe neuroprotective effects of EPO have used i.p. injections (van der Kooij et al. 2008).

In a recent study p7 rats were subjected to 90 min of HI and post-treated these animals with EPO using different doses and injection schedules after which neuroprotection was assessed. The rats were sacrificed at 2 and 7 days after insult. Either EPO (5 kU/kg) immediately after and at day 1 and 2 after insult or a single EPO injection at a 30 kU/kg dose immediately after HI were the most effective treatment schedules. Instead, a single injection immediately after insult or extended treatment immediately after and at day 1 and 2 after HI (EPO dose: 2.5 kU/kg) did not result in significant neuroprotection when assessed at 2 days after insult. However, a single EPO injection with 1 kU/kg i.p. immediately after HI has repeatedly been reported to exert neuroprotection in neonatal rats (Kumral et al. 2003, 2004, 2005). In these studies a relatively long period of hypoxia (2.5 h) was used. In contrast to our study, however, the ipsilateral tissue loss as a result from the insult as determined by conventional histology was reported to be only 16.8 ± 3.7% in vehicle treated animals (Kumral et al. 2003). Perhaps, the more extensive damage in our experimental paradigm (63 ± 9% loss of ispilateral MAP-2 staining) may explain why we did not observe neuroprotection after treatment with EPO.

Two different subtypes of recombinant human EPO are used clinically, i.e. EPO-α and EPO-ß. To our knowledge there are no differences in the clinical effect of these two isotypes on erythropoiesis. EPO-α is the most widely used subtype in studies on neonatal HI induced brain damage. For EPO-ß it has been described that pre-treatment using a dose of 2 kU/kg reduced HI-induced brain damage in the neonatal p7 rat (Spandou et al. 2005). In our study, we have used EPO-ß treatment after the insult, which is clinically more relevant, and used a lower dose. It is possible that these two differences explain the limited effect of EPO only on cleaved caspase 3+ cells in our study.

EPO has been described as protective for dopaminergic neurons in the VTA and SNc in the neonatal p7 rat when provided s.c. at 2.5 kU/kg on 3 consecutive days after HI and assessed at 3 weeks after insult (Demers et al. 2005). We did not observe significant loss of TH-intensity in the VTA/SNc regions. Perhaps, the loss of TH staining evolves gradually and may only be observed after a longer period; we sacrificed animals at 3 days after HI while in the study of Demers et al. animals were sacrificed 3 weeks after HI. In contrast, at striatal level, TH staining intensity was attenuated

after HI. TH staining intensity at striatal level however was not affected by DFO, EPO and DFO-EPO administration.

4.1 Conclusions

Despite indications of an anti-apoptotic effect of treatment after neonatal HI with DFO-EPO and a trend after EPO alone, our study could not confirm the described overall neuroprotective effects of DFO or EPO for neonatal HI as shown by others. Even though DFO or EPO treatment did not have an overt neuroprotective effect in our experimental model, we would at least have expected some neuroprotection as a result from the combined DFO-EPO treatment. We have chosen for an EPO dose of 1 kU/kg and a DFO dose of 200 mg/kg as these doses have been described to be neuroprotective. Higher doses of EPO (e.g. 5 kU/kg) may have yielded a higher neuroprotective effect but we anticipated that in that case, we would not have been able to demonstrate a synergistic effect between DFO and EPO.

Since such a protective effect was not observed, the results presented here do not suggest that the combination of DFO and EPO is beneficial for HI-induced brain injury in the neonatal rat.

We cannot exclude however that even in the absence of gross brain morphology improvements the behaviour and cognitive aspects may normalize. We aim to further elucidate the short- and longterm outcome after HI with EPO treatment using a functional approach.

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Chapter 4

Mild neonatal hypoxia-ischemia induces long-term motor- and cognitive impairments in mice.

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ABSTRACT

To understand and potentially treat the lifelong cognitive and motor deficits in humans resulting from perinatal mild cerebral hypoxic-ischemic (HI) events, valid animal models are of high importance. Nowadays the murine model of neonatal cerebral HI-injury (unilateral carotid artery occlusion followed by hypoxia) is applied more frequently. In the present study we investigated motor, behavioral and cognitive functioning in mice with mild cerebral HI-injury (45 min of hypoxia; HI-45) in comparison to mice exposed to severe HI (HI-75) and sham-control mice. Lateralizing motor disturbances as measured using the cylinder rearing test developed in both HI-45 and HI-75 mice and was significantly more severe in HI-75 animals. To assess behavior and cognitive functions, we used the modified hole board (mHB) test in two stages. First, the ability of the animals to find the 3 food rewards in cued holes over time was determined. The results revealed an overall learning impairment in HI-75 mice, while HI-45 mice were not different from sham controls. In the second stage, a reversal test was performed with rewarded cylinders being non-cued and non-rewarded cylinders being cued. This reversal task revealed impairments in cognitive flexibility in HI-45 mice as compared to sham control animals. Our data indicate that both the cylinder rearing task and the two stages of the mHB are suitable behavioral approaches to differentiate consequences of neonatal mild and severe brain damage on executive functioning.

1. INTRODUCTION

Mild hypoxia-ischemia (HI) induces significant cerebral injury in neonates and is frequently accompanied by motor and cognitive impairments throughout life (Lindstrom et al., 2006; van Handel et al., 2007). Neither the mechanisms determining the severity of long-term consequences, nor treatment options are sufficiently understood. To reach a better understanding of these processes, research on valid animal models is of great importance. Until now, however, few studies have dealt with functional consequences after mild cerebral HI in animal models (McAuliffe et al., 2006; Ten et al., 2004). Depending on the hypoxic duration after unilateral carotid artery occlusion in the mouse, mild to severe unilateral brain damage will develop. An experimental approach that can discriminate between the consequences of mild and severe HI at the executive level, i.e. behavioral and cognitive performance, may prove pivotal for the research on novel neuroprotective strategies.

In the current study, the cylinder rearing test (CRT) (Schallert et al., 2000) and two stages of the cognitive version of the modified hole board (mHB) test (Ohl et al., 2003) were used to investigate motor, behavioral and cognitive functions in adult mice that were subjected to mild or severe HI during the neonatal period.

The CRT has originally been described to detect motor disturbances in adult rat models of central nervous system injury (e.g. middle cerebral artery occlusion as a model for adult stroke) (Schallert et al., 2000) and was later on successfully applied to test mice after neonatal stroke (Chang et al., 2005).

Cognitive testing in animals usually is based on their behavioral performance. Therefore, cognitive parameters are potentially confounded by behavioral domains, such as exploratory motivation, anxiety-related behavior, locomotor activity and/or arousal (Brinks et al., 2007). The mHB test is an integrative behavioral paradigm which has been validated for both rats and mice (Ohl et al., 2003; Ohl et al., 2001). The test allows for taking into account the above mentioned behavioral domains in one test, thereby limiting inter-test interference (Belzung and Pape, 1994; Ohl et al., 2001). In addition to behavioral domains, parameters indicating long- or short-term memory, respectively, as well as overall cognitive performance were measured. Finally, the animals' cognitive flexibility was tested by performing a reversal test.

2. MATERIAL & METHODS

2.1 Animals

Experiments were performed in accordance with international guidelines and approved by the Experimental Animal Committee of the University Medical Center Utre-

cht. C57Bl/6J male mice were bred at the animal facility and surgery was performed at nine days of age (P9) (section 2.2). Mice 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*. Starting at 10 weeks of age, mice were housed in a behavioral observation room on a reversed 12h:12h dark-light cycle with lights on from 7pm-7am. During the dark-phase, mice were handled every other day to habituate to the experimentator and were subsequently allowed to consume 2 small almond pieces (0.01-0.02 g) placed in the home cage. After 2 weeks of habituation, the animals were tested in the mHB test. All analyses were performed in a blinded set-up.

2.2 Experimental model

P9 pups were anesthetized (isoflurane, 5% induction/2% maintenance in 0_2 in N_2) and the right common carotid artery was ligated. After 1-2 h of recovery, mice were subjected to 10% O_2 in N_2 for 45 (HI-45) (n=14) or 75 min (HI-75) (n=6) at a temperature of 36ºC. Sham-treated controls underwent anaesthesia and incision but no artery occlusion or hypoxia (n=19). The mortality rate was 6.7% (1/15) for HI-45 animals and 33.3% (3/9) for HI-75 animals.

2.3 Histopathology

Mice were sacrificed at 14 weeks 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 (8 µM) were cut. Brain sections were stained with hematoxylin-eosin (HE). Both hemispheres were outlined on full section images and the ratio of ipsi- and contralateral areas was calculated.

Deparaffinized sections were incubated with mouse-anti-myelin basic protein (MBP) (1:1,600) (Sternberger Monoclonals, Lutherville, MD) followed by biotin-labeled secondary antibodies and staining was revealed using Vectastain ABC kit (Vector laboratories, Burliname, CA) and diaminobenzamidine. MBP-staining was quantified using ImageJ software (http://rsb.info.nih.gov/ij/, 1997-2006).

2.4 Cylinder rearing test

At 18 days and 10 weeks of age between 1-3 pm, animals were individually placed in a Plexiglas transparent cylinder (18 days; 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: (right – left)/(right + left + both)*100 (Chang et al., 2005). The number of left-forepaw initiations was subtracted from the number of right-forepaw initiations in the numerator to take

into account the possibility that animals can prefer to use one forepaw (with no preference to use either left/right) per se instead of both forepaws. Non-injured animals will have no preference for either right- or left forepaw initiation which will increase towards a right paw preference depending on the severity of the insult.

2.5 Modified hole board test

At 12 weeks of age, animals were introduced to the mHB test according to a protocol described earlier (Ohl et al., 2003). In short, the mHB setup consisted of an opaque grey PVC box (50 x 50 x50 cm) containing a board (37 x 20 cm) placed in the middle of the box on which 10 grey cylinders (3 cm ø * 4 cm height) were staggered in two lines. All cylinders were flavoured with vanilla to attract the animals to the cylinders. Stage 1 consisted of 4 trials per day performed each with duration of maximum 5 min or until completion of the task on 5 consecutive days. During stage 1 three of the cylinders were cued (small white ring) and baited with a small almond piece (0.01-0.02 g) upon a metal grid representing the food reward (baited holes) whereas the remaining seven cylinders contained non-retrievable almond pieces placed under the metal grid (non-baited holes) (Fig. 1a). The reversal task (stage 2) was performed on the sixth day and consisted also of 4 trials with maximum duration of 5 min. During this task the baited cylinders were non-cued and the sequence was scrambled (Fig. 1b)

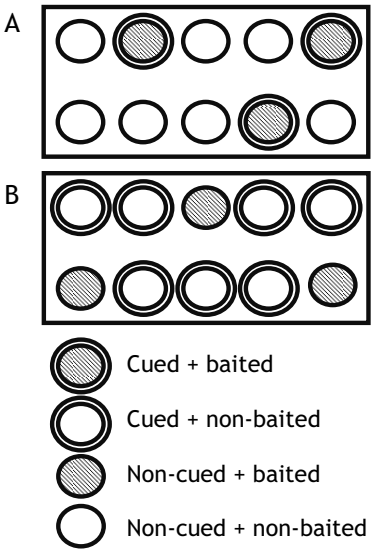

Figure 1. Setup of the modified hole board.
(A) For the first 5 days of testing, the hole board contained 3 cued and baited cylinders while 7 non-cued cylinders were non-baited (stage 1). (B) During stage 2 (the reversal-task) 3 non-cued cylinders were baited while 7 cued cylinders were non-baited. The sequence of baited cylinders was scrambled compared to the first 5 days of testing.

Between 10 am and 5 pm animals were scored live by a trained observer under red light conditions in the same room as the animals were housed for parameters indicative for cognition, exploration, anxiety, arousal and risk assessment (Table 1). The latency to complete the trial, the number of food rewards not retrieved by the animals (omission errors), the frequencies of non-baited hole visits (visits to cylinders containing a non-retrievable almond piece indicating long-term memory errors) and revisits of baited holes (indicating short-term memory errors) were analyzed as indicators of cognitive function.

Video-taping enabled further analysis (locomotor behavior). The video equipment was installed before arrival of the animals. Between trials, the arena was wiped clean with water and defecation was removed while the intertrial duration for each animal was 30-60 min.

Table 1. Parameters measured in the modified hole board.

Parameters measured	Dimension
Cognition	
Latency to find all 3 food rewards	Time (s)
Omission errors	Number
Non-baited hole visits (long-term memory errors)	Number
Revisit baited holes (short-term memory errors)	Number
Behavior	
Exploratory-related behavior	
Hole exploration	Number
Rearing in the box	Number
Anxiety-like behavior	
Board occurrence	Percentage
Latency to enter board zone	Time (s)
Duration in RIM-zone	Time (s)
Physiological arousal	
Latency to first self-grooming	Time (s)
Defecation	Number
Risk assessment behavior	
Stretched body posture	Number
Locomotor activity	
Distance moved	cm/s

2.6 Statistical analysis

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 was used to analyze group differences in area loss of ipsilateral hemispheres, the results from the CRT and risk assessment in the mHB.

The results obtained from the first 5 days of mHB testing are presented as a mean of 4 trials per day ± SEM. To analyze treatment, time, and interaction effects in the mHB two-way ANOVA with repeated measures was used with Bonferroni post-test. First, sphericity was tested using the Mauchly's test. A significant result in the Mauchly's test was corrected by either a Huynh-Feldt correction (estimates of sphericity >0.75) or by a Greenhouse-Geisser correction (estimates of sphericity <0.75) as suggested in the literature (Girden, 1992).

3. RESULTS

3.1 Histopathology

Mice at P9 were subjected to unilateral carotid artery occlusion followed by 45 or 75 min of hypoxia (10% O_2). Brain damage was assessed at 14 weeks of age. Representative examples of HE-stained sections of sham, HI-45 and HI-75 mice are shown in Fig. 2a. In the HI-45 group, the ipsilateral hippocampus was primarily affected and enlargement of the lateral ventricle was observed while other brain areas did not show overt signs of damage. The area of damage was quantified at -1.58mm from bregma ('hippocampal' level) and was significantly smaller in the HI-45 group as compared to HI-75 animals (Fig. 2b). Cerebral damage in the HI-75 group was much more extended and involved almost the entire ipsilateral hemisphere.

Loss of MBP staining, as a measure of white matter injury, was demonstrable in HI-45 and HI-75 animals but HI-75 mice were significantly more affected then the HI-45 group (Fig. 2c,d). Histological assessment did not reveal damage in the contralateral hemisphere of the animals.

3.2 Cylinder rearing test

At 18 days and 10 weeks of age we assessed the animals for motor disturbances in the CRT. Both HI-45 and HI-75 mice were significantly impaired compared to sham-control animals at 18 days of age (Fig. 3). However, at this early timepoint after the insult we did not observe a difference between HI-45 and HI-75 mice in the CRT. Retesting the animals at the age of 10 weeks revealed that the impairment had markedly increased in the HI-75 group whereas impairment did not progress in the HI-45 group. At 10

weeks of age, the preference to use the unimpaired forepaw was more pronounced in the HI-75 than in the HI-45 group and both groups differed from sham controls (Fig. 3).

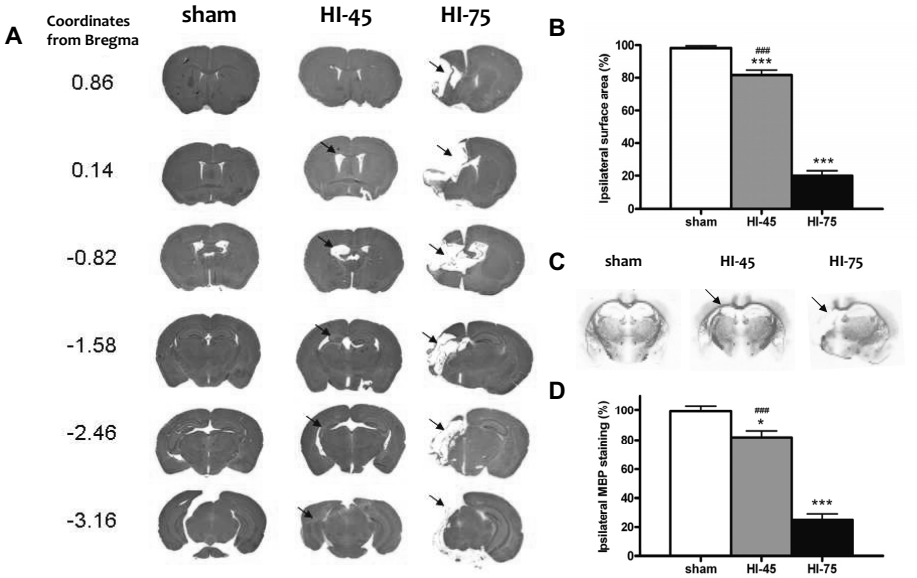

Figure 2. Brain injury after mild and severe HI.
(A) Representative examples for sham, HI-45 and HI-75 animals showing the regions affected (arrows) when comparing to sham. Distance from bregma (in mm) is depicted on the left. (B) Quantified analysis of ipsilateral surface area in sham, HI-45 and HI-75 animals. (C) Representative examples of MBP stained sections at -1.58mm from bregma for sham, HI-45 and HI-75 animals showing the regions affected (arrows) when comparing to sham. (D) Quantified analysis of ipsilateral MBP-stained area in sham, HI-45 and HI-75 animals. *, p<0.05; ***, p<0.001; HI-45 and HI-75 compared to sham, ###, p<0.001; HI-45 compared to HI-75.

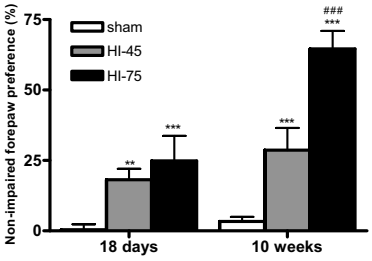

Figure 3.
Cylinder rearing test as an indication for sensory motor function shows the non-impaired forepaw preference for sham, HI-45 and HI-75 animals tested at 18 days and 10 weeks of age. **, p<0.01, ***, p<0.001 compared to sham; ###, p<0.001 compared to HI-45.

3.3 Modified hole board test

3.3.1 Cognitive performance (stage 1)

Sham-treated mice reduced the latency to complete the trial from d1 to d5 in the mHB (Fig 4a), which is consistent with decreasing numbers of omission errors (Fig 4b). The numbers of non-baited hole visits for sham animals was decreased from >2 on day 4 to <1 on day 5 indicating that sham animals have learned to find the food reward well (Fig. 4c). In the group of sham-treated animals, the number of revisits of baited holes increased from day 1 to day 2 and averaged around 1 per trial up to day 5 (Fig. 4d). Overall, HI-45 treated animals did not differ from sham mice in cognitive performance in this set up (Fig. 4).

In contrast, HI-75 mice retrieved less food rewards (Fig. 4a) and were slower to visit the three baited cylinders compared to both sham and HI-45 (Fig. 4b). Initially, the number of non-baited hole visits was significantly higher for sham and HI-45 than those observed in HI-75 (day 1 and 2; Fig. 4c). Subsequently, the number of non-baited hole visits decreased in sham and HI-45 animals while these numbers increased for HI-75 mice up to a significantly higher level on day 5 compared to sham and HI-45

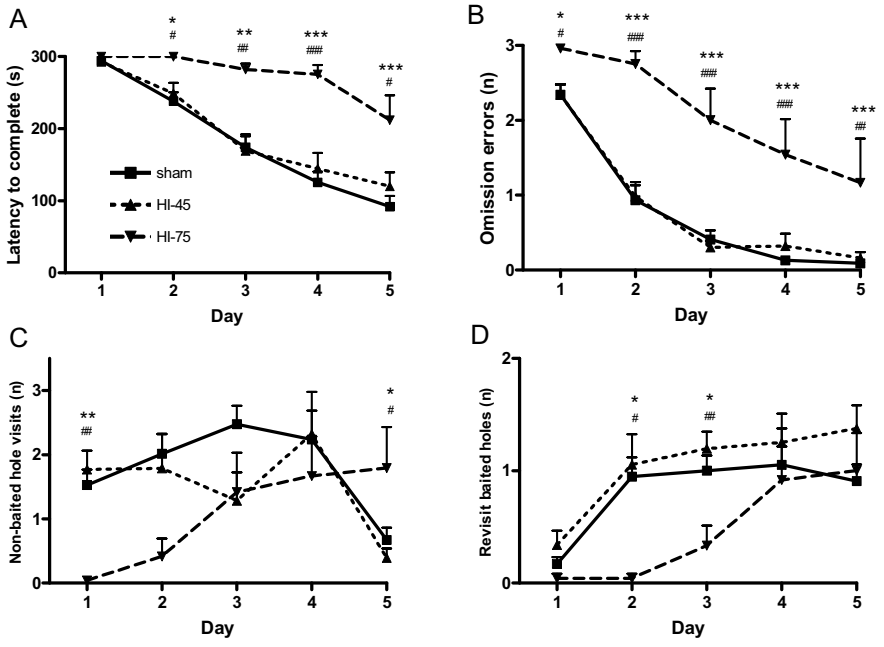

Figure 4.
Cognitive parameters measured for sham, HI-45 and HI-75 animals in the mHB during stage 1. Cognition was measured by (A) latency to complete the trial, (B) number of omission errors, (C) non-baited hole visits and (D) revisits for baited holes. *, p<0.05; **; p<0.01; ***, p<0.001 sham vs. HI-75 animals, #, p<0.05; ##, p<0.01; ###, p<0.001 HI-45 vs. HI-75 animals.

animals. These data indicate increased long-term memory errors for HI-75 animals. The number of revisits of baited holes was lower on day 2 and day 3 in HI-75 animals compared to sham- and HI-45 treated animals (Fig. 4d).

3.3.2 Behavioral activity (stage 1)

The number of holes explored on day 1-2 was higher in sham and HI-45 mice than in the HI-75 group, indicating reduced exploratory behavior in HI-75 animals on day 1-2 (Fig. 5a). Box-rearing frequency did not decrease in HI-75 animals on day 3-5 (in contrast to sham and HI-45 animals) but due to large variability, differences between groups were not statistically significant (Fig. 5b).

A significant group-effect was found for board occurrence; HI-75 mice did not spend as much time on the board as sham and HI-45 animals at days 1-4 (Fig. 5c). However, both the latency to enter the board zone and the duration of time the animals spent in the rim-zone (defined as the zone 5 cm from the edge of the arena to measure thigmotaxis) was not different between groups (data not shown).

The latency to first self-grooming and the number of boli for sham, HI-45 and HI-75 did not differ (data not shown).

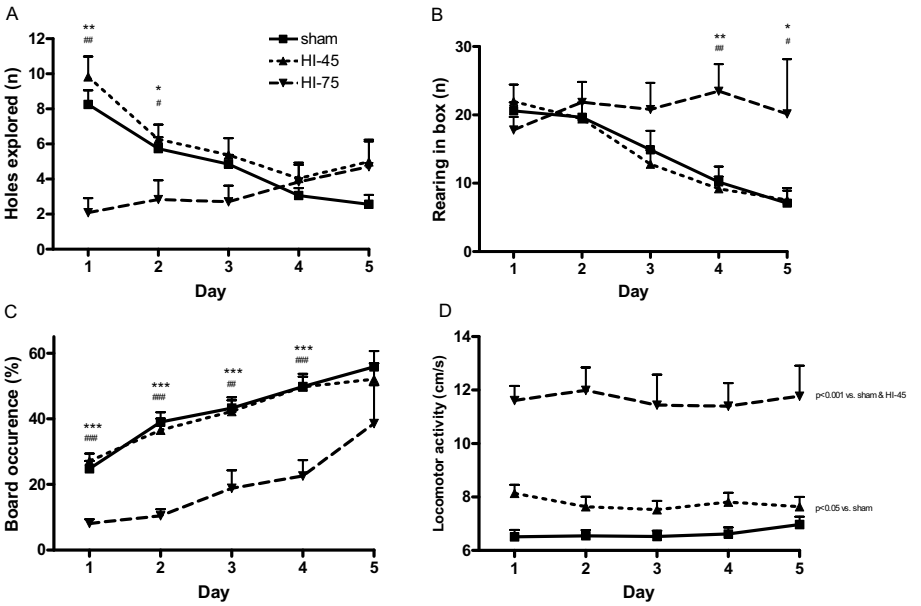

Figure 5.
Behavioral parameters measured for sham, HI-45 and HI-75 mice in the mHB during stage 1. Exploration was measured by (A) the number of holes explored and (B) box-rearing frequency. The relative time spent on the board (C) was taken as an indication for anxiety. (D) Locomotor activity. *, p<0.05; **; p<0.01; ***, p<0.001 sham vs. HI-75 animals, #, p<0.05; ##, p<0.01; ###, p<0.001 HI-45 vs. HI-75 animals.

No differences between the groups were found for the number of stretched attends as an indication for risk assessment; this behavior was only observed during first trial on the first day, (data not shown).

Locomotor activity was increased in both HI groups as compared to sham controls. The increased locomotion as seen for HI-45 and HI-75 animals persisted during the days tested in the mHB test (Fig. 5d). In addition, locomotor activity of HI-75 mice was significantly increased compared to HI-45 animals.

3.3.3 Cognitive performance and behavioral activity (stage 2)

To verify the absence of cognitive or behavioral impairments in HI-45 animals, we additionally tested this experimental group in comparison to sham animals by means of a reversal mHB test (Fig. 1b).

During the first trial of the reversal task the latency to complete the trial was increased in both groups as compared to the last trial of the first stage (Fig. 6a). Importantly, the latency to complete the trial was decreased more rapidly in sham than in HI-45 mice indicating an impaired cognitive flexibility (re-learning) in the HI-45 mice (Fig. 6a).

As compared to the previous trial we observed a strong transient increase in the number of non-baited hole visits for both sham and HI-45 animals (Fig. 6b). During the reversal task the number of non-baited hole visits (Fig. 6b) as well as the number of revisits of baited holes (data not shown) were not different between sham and HI-45 animals.

An overall increase in hole exploration in both sham and HI-45 mice was observed when comparing the first trial of the reversal task to the last trial of the first stage. Interestingly, during the reversal task, more holes were explored by HI-45 mice than by sham mice indicating increased exploratory behavior for HI-45 animals (Fig. 6c). Box-rearing behavior was increased for both sham and HI-45 animals during the first trial of the reversal task compared to the previous trial. The increased box-rearing behavior was not significant after post-test partly because of the high variability on this parameter (Fig. 6d). During the reversal task the amount of box-rearing decreased in both sham and HI-45 animals but remained higher in the HI-45 group although statistical significance was not reached.

4. DISCUSSION

In this study we show that mild and severe neonatal cerebral HI injury in mice induces differential effects in motor and cognitive function at adult age. We are the first to show that both the CRT and the mHB test can be employed to distinguish between the

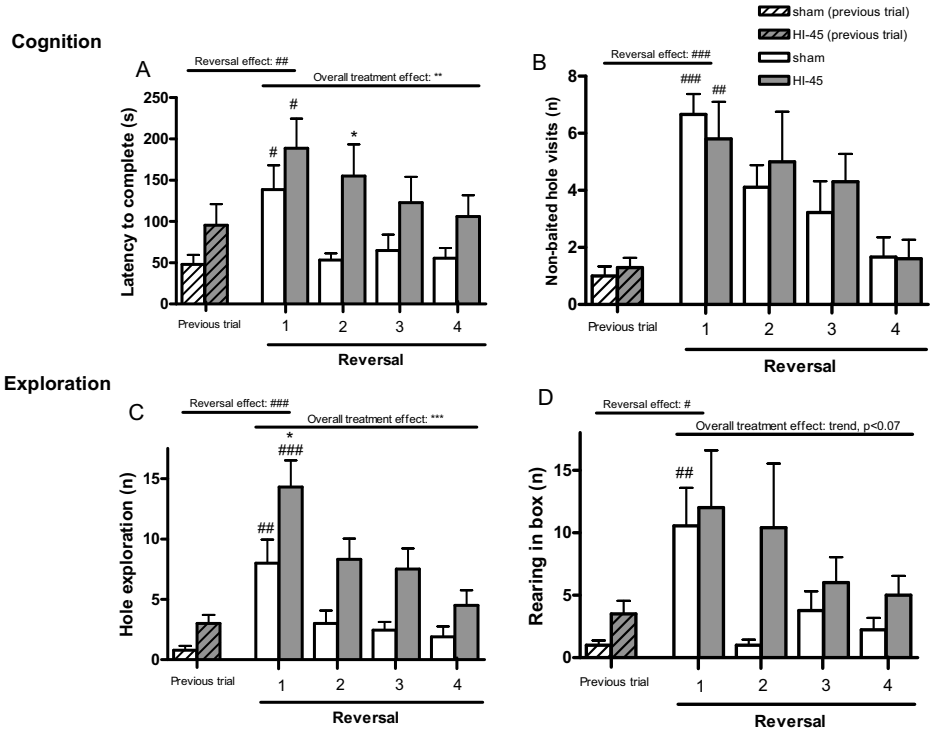

Figure 6.
Cognitive and explorative parameters measured for sham and HI-45 animals in the mHB during the reversal task. Cognition was measured by (A) latency to complete the trial and (B) the number of non-baited hole visits. Exploration was measured by (C) the numbers of holes explored and (D) box-rearing frequency. Trial effects: #, $p<0.05$; ##, $p<0.01$; ###, $p<0.001$ first trial reversal task vs. previous trial. Treatment effects: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ HI-45 animals vs. sham.

consequences of mild and severe neonatal HI injury at the executive level, which is motor movements and cognitive performance, respectively.

The duration of hypoxia is the major determinant for the amount of cerebral injury in mice after HI at a given age (McAuliffe et al., 2006; Sheldon et al., 1998). However, the variation in brain damage after a relatively short hypoxic duration is generally large, in part due to the formation of porencephalic cysts (Ikeda et al., 2001; Ten et al., 2004). In our study histopathological analysis proved that we successfully induced severe (HI-75) as well as mild (HI-45) cerebral injury in mice after HI with a minimal range of infarct variation within groups (Fig. 2c). A stable neonatal HI model for mild cerebral injury is crucial for the investigation of neuroprotective strategies since some treatment effects may not be observed after severe brain injury (van der Kooij et al., 2009).

To analyze the effects of severe vs. mild cerebral injury, we first tested whether the CRT was a valid discriminative tool for the assessment of sensorimotor function in mice. In the literature, detection of sensorimotor deficits in rodents after neonatal HI are dependent on the test used; e.g. impairment on the rotarod was demonstrated only in severely affected animals (Åden et al., 2002; Balduini et al., 2000; Ikeda et al., 2001; Jansen and Low, 1996; Ten et al., 2004) whereas the adhesive removal task was reported not to be sensitive enough to distinguish HI-affected rats from sham animals (Grow et al., 2003). By applying the CRT at 18 days of age similar motor impairments were observed in the HI-45 and HI-75 group. At 10 weeks, the motor impairment was more pronounced in the HI-75 animals when compared to the HI-45 animals, proving that the CRT test allows for the differentiation of sensorimotor dysfunctions of either severe or mild cerebral injury in mice.

At the cognitive level, HI-75 animals differed from sham- and HI-45 animals as it took HI-75 animals significantly more time to complete the trial. The average time to complete the task reduced over time in the HI-75 group, indicating that at least part of the animals retrieved all 3 food pellets. The finding that on average there are still food pellets that were not retrieved by HI-75 animals indicates that at the same time there are still animals who did not complete the task. Furthermore, HI-75 animals showed more non-baited hole visits on day 5 as compared to both sham- and HI-45 treated animals indicating a delayed learning effect for HI-75 animals. These results indicate that HI-75 animals were cognitively impaired as compared to sham- and HI-45 mice. In contrast to the sham and the HI-45 group, exploratory behavior in HI-75 animals was low at the beginning and increased over time, indicating that the cognitive impairment in HI-75 animals was not secondary to a lack of overall motivation. This suggestion is further confirmed by the fact that no differences in anxiety-related behavior, overall activity or other behavioral domains between groups were detected. We cannot exclude, however, that the enhanced locomotor activity observed in HI-75 animals has confounded our results with respect to the cognitive impairments and the results need therefore be interpreted with care.

Irrespective of the significant cerebral damage in HI-45 animals, no cognitive impairment was found during the first stage of the mHB test. To either validate the absence of cognitive impairments or detect more subtle effects in HI-45 animals, we additionally performed a reversal task of the mHB test. In re-learning of the previously obtained information, i.e. learning that the previously baited cylinders now are unmarked while the non-baited cylinders are marked, the hippocampus plays a pivotal role. Since the hippocampus was the brain region most severely affected in HI-45 animals, we hypothesized that a challenge of this type of cognitive flexibility could reveal additional information on the effects of a mild lesion (Fig. 1b). We found that during the reversal-task, the latency to complete the trial was significantly higher for

HI-45 as compared to sham animals, indicating an impaired cognitive flexibility after mild HI injury. The extended duration to complete the task by HI-45 was not caused by increased non-baited hole visits as these were increased to the same extent for sham control animals during the reversal task. Instead, compared to sham, HI-45 explored more holes and showed a trend for enhanced rearing behavior, indicating a less directed exploration strategy in these animals. It might be hypothesised that HI-45 mice were more distracted by the new situation and found it more difficult to adapt to it.

Since different behavioral domains and especially the emotional state of an animal may affect its cognitive performance, we investigated whether anxiety-related behavior, arousal and risk assessment behavior were affected by HI as well. HI-75 animals showed a higher avoidance of the central area of the experimental environment in comparison to sham and HI-45 mice, a behavior which might at the first sight be interpreted as increased anxiety (Ohl et al., 2001). However, no differences between groups tested were found for the latency to first self grooming, the latency to enter the central area, arousal or risk assessments. In contrast, individuals of the HI-75 group showed a persistent hyperactivity throughout the testing period and therefore conclude that the reduced time spend in the central area is likely to be secondary to a primary increase in undirected hyperactivity in HI-75 animals. The hyperactivity seen in HI-75 animals may have been caused by motoric disinhibition due to the severe injury inflicted to the basal ganglia after HI-75 (Chevalier and Deniau, 1990).

A more modest (but significant) increase in locomotor activity was found in the mildly affected HI-45 animals throughout day 1-5 of testing in the mHB. In this respect, hyperactivity has been reported for children with moderate neonatal encephalopathy (Marlow et al., 2005; Moster et al., 2002; Robertson and Finer, 1988). Moreover, some reports describe children diagnosed with attention-deficit/hyperactivity disorder (ADHD) after a HI-history (Lindstrom et al., 2006; Moster et al., 2002). In fact, neonatal hypoxia in rats has been proposed as an animal model for ADHD although a thorough evaluation of its validity is lacking (van der Kooij and Glennon, 2007).

Distinguishing animals with relatively mild brain injury from controls on a neurobehavioral level is pivotal for development of future therapies. Currently, only a limited number of studies have been published that involve neurobehavioral assessment of animals that sustained mild cerebral damage due to neonatal HI at an adult stage. In our study, differential motor deficits in HI-45 and HI-75 mice were clearly demonstrated in the CRT. At the cognitive level, both HI groups showed cognitive impairments but notably, the effects at the executive level in mildly damaged HI-45 animals are much more subtle than those in severely damaged HI-75 individuals. Careful cognitive-behavioral testing thus is required in order to reveal the neurobehavioral consequences of mild neonatal cerebral injury.

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Chapter 5

NF-κB inhibition after neonatal cerebral hypoxia-ischemia improves long-term motor and cognitive outcome in rats.

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ABSTRACT

We recently demonstrated that inhibition of the NF-κB-pathway by the specific peptide inhibitor TAT-NBD markedly reduced cerebral injury in a rat model of perinatal hypoxic-ischemic (HI) brain damage. The aim of the current study was to assess whether neuroprotection by TAT-NBD is associated with long-term functional improvements after neonatal HI. Postnatal-day 7 rats subjected to HI showed motor deficits in the cylinder-rearing test and adhesive removal task. HI-treated animals also showed cognitive impairments in a visuo-spatial learning task (modified hole board) as defined by an increased latency to complete this task and increased numbers of short- and long-term memory errors. HI-animals treated with TAT-NBD [20mg/kg i.p.] at 0 and 3h post-HI did not show impairments in the cylinder rearing test, adhesive removal task and modified hole board. In conclusion, the almost complete reduction in lesion size observed after TAT-NBD treatment was associated with long-lasting normalization of sensorimotor and cognitive functions.

1. INTRODUCTION

Neonatal hypoxia-ischemia (HI) severely affects brain integrity with long-term functional consequences including motor impairment and reduced intellectual, educational, and neuropsychological performance of affected children (Cowan et al. 2003; Ferriero, 2004; van Handel et al. 2007).

An adequate intervention to treat neonatal HI brain damage is not available at present. Recent clinical studies showed that hypothermia had modest neuroprotective effects after injury, but did not reduce death or severe disability (Gluckman, 2005; Shankaran et al. 2005; Azzopardi et al. 2009). Similarly, erythropoietin was found to be safe but the neurological outcome in infants improved only after moderate HI (Juul et al. 2008; Zhu et al. 2009). Therefore, development of additional therapies for severe neonatal HI is highly desirable.

We recently showed that inhibition of the activation of the transcription factor Nuclear Factor-κB (NF-κB) had a marked neuroprotective effect in a rat model of neonatal HI brain damage. NF-κB activation is dependent on phosphorylation of IκB by a complex of IKKα, IKKß and the regulatory subunit NEMO (IKKγ) (Yamaoka et al. 1998). In our studies, we used the specific NF-κB inhibitor NEMO-Binding Domain peptide (NBD) (May et al. 2000) coupled to a TAT-sequence to facilitate cerebral uptake. We showed in a neonatal rat model of cerebral HI that after intraperitoneal administration TAT-NBD peptide rapidly distributes to the brain and inhibits cerebral NF-κB activity as determined 3h after the insult. We also showed that TAT-NBD treatment provides marked neuroprotection as it reduced loss of MAP2 staining when applied up to 6h after HI (Nijboer et al. 2008). The most effective neuroprotective effect was obtained by administration of TAT-NBD immediately after and 3h after HI. The neuroprotective effect after TAT-NBD treatment was long-lasting as determined by histopathological analysis at 6 weeks after the insult.

We do not know, however, whether the neuroprotective effect of TAT-NBD treatment is associated with improved functional outcome. Earlier studies indicated that the severity of brain injury is not always directly related to functional performance. For example, dexamethasone pre-treatment completely prevented histological brain damage in rats after neonatal HI but only partially improved performance when tested in the water maze (Ikeda et al. 2002). Furthermore, rehabilitative training tasks and daily environmental enrichment did not affect the histological outcome after neonatal HI in rats but improved memory impairment in the water maze (Ikeda et al. 2006; Pereira et al. 2007).

In the current study, we therefore evaluated whether the marked reduction in HI brain damage that we observed after treatment with TAT-NBD was associated with restoration of long-term motor- and cognitive function. We used the cylinder rear-

ing test and the adhesive removal task (Nijboer et al. 2009) to investigate effects of treatment on sensorimotor function. Cognitive performance and behavior indicative of arousal, anxiety and exploration was evaluated using the modified hole board (mHB) test (Ohl et al. 2002), a multidimensional behavioral test which we recently validated for detection of functional impairment in adult mice and rats after neonatal HI (van der Kooij et al. 2009, Nijboer et al. 2009).

2. MATERIALS AND METHODS:

2.1 Animals

Experiments were performed in accordance with international guide lines and the animal care committee of the Academic Biomedical Center Utrecht (DEC-ABC) approved all experiments. HI was induced at postnatal day 7 (P7) in rat pups of both genders as described before (Nijboer et al, 2007). Pups were anesthetized (isoflurane, 5% induction/1.5% maintenance in 50% O_2 in N_2) and the right common carotid artery was ligated. After 1h of recovery, pups were subjected to 120 min of hypoxia (FiO_2 0.08). Sham-controls underwent anesthesia and incision but no artery occlusion or hypoxia. After the hypoxic period all animals returned to their dam. At 4 weeks of age, animals were weaned and group-housed per gender on a normal day-night cycle. At 10-12 weeks of age (one week prior to mHB testing), animals were placed in a separate room on a reversed 12h:12h dark-light cycle (lights on 7 pm- 7 am). In the week prior to mHB-testing animals were allowed to consume 3 small almond pieces (0.02-0.03 g) every other day to facilitate food-reward recognition in the mHB the subsequent week. During the whole duration of the experiment food and water was available *ad libitum*.

2.2 Treatment

TAT-NBD (YGRKKRRQRRR-TALDWSWLQTE;) (W.M. Keck facility, Yale University, New Haven, Ct) was dissolved in DMSO (40 mg/mL), diluted to 2 mg/ml in PBS and injected intraperitoneally immediately after and at 3h post-HI at a dose of 20 mg/kg. 5% DMSO in PBS was used as vehicle-solution.

2.3 Histology

Animals were sacrificed at 14 weeks of age with overdose pentobarbital (300 mg/kg i.p.). Rats were perfused with 4% paraformaldehyde in phosphate-buffered saline. Brains were post-fixed, embedded in paraffin and coronal sections (8 µm) were cut. Deparaffinized sections were incubated with mouse-anti-MAP2 (1:1,000 Sigma-Aldrich, Steinheim, Germany) or mouse-anti-myelin basic protein (MBP) (1:1,600 Sternberger Monoclonals, Lutherville, MD) followed by biotin-labeled horse-anti-mouse second-

ary antibodies and staining was revealed using Vectastain ABC kit (both Vector-labs, Burlingame, CA) and diaminobenzamidine. Full section images were captured with a Nikon D1 digital camera (Nikon, Tokyo, Japan). Ipsilateral area loss was determined on haematoxylin-eosin (HE)-stained sections. Ipsilateral MAP2 positive area loss was determined as a measure of neuronal damage and ipsilateral MBP positive area loss was determined to evaluate white matter damage. HE- and MAP2 stained sections 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. The area of MBP-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).

2.4 Cylinder rearing test

At 18 days, and 4 and 6 weeks of age, animals were individually placed in a Plexiglas transparent cylinder and observed for 3 min. Initial forepaw (left/right/both) preference of weight-bearing contacts during full rear were scored. The relative proportion of right (ipsilateral) forepaw contacts were calculated as: (right - left) / (right + left + both) x 100 (Chang et al. 2005). The cylinder rearing test was performed between 1-3 pm by a trained observer blinded to treatment.

2.5 Adhesive removal task

At 5 and 7 weeks of age, stickers (tough-spots, Diversified Biotech, Boston MA) were placed on the forepaw and the latency to removal was recorded. The order for left/right forepaw sticker placement was alternated between and within animals. We determined the mean removal latency of three sticker placements per forepaw after one training sticker per forepaw. The time to remove the training sticker for both left and right forepaws was not analyzed but served to habituate the animal to the test (Nijboer et al. 2009). The adhesive removal task was performed between 1-3 pm by a trained observer blinded to treatment.

2.6 Modified hole board test

At 11-13 weeks of age, animals were introduced to the mHB test as described by Ohl et al. (2002). In short, the mHB setup consisted of an opaque gray PVC box (100 x 50 x50 cm) containing a board

(60 x 20 cm) placed in the middle of the box on which 20 gray cylinders (3 cm ø * 4 cm height) were staggered in three lines (Fig. 1). The PVC box is enlarged by an additional 50-cm square compartment, in which the group mates of the experimental rats are placed during the test-period. This compartment is separated from the test area by a transparent perforated Plexiglas partition.

All cylinders were flavored with vanilla to attract the animals to the cylinders.

Animals were monitored during 4 trials per day for 5 consecutive days between 10 am and 5 pm, that is, during the activity phase of the animals. During testing trials all cylinders contained non-retrievable almond pieces placed under a metal grid (Fig. 1). In addition, 3 out of the 20 cylinders were baited with a small almond piece (0.02-0.03 g), which was placed on top of the metal grid. These almond pieces thus were retrievable and represented the food reward. The trials lasted maximally 5 min or until retrieval of all 3 food pellets. On the first 3 sequential days (stage 1, T1-T3) the baited cylinders were cued (small white ring) while the non-baited cylinders were non-cued. During the 4th day the location of the three cued and baited cylinders was scrambled (stage 2, cue switch). On the 5th testing day we introduced the animals in the mHB to a reversed setup (stage 3, reversal task); food rewards were placed in 3 non-cued cylinders while 17 non-baited cylinders were now cued. During the reversal task the baited cylinders were also at a novel position relative to the previous day.

Animals were scored live by a trained observer blinded to treatment under red light conditions for parameters indicative for cognition, exploration, anxiety and arousal as described previously (Table 1). Video-taping enabled further analysis (locomotor

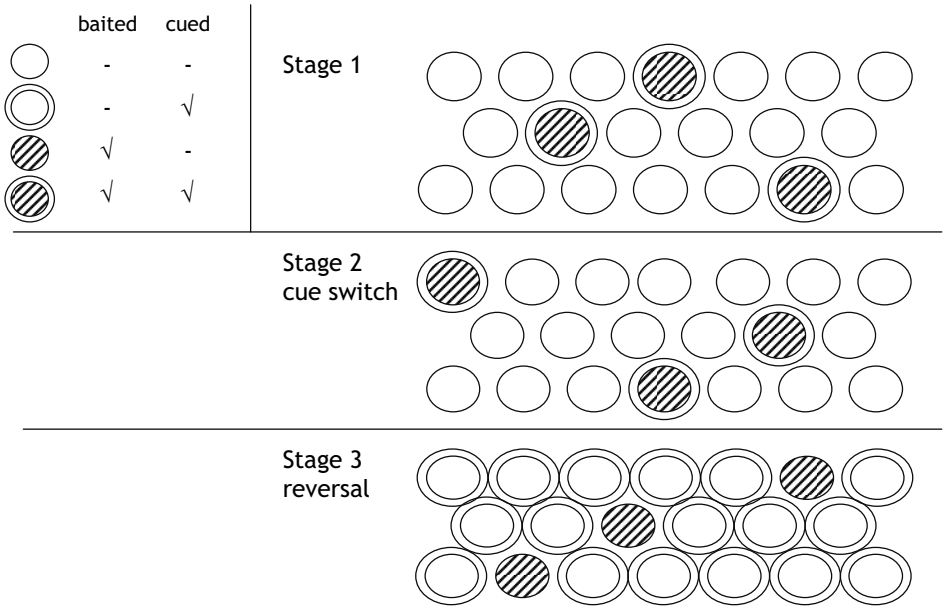

Figure 1.
Setup of the modified holeboard during training (T) day 1-3; 3 cued cylinders were baited while 17 non-cued cylinders were non-baited. During the cue switch the location of the cued and baited cylinders was scrambled compared to T1-3. During the reversal task the sequence of the 3 baited cylinders was scrambled again. The baited cylinders during the reversal task were non-cued while the remaining cylinders were cued but non-baited.

Table 1.

Parameters measured	Dimension
Cognition	
Latency to find all 3 food rewards	Time (s)
Omission errors	Number
Non-baited hole visits (long-term memory errors)	Number
Revisit baited holes (short-term memory errors)	Number
Exploratory-related behavior	
Hole exploration	Number
Rearing in the box	Number
Anxiety-like behavior	
Latency to enter board zone	Time (s)
Group contact	Number
Physiological arousal	
Latency to first self-grooming	Time (s)
Locomotor activity	
Distance moved	cm/s

activity). For cognitive performance in the mHB we measured the number of omission errors (food rewards not retrieved), the latency to find all three food rewards, the number of non-baited hole visits (visits to cylinders with a non-retrievable food reward indicating long-term memory errors) and revisits of baited holes (visits to cylinders from which food had already been retrieved, indicating short-term memory errors).

2.7 Statistical Analysis

Data are expressed as mean ± SEM and regarded statistically significant if $p<0.05$. Data were analyzed using one- or two-way ANOVA with LSD post-test.

3. RESULTS

3.1 Histology

Exposure of mice to HI at P7 induced a pronounced loss of ipsilateral brain area as determined at 14 weeks of age. In line with our previous data, HI/TAT-NBD-treated animals were almost completely protected from HI-induced brain damage (Fig. 2A).

We observed marked reductions in HI-induced ipsilateral gray and white matter loss after TAT-NBD treatment and in fact, we did not observe differences in the amount of gray or white matter between sham-controls and HI/TAT-NBD-treated animals (Fig. 2B & 2C). Histological assessment did not reveal damage in the contralateral hemisphere of any of the animals.

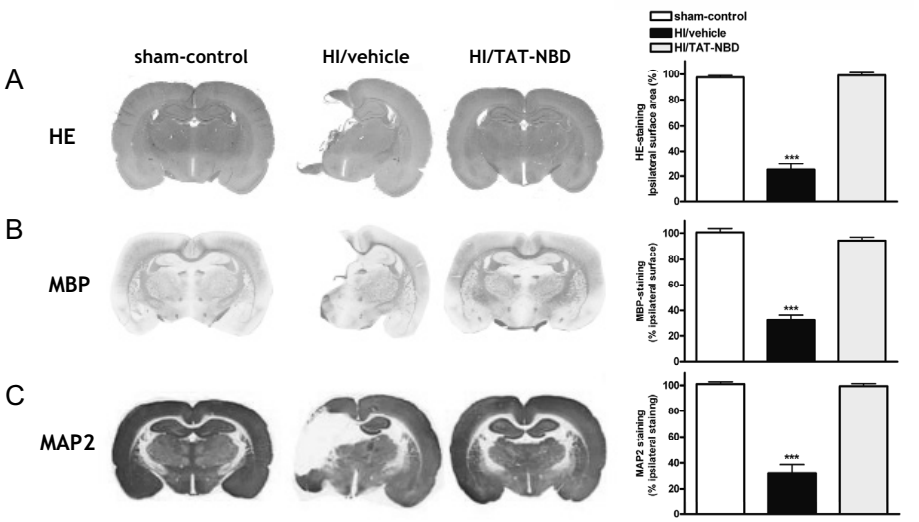

Figure 2.
Brain injury after HI and the effect of TAT-NBD treatment. Representative images and quantified ipsilateral staining analysis of (A) HE-, (B) MAP2- and (C) MBP-stained sections showing the infarct size in HI/vehicle-treated animals and the protective effect in HI/TAT-NBD-treated animals. ***, $p<0.001$ sham-controls vs. HI/vehicle-treated animals and HI/TAT-NBD-treated animals vs. HI/vehicle-treated animals.

3.2 Motor function after TAT-NBD-treatment

In the cylinder rearing test (Fig. 3A) sham-controls did not show a left- or right-forepaw initiation preference when rearing. In contrast, exposure of rats to HI resulted in a marked non-impaired forepaw preference at all ages (18d, 4 weeks and 6 weeks) tested. TAT-NBD treatment after HI significantly improved performance in the cylinder rearing test as compared to HI/vehicle animals at all timepoints; we could not detect a difference in performance in the cylinder rearing test between HI/TAT-NBD-treated animals and sham-controls.

Animals were tested in the adhesive removal task at 5 (Fig. 3B) and 7 weeks of age (data not shown.). In sham-control animals the latency for adhesive removal from either left- or right-forepaw was similar. It took HI/vehicle-treated animals longer to remove the sticker from the impaired (left)-forepaw compared to sham-controls. Treatment with TAT-NBD improved motor function as determined in the adhesive re-

moval task; HI/TAT-NBD-treated animals showed reduced latencies to complete removal of the adhesive from the left forepaw as compared to HI/vehicle rats. The latency to adhesive removal from the non-impaired (right)-forepaw was similar for sham-controls, HI/vehicle-treated animals and HI/TAT-NBD-treated animals. We observed no differences in performance within groups when comparing adhesive removal task performance at 5 and 7 weeks.

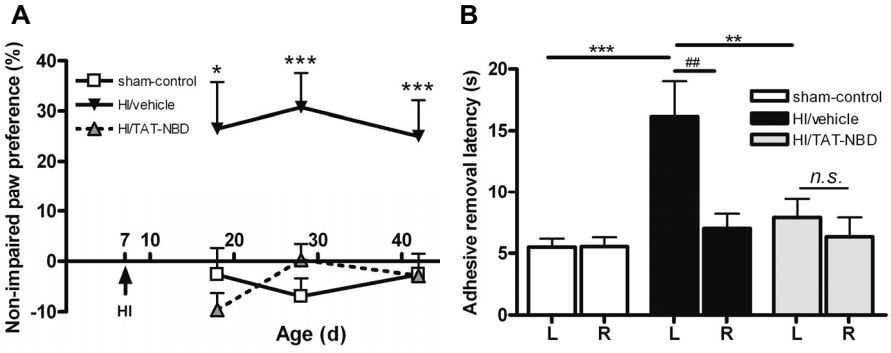

Figure 3.
Motor functions after HI and the effect of TAT-NBD treatment. (A) Non-impaired (right) paw preference during rear is diminished after TAT-NBD treatment in the cylinder rearing task. The effect of TAT-NBD-treatment in the cylinder rearing test was assessed 18d, 4w and 6w of age. *, $p<0.05$ and ***, $p<0.001$ comparing sham-controls and HI/NBD-treated animals to HI-vehicle-treated animals. (B) Adhesive removal latency at 5 weeks of age from the impaired left (L) forepaw is reduced after TAT-NBD-treatment in the adhesive removal task. The removal latency from the non-impaired right (R) forepaw did not differ between groups tested. N.S. denotes no statistically significant differences; **, $p<0.01$ vs. HI-vehicle left forepaw; ***, $p<0,001$ vs. HI/vehicle left forepaw and ##, $p<0.01$ left- vs. right forepaw in HI/vehicle-treated animals.

3.3 Cognitive performance in the modified hole board test after TAT-NBD treatment

During the training phase (T1-3), parameters indicative for cognitive functioning (see section 2.6) did not differ between the groups tested. Animals readily learned to find the food rewards as indicated by the decrease in the number of omission errors (fig. 4A) and the reduction in latency to retrieve all three available food rewards (Fig. 4B). During T1-3, the number of non-baited hole visits, indicating long-term memory performance, decreased gradually (Fig. 4C), while the number of revisits of baited holes, indicating short-term memory performance, increased (Fig. 4D).

After the cue switch, HI/vehicle-treated animals visited more non-baited holes as compared to sham-controls. In HI/TAT-NBD-treated animals this increase in the number of non-baited hole visits was not observed and the number of non-baited hole visits were not different from sham-controls (Fig. 5A). The number of omission errors,

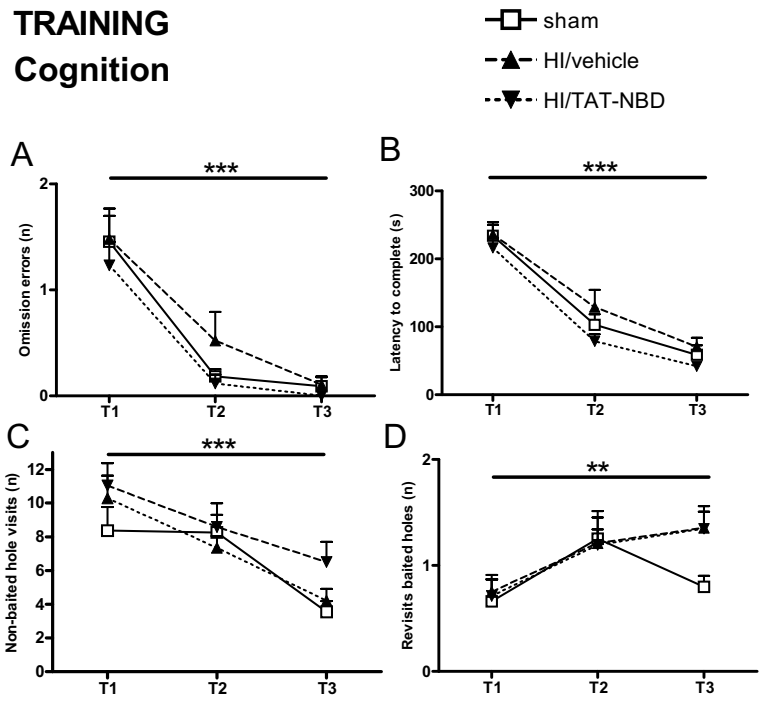

Figure 4.
Cognition for sham-controls, HI/vehicle and HI/TAT-NBD-treated rats in the mHB during training (T1-3). Cognition was measured by (A) the number of omission errors, (B) the latency to complete the trial, (C) non-baited hole visits and (D) revisits to baited holes. Time effects during T1-3: **, $p<0.01$ and ***, $p<0.001$.

the latency to complete the trial or the number of revisits to baited holes was not different between sham-controls, HI/vehicle and HI/TAT-NBD-treated animals during the four trials after the cue switch (Fig. 5B, C & D).

The subsequent day both location and cue of the baited holes were changed to test cognitive flexibility. During this reversal task, cognitive impairments in HI/vehicle-treated animals as compared to sham-controls became evident in multiple aspects of the task. All animals found the 3 food rewards but it took HI/vehicle-treated animals longer to complete the trial than sham-controls (Fig. 6A & B). In addition, HI/vehicle-treated animals visited more non-baited holes than sham-controls (Fig. 6C). Importantly, animals that received TAT-NBD treatment after HI did not show the increased latency to complete the task or the increased numbers of non-baited hole visits that was observed in HI/vehicle-treated animals. The number of revisits to baited holes was also increased for HI/vehicle-treated animals. Although the HI/TAT-NBD-treated animals tended to perform better on this aspect, the difference did not reach statistical significance (Fig. 6D).

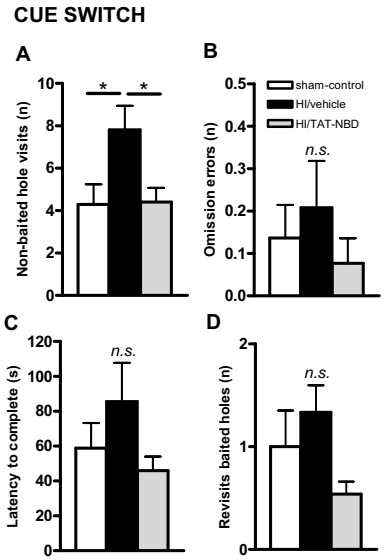

Figure 5.
Cognition for sham-controls, HI/vehicle- and HI/TAT-NBD-treated rats in the mHB after the cue switch. Cognition was measured by (A) the number of non-baited hole visits, (B) omission errors, (C) the latency to complete the trial, and (D) revisits to baited holes. N.S. denotes no statistically significant differences; *, p<0.05 as compared to HI/vehicle-treated animals.

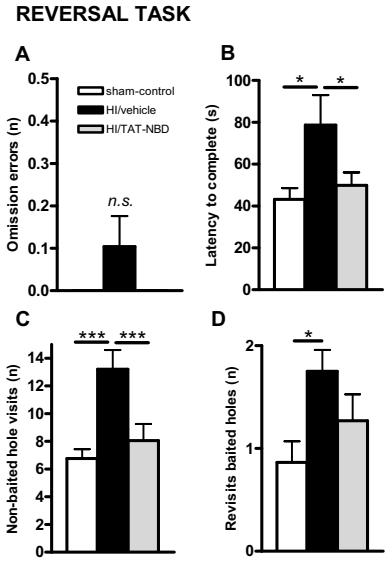

Figure 6.
Cognition for sham-controls, HI-vehicle- and HI-TAT-NBD-treated rats in the mHB during the reversal task. Cognition was measured by (A) the number of omission errors, (B) the latency to complete the trial, (C) non-baited hole visits and (D) revisits to baited holes. N.S. denotes no statistically significant differences; *, p<0.05 and ***, P<0.001 as compared to HI/vehicle-treated animals.

3.4 Anxiety, arousal, exploration and locomotor activity in the modified hole board after TAT-NBD treatment

Additionally, we analyzed behavior indicative for anxiety, arousal, undirected/directed exploration and locomotor activity in the modified hole board set up. HI or TAT-NBD treatment did not affect any of the parameters during T1-3, after the cue switch or during the reversal task as HI/vehicle- and HI/TAT-NBD-treated animals did not differ in this respect from sham-controls. From T1-3, the latency to enter the board and the number of group contacts decreased in all groups, indicating a reduction in the level of anxiety (Fig. 7A & B). The latency to first self-grooming increased from T1-3 indicating reduced arousal (data not shown). The frequency of rearing behavior as well as hole exploration (indicative for undirected and directed exploration respectively) decreased in all groups from T1-3 (Fig. 7C & D). As compared to T1, locomotor activity decreased at T2 and remained stable at T3 in all groups (data not shown). After the cue switch and during the reversal task all of the above-described behavioral patterns did not differ as compared to T3 (data not shown) and were similar in all groups.

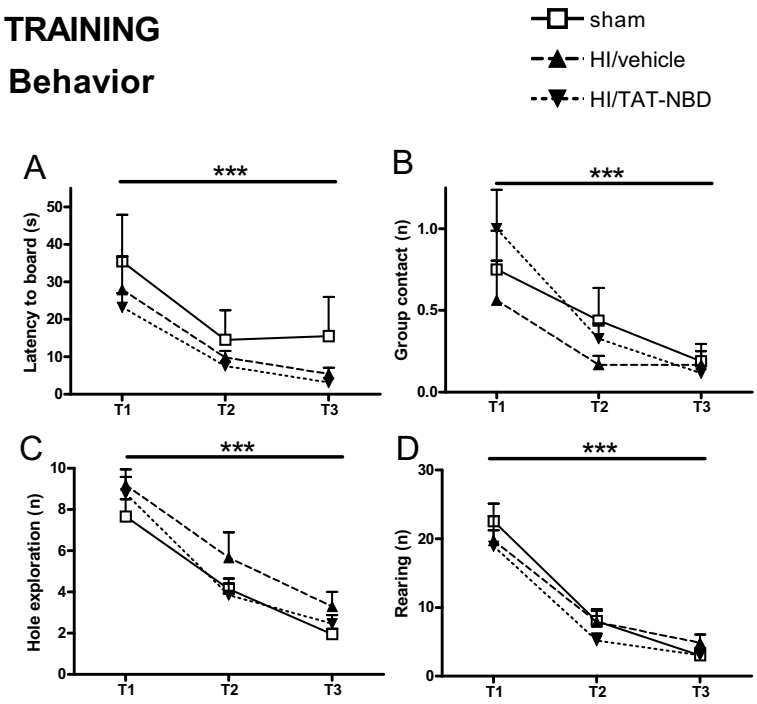

Figure 7.
Behavior for sham-controls, HI/vehicle and HI/TAT-NBD-treated rats in the mHB during training (T1-3). Anxiety was measured by (A) the latency to enter the board and (B) the frequency of group-contact. Exploratory behavior was measured by (C) rearing frequency (undirected exploration) and (D) hole exploration (directed exploration). Time effects during T1-3: ***, $p<0.001$.

4. DISCUSSION

The current study demonstrates for the first time that the strong neuroprotective effect of NF-κB inhibition by the TAT-NBD peptide after neonatal HI in rats is accompanied by long-lasting motor- and cognitive improvements. Animals treated with TAT-NBD at 0 and 3h post-HI improved sensorimotor function as determined in the cylinder rearing test and adhesive removal task as well as cognitive functioning as determined using the mHB test to such an extent that performance in HI/TAT-NBD-treated was comparable to that in sham-controls. The latter implies that the impressive reduction in lesion volume goes hand in hand with an almost complete restoration of motor and cognitive functions

Few studies have assessed neurobehavioral performance after NF-κB inhibition for the treatment of central nervous system injury. An earlier study showed that rota-rod performance, a test for motor-performance, was decreased in mice injected with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (an animal model of Parkinson's disease) and that rota-rod performance improved after TAT-NBD treatment in MPTP-treated mice (Ghosh et al. 2007). Moreover, in a murine model of spinal cord injury transgenic mice with selective astroglial NF-κB-inactivation displayed long-lasting functional recovery measured by hindlimb locomotor function which was accompanied by sparing of the white matter in the spinal cord (Brambilla et al. 2005).

We recently demonstrated that specific targeting of the JNK-pathway with a TAT-coupled JNK inhibiting peptide was moderately neuroprotective (53.7% reduction in lesion volume) but resulted in long-term motor improvements as measured in the adhesive removal task, rota-rod, and in cognitive improvements as assessed in the mHB test after neonatal HI in rats (Nijboer et al. 2009). The robust neuroprotection induced by TAT-NBD in the present study also translated into long-lasting motor improvements as indicated by the results from cylinder rearing test and adhesive removal task. An earlier study (Grow et al. 2003) evaluated motor-deficits in the cylinder rearing test and adhesive removal task after neonatal HI in P7 rats at 5 weeks of age. In that study, it was reported that HI-treated animals displayed impaired sensorimotor functioning in the cylinder rearing test, while the performance in the adhesive removal task was not affected. We observed impaired behavior in both tests for sensorimotor function. It is likely that differences between the models of HI used are responsible for these differences: the hypoxic duration in our study was longer (120' vs. 90') and a different strain of rats was used (Wistar vs. Sprague Dawley). Moreover, Grow et al. reported predominantly ipsilateral cortical damage (31% cortical atrophy), whereas the hippocampal region was not seriously affected (<1% atrophy). In our present study, ipsilateral cortical area loss in HI/vehicle-treated animals was 69% and the ipsilateral hippocampus was almost completely lost in all HI/vehicle-treated animals.

In the mHB test all animals learned to complete the task from T1-3 as indicated by the decreasing numbers of omission errors and the reduction in latency to complete the trial. Thus, despite the severe cerebral insult seen in HI/vehicle-treated rats, learning during the training phase of the mHB test was not impaired. Since brain damage in vehicle treated rats was extensive but unilateral, we cannot directly relate damage in specific regions of the brain to functional outcome. The increased number of non-baited hole visits of HI/vehicle-treated animals compared to sham-controls and HI/TAT-NBD-treated animals after the cue switch may indicate that HI/vehicle-treated animals relied heavier on spatial navigation than on the available cues. Possibly spatial memory in HI/vehicle-treated animals was intact, while cue based learning was impaired. This suggestion contrasts with a previous study testing adult mice in the water maze after severe neonatal HI brain damage. It was reported that severe neonatal HI did not affect cued learning but impaired spatial learning (McAuliffe et al. 2006). In addition, the number of non-baited hole visits at T3 indicative of long term memory errors, tended to be higher for HI/vehicle-treated animals compared to sham-controls and HI/TAT-NBD-treated animals although statistical significance was not reached.

To further test cognitive flexibility we changed both the location and cue of the baited holes during the reversal task. During this reversal task significant cognitive deficits were detected in HI/vehicle- treated animals while no deficits were observed in HI/TAT-NBD-treated rats. The finding that cognitive impairments occurred during the reversal task while being absent during training is in accordance with our previous study in which mice after mild neonatal HI injury showed normal learning in the mHB but were impaired during the reversal task (van der Kooij et al. 2009). The most prominent cognitive difference between sham-controls and HI/vehicle-treated animals during the reversal task in the current study was the increased number of non-baited hole visits, i.e. long-term memory errors, of HI/vehicle-treated animals. The increased latency to complete the trial and the increased number of non-baited hole visits during the reversal task indicated that HI/vehicle- treated animals developed a form of cognitive rigidity. The absence of such cognitive impairments in HI/TAT-NBD-treated animals prove that the histological improvement observed after TAT-NBD treatment (ipsilateral brain volume did not differ from sham-controls) was associated with complete functional recovery.

Importantly, the increased number of revisits of baited holes (short-term memory errors) during the reversal task by HI/vehicle-treated animals compared to sham-controls indicated that these animals were highly motivated to retrieve the food pellets irrespective of the increased latency to complete the task. Furthermore, the absence of behavioral alterations (including anxiety, arousal, exploration and locomotor activity) in HI animals indicated that the improved performance of HI/TAT-NBD-treated

animals mirrored a cognitive process which was not confounded by alterations in general behavior after TAT-NBD treatment.

Earlier we reported a neuroprotective effect of TAT-NBD treatment of 77.5% at 48h post-HI as measured with MAP2 staining (Nijboer et al. 2008). In the current study, the ipsilateral MAP2-positive area at 13 weeks post-HI did not differ from sham-controls. Possibly, TAT-NBD treatment did not only protect neurons but also protected progenitors that could lead to restoration of neuronal tissue over time. However, relative MAP2 loss in HI/vehicle-treated animals was also reduced comparing 48h (79.3%) to 6 weeks (75.7%) and 13 weeks post-HI (68.4%) and we did not observe time x group interactions. Therefore, it is unlikely that TAT-NBD treatment further enhanced neuroregeneration. It remains to be determined whether the battery of behavioral tests stimulated repair in both groups (Bruel-Jungerman et al. 2005) or whether this apparent repair is part of a natural process that occurred independently of the behavioral tests.

We demonstrated previously (Nijboer et al. 2008) that TAT-NBD treatment reduced neonatal HI cerebral injury with a therapeutic window of up to 6h after the insult. The present study confirms the strong neuroprotective effect of TAT-NBD treatment and demonstrates that the sparing of brain tissue was associated with normalization of functional outcome on both motoric and cognitive level. We propose that TAT-NBD peptide may become an important therapeutic strategy to diminish the devastating consequences of neonatal HI.

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Chapter 6

Inhibition of the JNK/AP-1 pathway reduces neuronal death and improves behavioral outcome after neonatal hypoxic-ischemic brain injury

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ABSTRACT

Perinatal hypoxic-ischemic (HI) brain damage continues to be a major clinical problem. We investigated the contribution of the MAP-kinase c-Jun N-terminal kinase (JNK), to neonatal HI brain damage. JNK regulates several transcriptional (via AP-1 activation) and non-transcriptional processes involved in brain damage such as inflammation and cell death/survival.

P7 rats were subjected to HI by unilateral carotid artery occlusion and hypoxia. HI-induced activation of cerebral AP-1 peaked at 3-6 hours post-HI. Intraperitoneal administration of the JNK inhibitor TAT-JBD immediately after HI prevented AP-1 activation. TAT-JBD treatment within 3h after HI reduced early neuronal damage by ~30%. JNK/AP-1 inhibition did not reduce HI-induced cytokine/chemokine expression. Analysis of indicators of apoptotic cell death revealed that TAT-JBD markedly reduced the HI-induced increase in active caspase 3. However, the upstream mediators of apoptosis active caspase 8, cleaved Bid, mitochondrial cytochrome *c* release and caspase 9 cleavage were not reduced after TAT-JBD. TAT-JBD inhibited the HI-induced increase in Smac/DIABLO, an inhibitor of IAPs that prevent activation of caspase 3. TAT-JBD treatment also reduced cleavage of α-fodrin, indicating that calpain-mediated brain damage was reduced. Neuroprotection by TAT-JBD treatment was long-lasting as gray- and white matter damage was diminished by ~50% at 14 wks post-HI concomitantly with marked improvement of sensorimotor behavior and cognitive functioning.

In conclusion, JNK inhibition by TAT-JBD treatment reduced neonatal HI brain damage with a therapeutic window of 3 hours and long-lasting anatomical and behavioral improvements. We propose that inhibition of mitochondrial Smac/DIABLO release and calpain activation contribute to neuroprotection by TAT-JBD.

1. INTRODUCTION

Perinatal hypoxia-ischemia (HI) is a major risk factor for human neonatal mortality and development of major neurodevelopmental disabilities (Volpe, 2001; Ferriero, 2004). At present, no effective pharmacological treatment to combat perinatal HI brain injury is available for these infants (Perlman, 2006). Excitotoxicity, inflammation and apoptosis are the major pathophysiological mechanisms that contribute to cerebral injury after HI (Vexler and Ferriero, 2001).

Mitogen-activated protein (MAP) kinases are essential kinases that respond to a plethora of extracellular stimuli and play a critical role in the regulation of several cellular processes like growth, proliferation, differentiation and cell survival/apoptosis (Cobb, 1999). c-Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs) as their activation is associated with a wide variety of environmental stressors, represent an important subfamily of the MAP kinase group (Bogoyevitch et al., 2004; Karin and Gallagher, 2005). JNKs were originally identified by their ability to phosphorylate c-Jun, the dominant component of the transcription factor AP-1, which is a DNA binding dimer composed of c-Jun combined with c-Fos, Maf or ATF subunits. JNK-mediated phosphorylation of c-Jun enhances the ability of c-Jun/AP-1 to induce transcription of numerous target genes involved in proliferation, cell survival, cell death, DNA repair, metabolism and inflammation (Bogoyevitch and Kobe, 2006). Nowadays, JNKs are also acknowledged for their capacity to regulate cellular function through phosphorylation of non-nuclear proteins. For example, JNKs have been suggested to play a key role in regulation of apoptosis by directly phosphorylating and modifying the activity of proteins residing at the mitochondria (Dhanasekaran and Reddy, 2008; Ameyar et al., 2003).

In mammals there are three known JNK isoforms: JNK1, JNK2 and JNK3. Whereas JNK1 and JNK2 are found in all cells and tissues, expression of JNK3 is mainly found in the brain (Bogoyevitch et al., 2004; Karin and Gallagher, 2005). The importance of JNKs for cerebral development and function has been revealed using mice with targeted deletion of the JNK isoforms. Neuronal development was greatly affected in $JNK1^{-/-}$ and $JNK2^{-/-}$ knockout mice that die around E11 due to hind-brain exencephaly. Loss of JNK3 is not embryonically lethal but rather protects the brain from glutamate-induced excitotoxicity and kainate-induced seizures (Yang et al., 1997; Bogoyevitch et al., 2004). Pirianov et al. (2007) and Kuan et al. (2003) have shown reduced neuronal loss in $JNK3^{-/-}$ mice in models of neonatal HI and adult stroke respectively.

A potent induction of c-Jun and sustained activity of the AP-1 transcription factor has been associated with neuronal cell death *in vitro* (Karin and Gallagher, 2005). Cerebral activation of transcription factors and the consequent modulation of expression of target genes is an important part of the cerebral response to injury (Chang

and Huang, 2006), like we have shown previously for the transcription factor nuclear factor kappa B (NF-κB) (Nijboer *et al.*, 2008a, 2008b, 2009).

JNKs, like the other MAP kinases, are activated via a typical 3-step phosphorylation cascade: JNK is activated by the MAPK kinases MKK7 and MKK4 and the latter are activated by MKK kinases (MEKKs or MAP3Ks). Scaffold proteins like JNK interacting protein (JIP) facilitate JNK activation by binding all elements of the JNK activation pathway.

We recently described that intraperitoneal administration of the short-lived JNK-inhibitor TAT-JBD (also named L-JNKI (Barr et al., 2002)), consisting of the 20 amino-acid sequence of JNK Binding Domain of JIP-1 (JBD) coupled to the protein transduction sequence of HIV-TAT, reduced early neuronal damage in P7 rats when administered intraperitoneally at 0 and 3 h after HI (Nijboer et al., 2009). The aim of the present study was to further investigate the neuroprotective effect of TAT-JBD in the neonatal rat model of HI brain damage. We examined the therapeutic window for TAT-JBD treatment, analyzed its effects on cerebral upregulation of cytokines and chemokines and on mediators of (apoptotic) cell death. Furthermore, we investigated effects of TAT-JBD treatment on long-term brain damage, sensorimotor function and cognitive behavioral outcome.

2. METHODS

2.1 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.

TAT-JBD (L-JNKI: YGRKKRRQRRR-PP-RPKRPTTLNLFPQVPRSQDT) (W.M. Keck facility, Yale University, New Haven, CT) was dissolved in DMSO (40 mg/ml), diluted to 1 mg/ml in PBS and administered i.p. at a dose of 10 mg/kg. Vehicle-treated HI animals received an injection of 2.5% DMSO in PBS. TAT-JBD consists of the JNK binding domain of JNK-interacting protein-1 (JIP-1) coupled to the protein transduction sequence of HIV-TAT to facilitate cerebral uptake and acts as a specific JNK inhibitor (Barr et al., 2002; Bogoyevitch and Arthur, 2008).

2.2 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) or with mouse-anti-MAP2 (Sigma-Aldrich, Steinheim, Germany) or mouse-anti-MBP (Sternberger Monoclonals, Lutherville, MD) 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). The various brain areas (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 MBP 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).

2.3 Western blotting and EMSA

Rats were terminated by an overdose of pentobarbital and decapitated. Cytosolic and nuclear fractions were prepared as described (Nijboer et al., 2007). Cytosolic proteins were separated by SDS-PAGE, transferred to Hybond-C membranes (Amersham, Buckinghamshire, UK) and revealed using rabbit-anti-cleaved caspase 3, mouse-anti-caspase 9 (both Cell Signaling, Danvers, MA), mouse-anti-cytochrome *c* (BD Biosciences Pharmingen, San Jose, CA), rabbit-anti-fodrin (BioVision, Mountain View, CA), rabbit-anti-Smac/DIABLO (Chemicon, Temecula, CA), rabbit-anti-caspase 8, rabbit-anti-Bid or goat-anti-ß-actin (all 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).

Electromobility shift assays (EMSA) on nuclear brain extracts with ^{32}P labeled AP-1 probe (Promega, Madison, WA; sequence 5'-CGCTTGATGAGTCAGCCGGAA-3') were performed as described (Nijboer *et al.*, 2008a).

2.4 Quantitative Real Time reverse transcriptase (RT)-PCR

Total RNA was isolated with TRIzol® (Invitrogen, Paisley, UK). cDNA was synthesized with SuperScript Reverse Transcriptase (Invitrogen). The PCR reaction was performed with iQ5 Real-Time PCR Detection System (Bio-Rad) using primers for TNF-α, IL-1ß, IL-10, IL-4 (for primer sequences see Nijboer et al., 2008a), IL-6 forward: AACGAAAGTCAACTCCATCTG, reverse: GGTATCCTCTGTGAAGTCTCC; MIP1α (CCL3) forward: TTTGAGACCAGCAGCCTTTG, reverse GAAGAGTCCCTGGATGTGGC and MIP2 forward: TTGTCTCAACCCTGAAGCCC, reverse TGCCCGTTGAGGTACAGGAG. Data were individually normalized to the mean of the relative expression of ß-actin and GAPDH (for primer sequences see Nijboer et al., 2008a).

2.5 Cylinder Rearing Test (CRT)

At 2.5, 4 and 6 wks of age, rats were individually placed in a Plexiglas transparent cylinder and observed for 3 min. Initial forepaw (left/right/both) preference 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).

2.6 Adhesive Removal Task (ART)

At 5 wks of age, 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).

2.7 Rota-rod

At 10-12 wks of age, animals were tested on the rota-rod (Stoelting Europe, Ireland) according to a standardized protocol (Rozas et al., 1997). In short, animals were trained to remain on the rota-rod at a speed of 5 rpm for a maximum duration of 150 s for three subsequent days. On day 4 we tested performance of the animals to stay on the rota-rod by increasing the speed from 5-40 rpm for a maximum duration of 300 s with increasing steps of 5 rpm. The Overall Rod Performance (ORP) was calculated as the total area under the curve in a plot of time on the rod against rotational speed. The ORP is calculated as the sum of the individual trapezia (speed); an individual area would take the value of ((a+b)/2)*c where 'a' is time-on-the-rod at lower speed, 'b' the time at the higher speed, and 'c' the difference between the two speeds (Rozas et al., 1997). Since we tested for 8 different rod rotational speeds ORP could take any value between 0 and 10500.

2.8 Modified hole board (mHB)

At 11-13 wks of age, animals were introduced to the mHB test as described by Ohl et al. (2003). In short, the mHB setup consisted of an opaque gray PVC box (100 x 50 x50 cm) containing a board (60 x 20 cm) placed in the middle of the box on which 20 gray cylinders (3 cm ø * 4 cm height) were staggered in three lines. The PVC box is enlarged by an additional 50-cm square compartment, in which the group mates of the experimental rats are placed during the test-period. This compartment is separated from the test area by a transparent perforated Plexiglas partition.

All cylinders were flavored with vanilla to attract the animals to the cylinders. Three of the cylinders were cued (small white ring) and baited with a small almond piece (0.02-0.03 g) placed on a metal grid representing the food reward. The remain-

ing 17 cylinders contained non-retrievable almond pieces placed under the metal grid (Fig. 6A). Between 10am and 5pm animals were scored live by a trained observer under red light conditions for parameters indicative for cognition (time until retrieval of all food rewards), exploration, anxiety, arousal and risk assessment. Non-baited hole visits and revisits of baited holes from which food had already been retrieved are referred to as long-term and short-term memory errors respectively.

Animals were monitored during 4 sessions/per day lasting maximally 5 min or until retrieval of all three food pellets on 3 sequential days (stage 1, T1-T3). On the fourth day, the location of the three cued and baited cylinders was changed and time until retrieval of all three food rewards was monitored during four sessions (stage 2, CS). During the reversal task (stage 3, RT) time until retrieval of all food rewards in three non-cued baited cylinders in a novel position was determined during four sessions. All non-baited cylinders were now cued.

2.9 Statistical Analysis

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

3. RESULTS

3.1 JNK/AP-1 activation after HI and effect of i.p. TAT-JBD treatment

Analysis of the kinetics of cerebral JNK activation after neonatal HI showed that JNK/AP-1 activity peaked early; HI induced significant nuclear AP-1 activity as determined by EMSA on nuclear extracts of ipsi-and contralateral hemispheres. AP-1 activity was upregulated from 0.5-6 h after the insult in the ipsilateral hemisphere (Fig. 1A). At 12 h after the insult ipsilateral AP-1 activity had returned to base-line values. Intraperitoneal treatment with TAT-JBD immediately after HI almost completely blocked HI-induced AP-1 activation as determined at 3h post-HI (Fig. 1B).

3.2 Therapeutic window of TAT-JBD treatment

At 48h after induction of HI in P7 rats, we observed severe brain damage in vehicle-treated animals with ~83% loss of microtubule associated protein 2 (MAP2), as an early marker of neuronal damage (Fig. 2A, Fig. 2B). No MAP2 loss was detected in the contralateral hemisphere. Inhibition of JNK by treatment with TAT-JBD directly after HI and 3 h later (0/3h) significantly reduced ipsilateral MAP2 loss by ~30% (Fig. 2A, Fig. 2B). To determine the therapeutic window for this neuroprotective effect, TAT-JBD was administered as a single injection at only 3 h or only 6 h after the insult. The therapeutic window of i.p. TAT-JBD was at least 3 h post-HI as MAP2 loss was still

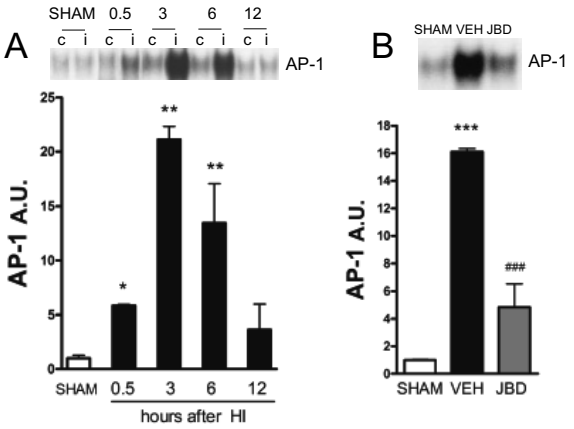

Figure 1. AP-1 activation after HI and effect of i.p. TAT-JBD treatment
A: Ipsilateral AP-1 activity was determined by EMSA on nuclear brain extracts of the ipsilateral hemisphere obtained at different time points after HI (n=4-6/timepoint). Inset shows representative examples. c: contralateral, i: ipsilateral. A.U.: Arbitrary density Units. *p<0.05 and **p<0.01 vs. AP-1 levels in sham-operated animals (SHAM). AP-1 activity in contralateral hemispheres was comparable to SHAM.
B: HI-induced ipsilateral AP-1 activity at 3 h post-HI as determined by EMSA on nuclear brain extracts after vehicle or TAT-JBD (JBD) treatment. ***p<0.001 vs. levels in sham controls; ###p<0.001 vs. levels in vehicle-treated (VEH) animals. Inset shows a representative example of n=6 animals/group. AP-1 activity in contralateral hemispheres was similar to levels in SHAM.

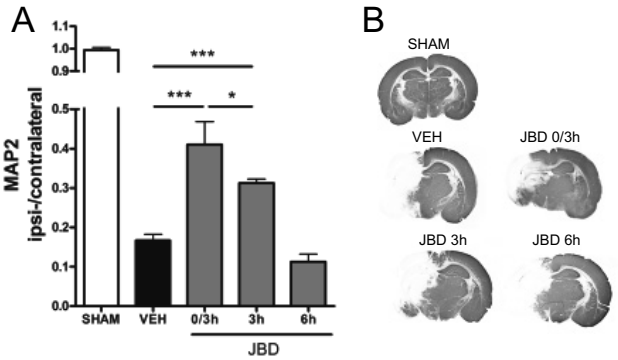

Figure 2. Therapeutic window of TAT-JBD treatment after HI
A: Ratio ipsi-/contralateral MAP2 positive area at 48 hours post-HI as a measure of damage. TAT-JBD was administered i.p. at 0 and 3 h post-HI (0/3h) or as a single injection at 3 h or 6 h after HI. No MAP2 loss was observed in the contralateral hemisphere. SHAM n=5, VEH n=13, TAT-JBD treated groups n=8. ***p<0.001 vs. vehicle-treatment, *p<0.05 TAT-JBD treatment at 0/3 h vs. 3h post-HI.
B: Representative photographs of MAP2 loss.

significantly reduced by ~18% after this treatment (Fig. 2A, Fig. 2B). Treatment with TAT-NBD at 6 h after HI did not reduce MAP2 loss.

3.3 Effects of TAT-JBD on cytokine and chemokine expression

The JNK/AP-1 pathway is thought to play an important role in regulating inflammatory processes in the brain. We have previously described that intraperitoneal administration of TAT-JBD did not significantly reduce the HI-induced increase in TNF-α mRNA or protein in this model of neonatal HI brain damage (Nijboer et al., 2009). Here we further expand these observations and show that TAT-JBD did not have a major effect on the HI-induced upregulation of cytokine or chemokine expression. We did not observe a statistically significant effect of TAT-JBD treatment on the level of mRNA encoding TNF-α, IL-1ß, IL-6, MIP2, MIP1α, IL-10 and IL-4 (MIP1α) (Table 1).

Table 1: Effects of TAT-JBD on cytokine and chemokine expression

Cytokine		VEHICLE		TAT-JBD		VEH vs. TAT-JBD
		contra	ipsi	contra	ipsi	
TNF-α	Mean	0.16	0.76**	0.13	0.58*	p=0.36
	(SEM)	(0.03)	(0.15)	(0.02)	(0.12)	
IL-1ß	Mean	0.20	0.33*	0.14	0.27*	p=0.28
	(SEM)	(0.04)	(0.05)	(0.07)	(0.02)	
IL-6	Mean	n.d.	0.43**	n.d.	0.41**	p=0.87
	(SEM)		(0.13)		(0.06)	
MIP2	Mean	n.d.	0.51*	n.d.	0.46*	p=0.86
	(SEM)		(0.22)		(0.17)	
MIP1α	Mean	n.d.	0.46*	n.d.	0.49*	p=0.94
	(SEM)		(0.25)		(0.15)	
IL-10	Mean	n.d.	0.42*	n.d.	0.28*	p=0.51
	(SEM)		(0.19)		(0.08)	
IL-4	Mean	0.16	0.52**	0.12	0.39*	p=0.20
	(SEM)	(0.05)	(0.07)	(0.07)	(0.06)	

Effect of HI and TAT-JBD treatment on mRNA expression of TNF-α, IL-1ß, IL-6, MIP2, MIP1α, IL-10 and IL-4 in contra- and ipsilateral hemispheres as determined by quantitative real time RT-PCR at 3 h post-HI. Expression levels in sham-operated animals were similar to contralateral levels in HI-treated animals. HI induced upregulation of all cytokines and chemokines in ipsilateral hemispheres *p<0.05, **p<0.001 contra- vs. ipsilateral levels. TAT-JBD treatment did not inhibit cytokine or chemokine expression: right column indicates p-values of vehicle-treatment vs. TAT-JBD treatment. n.d. indicates non-detectable: values < 0.005. n=7 animals for all groups.

3.4 Effect of TAT-JBD on cell death markers

We determined whether JNK inhibition had an effect on the executioner of apoptosis caspase 3. At 24 h post-HI, TAT-JBD treatment significantly decreased HI-induced ipsilateral levels of cleaved (active) caspase 3 (Fig. 3A). Caspase 3 can be activated by the *intrinsic* pathway of apoptosis that is stimulated by release of cytochrome *c* from the mitochondria to the cytosol leading to cleavage of pro-caspase 9 to form active caspase 9. At 24 h after the insult, HI induced a slight but statistically significant decrease in the level of pro-caspase 9, indicating caspase 9 activation (Fig. 3B). TAT-

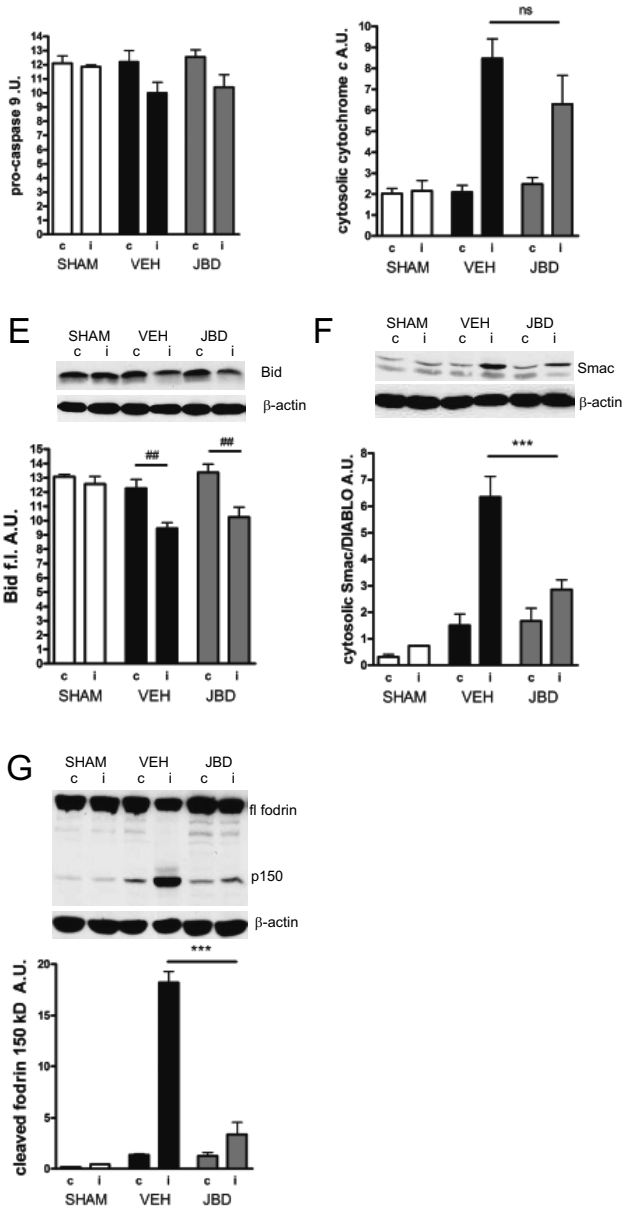

Figure 3. Effects of TAT-JBD treatment on markers of cell death after HI
P7 rats were subjected to HI, treated with VEH or TAT-JBD at 0/3h after HI and the following markers were quantified by Western Blot analysis of cytosolic brain fractions of contra- (c) and ipsilateral (i) hemispheres: (A) active (cleaved) caspase 3 at 24 h post-HI, (B) pro-caspase 9 at 24 h post-HI, (C) cytosolic cytochrome c at 24 h post-HI, (D) active (cleaved) caspase 8 at 3 h post-HI, (E) full length (f.l.) Bid at 3 h post-HI, (F) cytosolic Smac/DIABLO at 24 h post-HI and (G) (cleaved p150 kD) α-fodrin at 6 h post-HI. ***p<0.001 vehicle vs. TAT-JBD, ##p<0.01 or ###p<0.001 contra- vs. ipsilateral levels. ns in B: p=0.24. Sham controls n=4, vehicle- and TAT-JBD-treated n=9-10.

JBD treatment, however, did not affect the reduction in pro-caspase 9 (Fig. 3B). HI increased cytosolic cytochrome *c* levels in the ipsilateral hemisphere at 24 h post-HI (Fig. 3C). JNK inhibition by TAT-JBD slightly but not significantly reduced the level of released cytochrome *c* (Fig. 3C).

Caspase 8 is an important direct upstream activator of caspase 3 via the so-called *extrinsic* route of apoptosis in response to stimulation of death receptors. Exposure of rat pups to HI induced activation of caspase 8 observed as an increase in cleaved (p18) caspase 8 in the ipsilateral hemisphere 3-6 h after the insult. TAT-JBD treatment did not affect caspase 8 activation at both time points after HI (Fig. 3D and data not shown). Caspase 8 also induces the intrinsic route of apoptosis via cleavage of Bid, a BH3-only pro-apoptotic Bcl-2 family member. TAT-JBD treatment did not affect HI-induced Bid cleavage as the level of full length Bid was reduced equally in vehicle-treated or TAT-JBD-treated animals at 3 h post-HI (Fig. 3E).

Thus, although TAT-JBD treatment reduced HI-induced caspase 3 activation by ~ 57%, we did not detect a significant effect of treatment on caspase 8 activation, Bid cleavage, cytochrome *c* release and caspase 9 activation, the upstream regulators of caspase 3 activity.

In *in vitro* studies, it has been shown that JNK activation can induce the release of Smac (Second mitochondria-derived activator of caspase) also known as DIABLO (Direct Inhibitor of Apoptosis-Binding protein with Low pI) (Deng et al., 2003). Smac/DIABLO release to the cytosol contributes to apoptosis by interacting with endogenous inhibitors of apoptosis (IAPs), thereby enhancing caspase 9 and caspase 3 activity (Duckett, 2005; Shiozaki and Shi, 2004). Since it is unknown whether *in vivo* JNK activity also contributes to Smac/DIABLO release, we analyzed cytosolic levels of this protein. At 24h post-HI, HI induced release of Smac/DIABLO to the cytosol. Importantly, treatment with TAT-JBD significantly reduced the release of Smac/Diablo to the cytosol (Fig. 3F).

There is evidence that neonatal cerebral HI also triggers necrotic/excitotoxic cascades, including calcium-dependent activation of enzymes like calpain leading to cleavage of α-fodrin and subsequently membrane malfunction and cell shrinkage associated with necrotic cell death (Northington et al., 2007). Consistently, Fig. 3G shows that HI induced a large increase in calpain-mediated cleavage of α-fodrin (p150 band) at 6 h post-HI. Interestingly, cleavage of α-fodrin was strongly reduced after TAT-JBD treatment compared to vehicle treatment (Fig. 3G).

3.5 Long-term effects of TAT-JBD treatment on cerebral damage

To assess the long-term effects of JNK inhibition by TAT-JBD at 0/3h post-HI on brain damage, rats were terminated at 14 wks after HI and lesion size was measured as a reduction in volume of the ipsilateral hemisphere. Treatment with TAT-JBD significantly

reduced lesion size by ~56% compared to vehicle-treatment (Fig. 4A, Fig. 4B). White matter loss as determined by loss of myelin basic protein (MBP) staining was also significantly decreased after TAT-JBD treatment (~47%) (Fig. 4C, Fig. 4D).

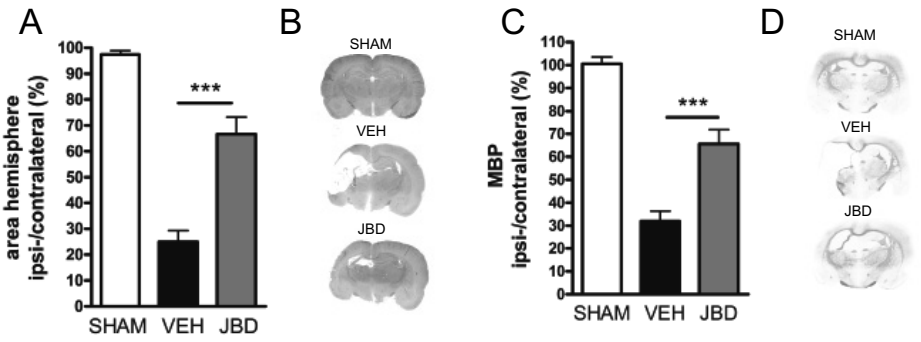

Figure 4. Long-term effects of TAT-JBD on cerebral gray and white matter damage
A: HI-induced ipsilateral cerebral area loss expressed as area of the ipsi-/contralateral hemisphere x 100% at ~-3.20 mm from bregma at 14 wks post-HI. B: representative examples of HE staining. C: HI-induced white matter damage expressed as the area of ipsi-/contralateral MBP staining x 100% at ~-3.20 mm from bregma at 14 wks post-HI. D: representative examples of MBP staining. Sham controls n=14, vehicle n=12, TAT-JBD n=14; ***p<0.001 vehicle vs. TAT-JBD treatment.

3.6 Motor function after TAT-JBD treatment

At 2.5, 4 and 6 wks of age, sham-operated rats and rats that underwent HI at P7 were tested in three different tests of sensory motor function; the cylinder rearing test (CRT), the adhesive removal task (ART) and the rota-rod (for detailed description see Methods). Sham control rats did not show any paw preference during rearing in the CRT (Fig. 5A). HI caused a ~30% paw preference in using the unimpaired forepaw in vehicle-treated animals (Fig. 5A). Paw preference in vehicle-treated rats did not alter over time. However, neuroprotective treatment with TAT-JBD did not significantly reduce paw preference in the CRT at any of the time-points measured.

At 5 wks of age, all animals were tested in the adhesive removal task (ART). Sham-treated animals did not show a difference in adhesive removal latency between left- and right forepaw (Fig. 5B). After HI, animals were impaired in the ART: latency to remove the sticker from the left (impaired) forepaw was significantly higher than the latency to remove the sticker from the right (unimpaired) forepaw (Fig. 5B). Importantly, TAT-JBD-treated animals showed a reduced latency for adhesive removal from the impaired forepaw as compared to vehicle-treated animals indicating functional motor improvement after TAT-JBD treatment (Fig. 5B). The removal latency from the non-impaired forepaw did not differ between groups.

At 10-12 wks of age, rats were tested on the rota-rod for motor coordination. The Overall Rota-rod Performance (ORP) was clearly affected by HI; the ORP of sham-operated rats was twice as high as the ORP of vehicle-treated animals (Fig. 5C). Treatment with TAT-JBD after HI improved performance on the rota-rod significantly; the ORP of TAT-JBD-treated rats did not differ from sham-operated animals (Fig. 5C).

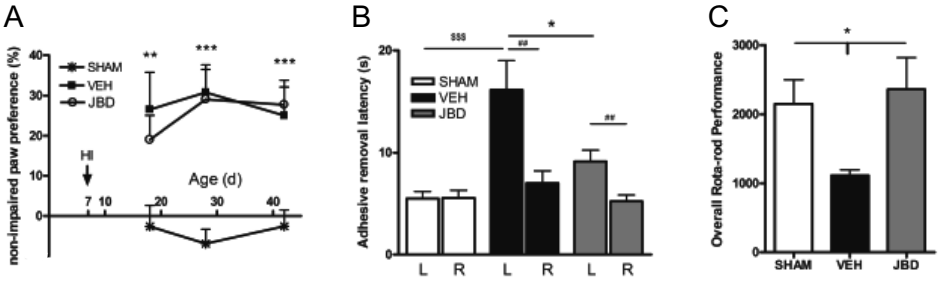

Figure 5. Effects of TAT-JBD treatment on sensorimotor function
Rats were subjected to HI at P7 and were tested for sensory motor function. Sham controls n=14, vehicle n=12, TAT-JBD n=14.
A: Rats were tested at 2.5, 4 and 6 wks of age in the cylinder rearing test (CRT) as an indication for laterizing sensory motor defects. Paw preference during full rears during the 3 min test period was determined for sham controls and HI rats treated with vehicle or TAT-JBD. $^{**}p<0.01$; $^{***}p<0.001$ sham controls vs. vehicle-treated or sham controls vs. TAT-JBD treated animals.
B: Rats were tested in the adhesive removal task (ART) at 5 weeks of age. 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. $^{\$\$\$}p<0.001$ L-paw of sham control vs. L-paw of vehicle-treated HI rat; $^{\#\#}p<0.01$ L vs. R forepaw; $^{*}p<0.05$ vehicle vs. TAT-JBD.
C: Rats were tested for their performance in the rota-rod test at 10-12 weeks of age. Overall rota-rod performance (ORP) was calculated as described in the Methods section. $^{*}p<0.05$ vehicle vs. sham controls or vehicle vs. TAT-JBD treated animals.

3.7 Cognition after TAT-JBD treatment

At 11-13 wks of age, rats were tested in the modified hole board (mHB; for detailed description see Methods) (Fig. 6A). The latency to complete the trial (find all three food pellets in the baited holes) reduced from day 1-day 3 for all groups with no differences between the groups. During the cue switch task (CS) on day 4, latency to complete the trial also did not differ between groups. During the reversal task (RT), we observed a trend towards increased latency to complete the trial in vehicle-treated animals compared to both sham-operated and TAT-JBD-treated littermates, indicating that TAT-JBD treatment might have improved cognition (Fig. 6B).

From day 1 to day 3, the number of non-baited hole visits significantly decreased in all groups (Fig. 6C). Vehicle-treated HI animals tended to visit non-baited holes more often than sham controls or TAT-JBD-treated HI animals during the first 3 days of testing

(p=0.074). During CS and RT, vehicle-treated animals visited significantly more non-baited holes, indicative of more long-term memory errors than sham control rats (Fig. 6C). Rats treated with TAT-JBD showed significantly fewer long-term memory errors than vehicle-treated rats, indicating improved cognition after TAT-JBD treatment (Fig. 6C).

The number of revisits to baited holes, an indicator of short-term memory errors, increased from T1-T3 with no differences between groups (Fig. 6D). During the RT, we observed that TAT-JBD-treated HI animals made significantly less short-term memory errors than vehicle-treated HI animals and were comparable to sham-operated littermates (Fig. 6D).

We controlled for potentially confounding factors like anxiety, arousal and exploratory behavior. No significant differences between sham controls, vehicle-treated HI animals and TAT-JBD-treated HI animals were observed (data not shown; all p-values between 0.14-0.84).

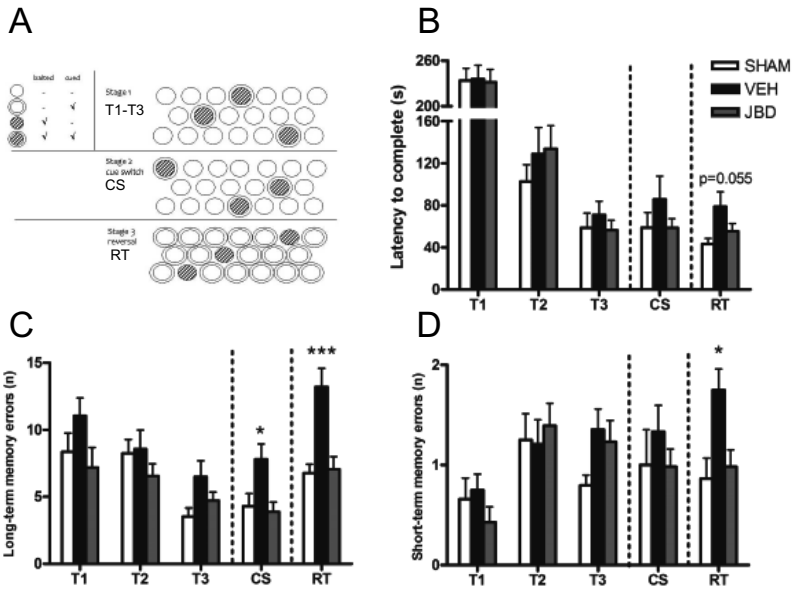

Figure 6. Effects of TAT-JBD on cognitive function

Rats were subjected to HI at P7 and were tested for cognitive function using the modified hole board (mHB) at 11-13 weeks of age. Sham controls n=14, vehicle n=12, TAT-JBD n=14

A: Setup of the mHB during training days 1-3 (T1-T3), cue switch (CS) and reversal task (RT). During T1-T3 3 cued cylinders were baited while 17 non-cued cylinders were non-baited. During CS the location of the cued & baited cylinders was changed. During RT 3 baited cylinders were cued and their position was changed while the remaining cylinders were cued but non-baited. Sham-controls, vehicle-treated and TAT-JBD treated animals were tested for latency to complete the trial (B), number of non-baited hole visits as an indicator of long-term memory errors (C) and revisits of baited holes as an indicator of short-term memory errors (D). *p<0.05, ***p<0.001 vehicle-treated animals vs. sham-controls and TAT-JBD-treated animals.

4. DISCUSSION

The present study provides evidence for the neuroprotective potential of JNK inhibition by the small peptide inhibitor TAT-JBD (L-JNKI) in a neonatal model of HI brain injury. Our data demonstrate that peripheral (i.p.) administration of the TAT-JBD peptide at 0 and 3 h after the insult resulted in a marked reduction of brain damage which was long-lasting up to 14 wks post-HI. Long-term anatomical cerebral improvements up to 50% were associated with both sensori-motor and cognitive behavioral benefits, which is highly relevant to therapeutic prospects in human neonates facing HI.

Protein transduction domain technology with the HIV-1 TAT-sequence as a potent example is a novel approach to shuttle biologically active peptides across the blood brain barrier and/or across the plasma membrane. We and others have shown effective and rapid intracerebral delivery and subsequent neuroprotection of e.g. TAT-Bcl-xL protein, TAT-GDNF, TAT-coupled NF-κB inhibiting NBD peptide and JNK-inhibiting JBD peptides (Yin et al., 2006; Kilic et al., 2003; Nijboer et al., 2008a; Borsello et al., 2003; Repici et al., 2007). TAT-coupled NF-κB- and JNK-inhibiting peptides were detectable in the brain parenchyma within 1 hour after i.p. administration (Nijboer et al., 2008a; Borsello et al., 2003; Repici et al., 2007). Here we show that i.p. administration of TAT-JBD largely prevents HI-induced cerebral AP-1 activation, indicating that JNK activity in the brain is inhibited effectively by TAT-JBD treatment. Therefore, we propose that the neuroprotective effect of TAT-JBD observed is mediated via inhibition of JNK activity within the brain. We cannot exclude the possibility, however, that inhibition of peripheral JNK activity by TAT-JBD also contributes to cerebral neuroprotection.

The early rapid activation of JNK/AP-1 which was observed 0.5-6 h after HI in our neonatal model, is in line with other studies showing JNK/c-Jun activation peaking at 3-6 h or from 0.5-8 h after focal ischemia in P14 rats and adult mice respectively (Repici et al., 2007; Gao et al., 2005). The observed therapeutic window of TAT-JBD of at least 3 h is closely associated with the rapid onset of JNK/AP-1 activation peaking at 3 h and returning to basal levels at 12 h.

Importantly, this study shows that TAT-JBD (L-JNKI) treatment is potently neuroprotective in the Vannucci and Rice model of global hypoxia using P7 rats. In contrast, Ginet et al. (2009) recently tested JNK inhibition with repeated treatment with D-JNKI in the same model using P7 rats. Although effects of D-JNKI on neuronal cell death could be demonstrated, there was no effect found on lesion volume and therefore the authors suggested that there might be a limited role of JNK in the *neonatal* brain compared to the adult brain in which D-JNKI was shown to be protective (Borsello et al., 2003; Hirt et al., 2004; Repici et al., 2007; Esneault et al., 2008). We suggest that it might be better to inhibit only acute HI-induced JNK activity by giving the *short-lived*

TAT-JBD (L-JNKI), especially because we have shown previously in our studies on NF-κB inhibition that there is a delicate balance between cell death and survival in the neonatal brain with respect to duration of treatment (Nijboer et al., 2008a, 2008b). In these previous studies, we demonstrated that early rapid inhibition of NF-κB had neuroprotective effects of >80%, whereas prolonged or late inhibition of NF-κB was detrimental to neonatal HI brain injury (Nijboer et al., 2008a, 2008b). Moreover, Bogoyevitch and Arthur (2008) have also discussed that usage of the protease-resistant retro-inverso D-JNKI peptide with an extended half-life is not required for the effect when rapid, acute JNK inhibition is desirable. Interestingly, a recent study by Fornoni et al. (2007) shows that L-JNKI but not D-JNKI provides protection in a model of pancreatic islet preservation.

JNK-mediated activation of the transcription factor AP-1 is thought to regulate several target genes involved in inflammation (Chang and Huang, 2006). To our knowledge the other studies using JNK inhibiting strategies after cerebral ischemia did not show any data on inflammatory targets. Importantly, neuroprotection by TAT-JBD in our model was not associated with reduced expression of cytokines and chemokines, similar to what we observed in our previous study using the NF-κB inhibiting NBD peptide (Nijboer et al., 2008a, 2009). We have demonstrated that JNK/AP-1 activity becomes imperative in taking over early cytokine regulation when NF-κB is inhibited. Only inhibition of both the NF-κB and the JNK/AP-1 pathway leads to abolishment of cytokine production in the neonatal brain after HI (Nijboer et al., 2009). Therefore, even when one of the main regulators of inflammatory target gene transcription (e.g. NF-κB or JNK/AP-1) is absent, cerebral cytokine/chemokine production will be maintained. Furthermore, the data obtained in the present study confirm that neuroprotection is preserved regardless of cytokine/chemokine expression which indicates that production of early cytokines is not detrimental as such and might even contribute to neuroprotection as suggested previously to be the case for TNF-α (Nijboer et al., 2009).

Our data show that TAT-JBD treatment after HI strongly reduced the release of Smac/DIABLO from the mitochondria. Deng et al. (2003) described in HeLa cells *in vitro* that JNK could induce caspase-8 independent cleavage of Bid into j-Bid, a novel Bid cleavage product, which translocates to the mitochondria and leads to preferential release of Smac/DIABLO without release of cytochrome *c*. Smac/DIABLO facilitates cell death by abrogating the caspase inhibiting actions of IAPs (Salvesen and Duckett, 2002; Shiozaki and Shi, 2004; Duckett, 2005). We show here for the first time that the proposed JNK-mediated release of Smac/DIABLO might be an important pathway in the neonatal brain after HI. We suggest that inhibition of Smac/DIABLO is a possible neuroprotective strategy for HI brain injury, since inhibition of Smac/DIABLO release is sufficient to inhibit activation of caspase 3 even when upstream activators

like cytochrome *c* or caspase 8 are still released or activated (see Fig. 7 for a model). Smac/DIABLO release from mitochondria into the cytosol has been shown in a few recent studies using transient focal ischemia models in adult mice and rats (Saito et al., 2003; Siegelin et al., 2005). These studies demonstrated that Smac/DIABLO was released from 12-48h after the insult in vulnerable regions of the brain and that enhanced cytosolic Smac/DIABLO correlated well both temporally and spatially with effects on XIAP and caspase 3 activation. Moreover, Saito et al. (2003) demonstrated co-immunoprecipitation of XIAP and Smac/DIABLO after MCAO in mice. In contrast to our study, Matsumori et al. (2005) did not detect Smac/DIABLO translocation after induction of HI in neonatal mice from 6-24 h.

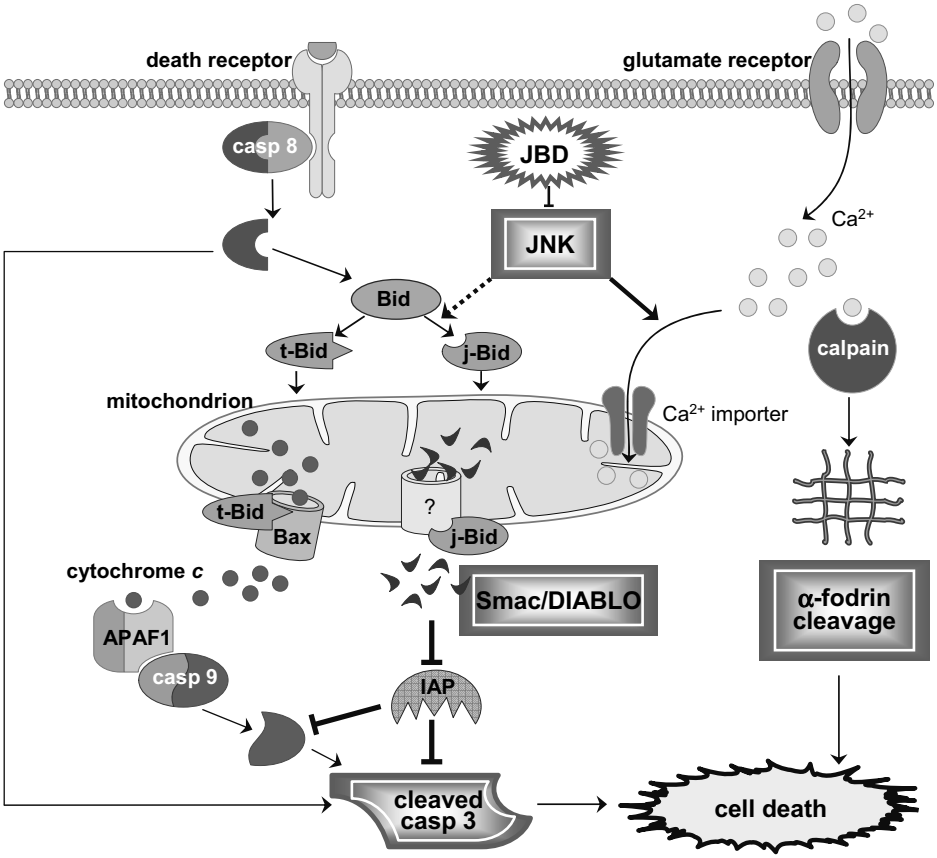

Figure 7. Simplified schematic overview of apoptotic and necrotic pathways of cell death and the effects of TAT-JBD.
We showed that inhibition of JNK by JBD treatment reduced Smac/DIABLO release from the mitochondria, activation of caspase 3 and cleavage of α-fodrin. We propose that these effects are the consequence of inhibition of JNK-mediated j-Bid cleavage by TAT-JBD (dotted line) and of increased calcium buffering capacity of the mitochondria when JNK is inhibited.

In *in vitro* models using primary cortical neuronal cultures, stimulation with glutamate clearly activates phosphorylation of MKK7 and JNK (Borsello et al., 2003; Arthur et al., 2007). Inhibition of JNK reduced excitotoxic cell death by mainly decreasing cytosolic calcium concentration and preventing mitochondrial dysfunction (Borsello et al., 2003; Arthur et al., 2007; Centeno et al., 2007). We showed that after neonatal HI, JNK inhibition by TAT-JBD strongly reduced calpain-dependent cleavage of α-fodrin as an indicator of necrotic cell death (see Fig. 7 for a model). However, as the strong inhibitory effect of TAT-JBD on α-fodrin cleavage does not proportionally relate to the observed net neuroprotective effect of TAT-JBD on brain damage, we suggest that in the neonatal brain calpain-mediated cell death might be less important compared to apoptotic cell death than in adult brain. This might explain why in some adult stroke studies higher percentages of reduction in lesion volume were obtained by using JNK inhibiting peptides. Northington et al. (2007) have discussed that at present there still is no consensus on the classification of cell death in the neonatal brain and show that after neonatal HI, apoptosis and necrosis can even occur in the same cell, leading to appearance of the apoptotic-necrotic continuum.

We are the first to show functional improvements of TAT-JBD (L-JNKI) in rats in which HI was induced during the neonatal period with a follow-up to 14 wks. We used several tasks: forepaw use during rearing (CRT), forepaw somatosensory and sensorimotor deficits (ART) and (sensori)motor coordination (rota-rod) as well as cognitive processes and behavior (mHB). A few studies using MCAO models in adult mice and rats addressed functional outcome after D-JNKI treatment. These studies demonstrated improved performance on one motor task at 1 or 14 days after the insult (Borsello et al., 2003; Hirt et al., 2004). Esneault et al. (2008) showed improvement of MCAO rats in the ART, beam walking test and object recognition test after D-JNKI from 6-10 days but without an effect on lesion size.

After TAT-JBD treatment, motor behavior improved in the ART and rota-rod but not in the CRT. The apparent discrepancy in outcome of these tests might be explained by the nature of the tasks. During the CRT, animals voluntarily reared in order to explore the environment and animals affected by HI preferred to use the non-impaired forepaw. In contrast on the rota-rod, rats were forced to remain on the rotating rod (and use both forepaws) or they would fall off. During the ART, both forepaws were used to remove the sticker. The animals initiated rearing behavior only after the sticker was removed likely indicative of a high motivation to remove the sticker. The impairment during voluntary movements and reduced impairment during forced movements is also observed in humans after brain injury. Especially therapy in young children with cerebral palsy should be geared at improving spontaneous use of the affected limb, to limit developmental disregard, although involuntary movements are performed normally (Hoare et al., 2007).

Notably, anatomical neuroprotective effects of TAT-JBD at 14 wks post-HI were associated with improved cognitive function of the rats in the mHB. HI significantly increased both long-term and short-term memory errors during CS and RT in the mHB and this effect was ameliorated by treatment with TAT-JBD. These results prove that TAT-JBD in fact rescued neuronal functioning of brain tissue after HI damage.

In conclusion, the present study shows that early JNK inhibition by the *short-lived* TAT-JBD peptide may be a promising therapy for neonatal HI by conferring long-term anatomical and behavioral improvements. Moreover, this study provides novel insights into the contribution of Smac/DIABLO to apoptotic cell death and calpain-mediated cell death in the newborn brain when challenged with a HI insult.

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Chapter 7

Summary and Discussion

SUMMARY AND DISCUSSION

Nowadays no effective therapeutic treatment is available for neonatal hypoxic-ischemic encephalopathy (HIE). Several *experimental* treatments, however, have a high potential to become an effective clinical approach to combat neonatal hypoxic-ischemic (HI) brain injury. Some of these strategies have been described in the current thesis. The most remarkable findings are discussed in the summary of this chapter.

In the first part of this thesis (Chapter 2-3) we focused on the role of erythropoietin (EPO) as a neuroprotectant. In Chapter 2 we described that EPO has proven to exert not only neuroprotection *in vitro* but also in *in vivo* in neonatal and adult models for HI-induced brain injury. As can be seen in table 2-3 from Chapter 2 a large number of EPO treatment regimens have been employed with mixed –but mostly beneficial- outcome based on histology.

It has been described before that the iron chelator Deferoxamine (DFO) has a neuroprotective effect after neonatal HI through a reduction of intracellular oxidative stress (Sarco et al. 2000). Furthermore, DFO is thought to stabilize HIF1α and may thereby stimulate endogenous EPO production (Chapter 1 and 3). Endogenous EPO production might be beneficial over systemic EPO administration since it will likely be produced at the site of action in the brain. We hypothesized that a combination of DFO with a relatively low EPO dose (1 kU/kg) may have a 'combined or synergistic' neuroprotective potential compared to the use of only DFO or EPO. In Chapter 3 we showed that cerebral apoptosis (caspase-3 expression) was modestly inhibited after EPO or the DFO-EPO combination treatment but both treatment schedules did not result in neuroprotection based on other histological parameters including white and gray matter loss. The lack of overt neuroprotection may perhaps be caused by the severity of the insult (90 min at 8% O_2 in N_2) in the rat HI model used in our study, leaving practically no 'matrix tissue' in the brain to start effective regeneration in comparison to Spandou et al. (2004) (who applied 60 min at 8% O_2 in N_2). Moreover, we did not observe a gender-specific effect of EPO treatment. Therefore, we have to conclude that the DFO-EPO combination is not a promising strategy to treat the devastating consequences of neonatal HI.

Recently it has been shown in our laboratory that EPO 5 kU/kg, when given 3 times after HI, has a neuroprotective effect with respect to reduction of white matter loss and improvement of motoric behavior. However, the improvement was only found in female mice (Fan,X. et al., unpublished results). Taken our results together, we have to conclude that our data are somewhat in contrast with data in the literature where EPO treatments most often resulted in major neuroprotection in animal models for neonatal HI under a wide variety of circumstances (Kellert et al. 2007; Spandou et al. 2004; Kumral et al. 2006) (Chapter 2). However, since 'negative' results are often not

published, we would like to suggest that EPO might have a modest effect as a neuroprotective agent, but that EPO will probably not become a major breakthrough in the pharmacotherapy of neonatal neuroprotection after brain injury.

In Chapter 4-6 we explored the behavioral outcome after neonatal HI with a particular emphasis on the long-term functionality.

We explored motor- and cognitive abnormalities seen after a severe and a relatively mild neonatal HI-insult. We found that animals after HI showed motor impairments in the cylinder rearing test. The severity of the motor impairment depended on the duration of the hypoxia which is associated with the severity of the cerebral lesion.

The unilateral (non-affected) forepaw preference in the cylinder rearing test is a direct consequence of the lesion induced in the affected hemisphere (Schallert et al. 2000). A similar rationale was used for the adhesive removal task; HI-affected animals are impaired to remove a sticker from the impaired forepaw. The motor impairments seen for the cylinder rearing test and adhesive removal task may relate to the hemiparesis often diagnosed in infants affected by neonatal HI (Volpe 2001).

Cognitive performance was assessed in a novel paradigm for neonatal HI, the modified holeboard (mHB) which is based on a modification as described by Ohl et al. (2003). In the mHB, animals are tested for their capability to retrieve food rewards located in 3 cued cylinders situated amongst 7 non-cued cylinders. Severely affected animals were highly impaired but mildly affected animals initially did not show any cognitive impairment during the training phase. During the subsequent *reversal task*, the baited cylinders were non-cued while now the remaining non-baited cylinders were cued. Remarkably, during this reversal task which required switching of the strategy, mice after mild HI did show cognitive deficits as if these animals were impaired to 'unlearn' the previous task requirements and to incorporate the new requirements demanded by the reversal of the cues. Mildly HI-affected mice experienced more difficulties adapting to the new setup of the mHB in comparison to sham-controls. Perhaps mildly HI-affected animals were more distracted than sham-controls by the altered setup (the reversal task) and impaired to create a new mental image of the novel setup (by exploring the mHB) required for effective retrieval of the food-rewards. Alternatively, mildly HI-affected animals could have been impaired to extinguish the initial strategy that was required to find the food rewards and continued to use the strategy employed during training sessions thereby increasing the number of long-term memory errors. Mildly HI-affected animals showed increased exploratory behavior during the reversal task in absence of increased long-term memory errors (visits to non-baited cylinders) as compared to sham-controls. Therefore it is more likely that mildly HI-affected animals were impaired in the reversal task as a result of decreased attention as indicated by increased exploratory behavior.

Our finding with regard to the impaired performance in animals adapting to a novel setup is in line with the findings of a previous paper using a murine model for stroke (Winter et al. 2004). Here animals with mild cerebral ischemia learnt well in the water maze but displayed distinct neurological deficits in strategy switching. The findings described by Winter et al. (2004) and by us in Chapter 4 regarding strategy switching may be reminiscent of the dysexecutive syndrome seen in patients in which the basal ganglia are affected (Inzelberg et al. 2001).

We continued the use of the cylinder rearing test and other motor tests as well as the mHB as a behavioral screening battery to assess motor- and cognitive impairments in rats after putative neuroprotective treatments (Chapters 5-6).

HI has been shown to activate cerebral NF-κB. Activation of this important transcription factor is dependent on phosphorylation of IκB by a complex of IKKα, IKKß and the regulatory subunit NEMO (IKKγ) (Yamaoka et al. 1998) (Figure 1). In Chapter 5 we used the specific NF-κB inhibitor NEMO-Binding Domain (NBD) peptide (May et al. 2000) coupled to a TAT-sequence to facilitate cerebral uptake. When the peptide in-

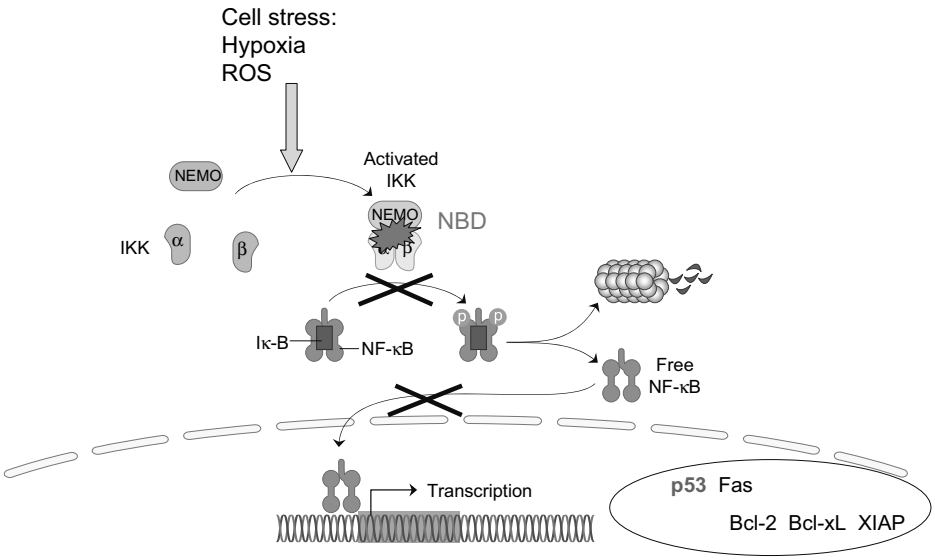

Figure 1. Simplified schematic overview of the NF-κB pathway and the effects of TAT-NBD. In resting cells, NF-κB is bound in the cytoplasm to inhibitory IκB proteins. Signal induced phosphorylation of IκBα by the IκB-kinase (IKK)-complex is a key step in NF-κB activation. The IKK complex consists of the kinases IKKα and IKKß and the regulatory protein NEMO (NF-κB essential modulator). Phosphorylated IκBα becomes ubiquitinated and is proteasome-degraded after which free NF-κB enters the nucleus to regulate transcription. The NEMO Binding Domain (NBD) peptide prevents binding of the IKK-complex to NEMO. Therefore, phosphorylation of the inhibitory IκB proteins will not take place. As a result the transcription of genes normally induced by NF-κB (such as p53, Fas, Bcl-2, Bcl-xL and XIAP) is prevented.

hibits the binding of NEMO to the complex of IKKα and IKKβ, IκB is not phosphorylated. In response, nuclear translocation of NF-κB is prevented and as a result transcription of NF-κB dependent genes will not take place (Figure 1). We confirmed the strong neuroprotective capacity of the NF-κB inhibiting TAT-NBD peptide treatment as previously shown by Nijboer et al. (2008a).

We now described that the impressive neuroprotective effect lasted up to 14 weeks of age. The treatment with TAT-NBD is extremely powerful since it nearly completely prevents the apoptotic storm initiated by the hypoxic-ischemic event. During development the milieu in the brain is tightly regulated represented by e.g. a balance between cellular apoptosis on the one hand and proliferation and differentiation on the other hand. A putative risk of such a 'strong' neuroprotectant might be that in the long-term the cells that should have died from natural causes during ontogeny will linger on and may even initiate tumour formation. However, gross brain morphology at 13 weeks after inhibition of NF-κB did not reveal abnormalities in HI/TAT-NBD-treated animals. It remains to be investigated whether or not these animals will be prone to the development of brain tumours during aging.

The robust neuroprotective improvements based on histology were associated with complete functional recovery in HI/TAT-NBD-treated animals. We measured motor functionality in the cylinder rearing test and the adhesive removal task. HI/vehicle-treated animals showed a right forepaw preference in the cylinder rearing test and a reduced latency to remove the adhesive from the impaired forepaw when compared to sham-controls indicating motor deficits. Motor behavior in HI/TAT-NBD-treated animals was completely restored; HI/TAT-NBD treated animals did not show a forepaw preference in the cylinder rearing task and removed the adhesive with latencies comparable to sham-controls.

In the mHB we observed that during the first 3 training-days all treatment groups readily acquired the task as indicated by the reduced number of food rewards that were omitted, the latency to complete the trial and the number of long-term memory errors. After switching the location of the cued cylinders HI/vehicle-treated animals showed increased numbers of long-term memory errors. HI/TAT-NBD-treated animals did not show an increase in the number of long-term memory errors after the cue switch. During the subsequent reversal-task, cognitive impairments for HI/vehicle-treated animals were evident; HI/vehicle-treated animals showed increased numbers of long-term memory errors, an increased latency to complete the task and increased numbers of short-term memory errors compared to sham-controls. The impairments of the severely HI-affected rats seen during the reversal task (Chapter 5-6) differed from those observed for mildly HI-affected mice (Chapter 4). Both mildly HI-affected mice (Chapter 4) and severely HI/vehicle-treated rats (Chapter 5-6) showed an increased latency during the reversal task. However, the severely affected HI/vehicle-treated

rats showed increased numbers of short- and long-term memory errors without enhanced exploratory behavior indicating that the severely affected HI/vehicle-treated animals persevered in their strategy employed during training sessions.

In HI/TAT-NBD-treated animals the latency to complete the task as well as the number of long- and short-term memory errors did not differ from those found in sham-controls. The neuroprotective properties of early NF-κB inhibition by TAT-NBD treatment after neonatal HI (80% neuroprotection at 48h after insult and total recovery at 13 weeks after insult based on MAP2 staining) in rats are therefore associated with complete motor and cognitive recovery in the long-term. The latter data suggest that NBD may represent an attractive therapeutic strategy for neonatal HI in humans.

However, timing of TAT-NBD administration appeared to be crucial; administration early after the insult resulted in strong neuroprotection. In about 50% of the cases, neonatal HIE is caused by an acute complication which allows the neonatologist to determine the time of the insult (Cowan et al. 2003). For these acute complications TAT-NBD treatment may prove to be very effective. However, extended treatment or late administration of the TAT-NBD peptide did not work or even exacerbated cerebral injury a little (van den Tweel et al. 2006; Nijboer et al. 2008). The risk of aggravating brain injury when given (too) late is a major clinical drawback. The complicated mode of action of TAT-NBD stimulated us to investigate other, safer therapeutics.

As NF-κB is known as a major transcription factor for the expression of several cytokines, we found it remarkable that the observed neuroprotection by TAT-NBD was not associated with inhibition of cytokine production. We hypothesized that during NF-κB inhibition the cell may switch to the use of other transcription factors such as the JNK/AP-1 pathway to produce cytokines. Indeed, Nijboer et al. have shown that under conditions of TAT-NBD, the cell switches to the use of AP-1. In addition inhibitors of the JNK/AP-1 pathway such as Gadd45β and XIAP (x-linked inhibitor of apoptosis) were downregulated in HI/TAT-NBD-treated animals (Nijboer et al. 2009) (Figure 2).

In Chapter 6 we investigated the potential neuroprotective potential of a peptide that inhibited the JNK-pathway (TAT-JBD) (Figure 2). We showed that inhibition of JNK using a TAT-JBD peptide moderately decreased (30% neuroprotection at 48h after insult based on MAP2 staining) neuronal damage in our model of neonatal HI in rats. TAT-JBD treatment was associated with reduced levels of the apoptotic executioner caspase 3. Surprisingly, the levels of known upstream activators of caspase 3, active caspase 8, mitochondrial cytochrome *c* release and cleaved caspase 9 were not affected by TAT-JBD treatment.

Mitochondrial cytochrome *c* release and cleaved caspase 9 are controlled by Bid cleavage into t-Bid. Bid may however be spliced into the j-Bid variant upon JNK stimulation. In addition, j-Bid is known to lead to preferential release of Smac/Diablo, a small mitochondrial protein that impairs IAP (Inhibitors of APoptosis)-functioning and

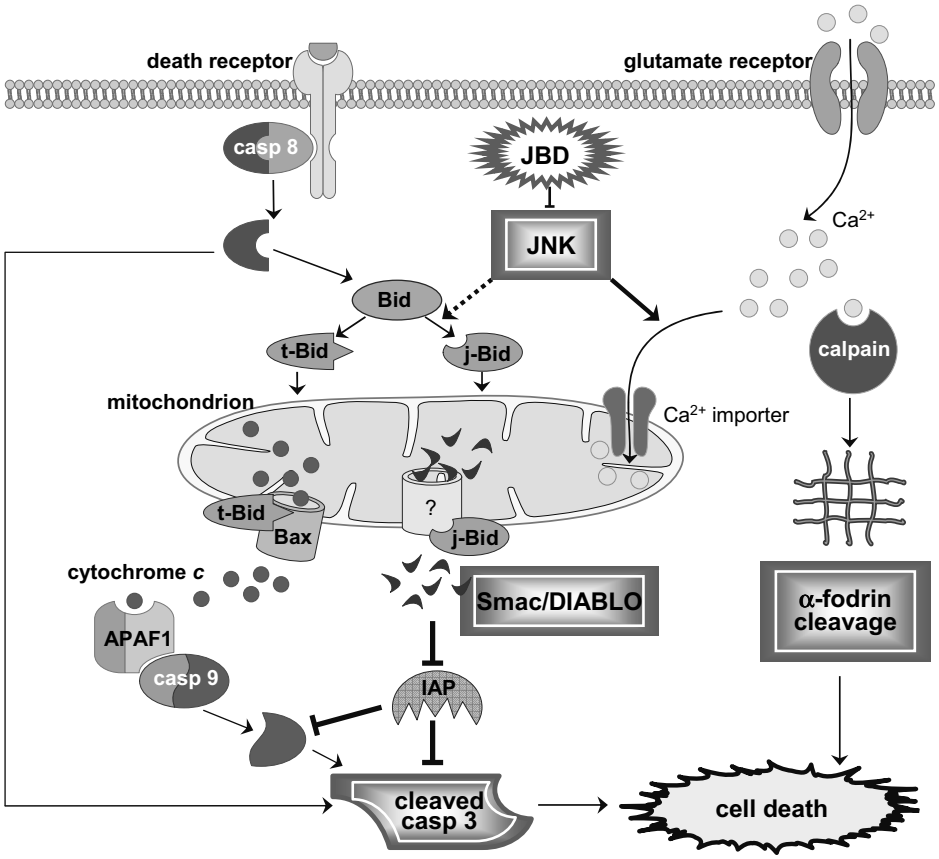

Figure 2. Simplified schematic overview of apoptotic and necrotic pathways of cell death and the effects of TAT-JBD.

We showed that inhibition of JNK by JBD treatment reduced Smac/DIABLO release from the mitochondria, activation of caspase 3 and cleavage of α-fodrin. We propose that these effects are the consequence of inhibition of JNK-mediated j-Bid cleavage by TAT-JBD (dotted line) and of increased calcium buffering capacity of the mitochondria when JNK is inhibited.

mediates apoptosis without release of cytochrome *c*. Most importantly, mitochondrial Smac/DIABLO was increased after HI but subsequently inhibited by TAT-JBD treatment. Likely, TAT-JBD treatment inhibits HI-induced j-Bid formation thereby reducing Smac/Diablo. Smac/Diablo decreases IAP functioning and enhances active caspase 3 formation in HI/vehicle-treated animals but this is prevented through TAT-JBD treatment (Figure 2).

As discussed in the introduction, apart from apoptotic-like cell death, necrosis is also a prominent feature after neonatal HI-brain injury and both types of cell death may co-occur in particular cells (Northington et al. 2007). Enzymes such as calpains are induced upon enhanced levels of extracellular calcium and activate α-fodrin

cleavage that lead to necrotic-like cell death (Figure 2). In this respect, TAT-JBD treatment was also found to reduce the cleavage of α-fodrin indicating that calpain-mediated brain damage was reduced as well. Therefore, TAT-JBD treatment not only inhibits apoptotic-like cell death but may also decrease necrosis.

For motor functioning we assessed the effect of TAT-JBD treatment in the cylinder rearing test (2.5, 4 and 6 weeks of age), the adhesive removal task (5 weeks of age) and rota-rod (10-12 weeks of age). HI/vehicle-treated animals were impaired on all three motor tasks in comparison to sham-controls. HI/TAT-JBD-treated animals did not show an improved performance on the cylinder rearing test but showed improved performance in comparison to HI/vehicle-treated animals in the adhesive removal task and rota-rod. The finding that HI/TAT-JBD-treated animals were impaired on all time-points measured in the cylinder rearing test but not in the adhesive removal task or the rota-rod may imply that the cylinder rearing test is a more sensitive tool to study motor impairments than the rota-rod and the adhesive removal task. The difference in nature between the tests, voluntary movements during the cylinder rearing test while the adhesive removal test and the rota-rod may be regarded as non-voluntary and/or even aversive stimuli, indicates that the impaired forepaw can be used when required. This is an interesting aspect since in human rehabilitation programs this diversity in voluntary vs. non-voluntary motoric behavior is often observed as well.

Finally we tested whether cognitive performance was improved in HI-animals after TAT-JBD treatment in the modified holeboard (11-13 weeks of age). During training sessions rats from all treatment groups showed no significant differences. However, HI/TAT-JBD-treated animals showed *less* long-term memory errors in comparison to HI/vehicle-treated animals during the *cue switch*. Moreover, during the reversal task HI/TAT-JBD-treated animals –in comparison to HI/vehicle-treated animals- tended to show a reduced latency to complete the trial and made less short- and long-term memory errors. The histology of the lesions with and without TAT-JBD treatment at 13 weeks after the insult showed that TAT-JBD had a neuroprotective effect of 56%. The relatively moderate neuroprotection seen after early JNK-inhibition in a rat model for neonatal HI thus results in partial preservation of motor functions and intact cognitive properties as assessed in the mHB. Our findings indicate therefore that a complete restoration of affected cerebral tissue after neonatal HI (such as seen after TAT-NBD treatment) is not required for normal motor behavior as observed in the adhesive removal task and rota-rod as well as for cognitive functioning in the mHB. Since the neurobehavioral performance is ultimately the most important outcome after neonatal HI, we feel that the results obtained after TAT-JBD treatment warrant further research to assess TAT-JBD as a putative neuroprotectant in the clinic.

Apart from impaired motor behavior and affected cognition, a number of clinical studies have shown that a history of mild perinatal HI is associated with a number of

affective disorders including attention-deficit hyperactivity disorder (ADHD), aggression, autism etc. (Rennie et al. 2007). In addition, behavioral disorders such as increased anxiety in animal models for neonatal HI can interfere with the results of the cognitive tests as a confounding factor and should thus be taken into account when testing animals for cognition.

To investigate if HI has behavioral consequences, we tested animals in a pilot study after mild neonatal HI on the elevated plus maze (unpublished results). The elevated plus maze consisted of 4 arms emanating from a central 'open' platform. Mice prefer to avoid open spaces. Therefore, the frequency and latency to enter a closed arm as well as the time spent in a closed arm were regarded as a measure for anxiety-like behavior.

When tested during adult life, mildly affected HI-treated mice (45 min of hypoxia at 10% O_2 after carotid artery occlusion at 9 days after birth) showed a reduced latency to enter a closed arm (Fig. 3A); visited more frequently the closed arms (Fig. 3B) and showed a reduced amount of time spent in the closed arms (Fig. 3C). Since we previously found no differences in the modified hole board for any of the exploratory measurements, we suggest that the differences are indeed due to differences in anxiety-like behavior and not secondary to differences in exploration. Perhaps neonatal HI in mice disrupts inhibitory processes normally restraining unaffected animals from exploring a dangerous environment. In behavioral paradigms that may induce fear, one should be aware therefore that anxiety-like factors as well as other factors may affect the behavioral outcome.

Positive experimental results for the use of EPO as a treatment for HI in animal models have been followed up by ongoing clinical trials in which EPO is evaluated as a possible therapeutic against neonatal HIE (Chapter 2). The current use of EPO (for other medical indications) in the clinic and its excellent safety favour the use of EPO as a neuroprotective strategy. The neuroprotective effects of EPO based on the results from animal studies are, however, limited (Chapter 3) and therefore not more than a modest neuroprotective effect should be expected when treating neonatal HIE. Since we still observed a significant amount of brain damage after EPO treatment, these animals will likely suffer from a substantial proportion of residual brain damage. Perhaps a combination therapy of EPO, to inhibit early apoptotic processes, combined to mesenchymal stem cell therapy, to enhance brain restoration, may prove to be a fruitful approach in order to attenuate HI-brain injury. However, more powerful treatment options to inhibit apoptosis are ready to be tested in humans such as TAT-JBD and TAT-NBD or peptides specifically targeting early apoptosis, such as p53 inhibitors that are currently tested in our laboratory with very promising results (Nijboer et al., unpublished results).

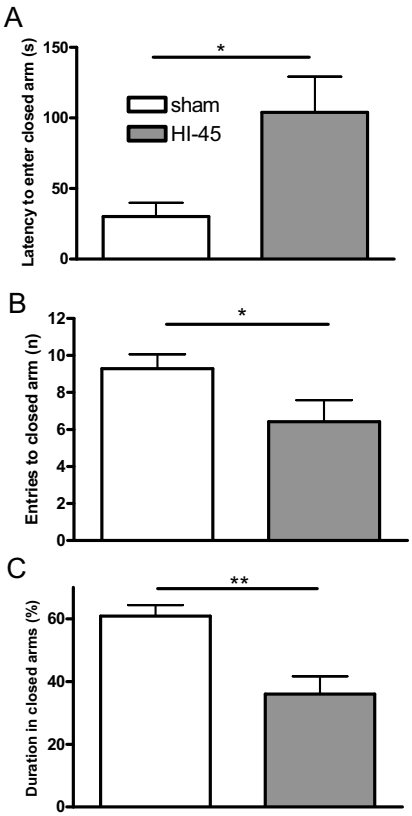

Figure 3. Elevated plus maze.
Lack of anxiety-like behavior is indicated by (A) reduced latency to enter a closed arm, (B) decreased entries to a closed arm and (C) overall reduced duration of time spent in the closed arms.

Only a limited number of behavioral tests are available to evaluate the functional integrity of animals after neonatal HI. This paucity of valid behavioral tests is especially relevant for the assessment of cognitive integrity of mice. In Chapters 4-6 we have shown that the modified hole board is an important addition to our arsenal of behavioral tests. The use of the modified hole board as a behavioral test for the cognitive integrity of rodents should not be limited to the assessment of neuroprotectants for the treatment of cerebral damage after neonatal HI. Researchers working with animals models for other brain disorders, such as Alzheimer's- , Parkinson's and Huntington's disease, Schizophrenia and Fragile X-syndrome, may very well benefit from application of the modified holeboard as a behavioral test.

We showed in Chapters 4-6 that the crucial differences between animals tested in the modified hole board were revealed only after alterations of the initial training-setup with the cue switch and the reversal task. As a result, initial learning in rodents

after neonatal HI may often be intact but we want to emphasize that cognitive flexibility may be impaired.

In this thesis we have established that early inhibition of both the NF-κB and JNK-pathway (Chapters 5-6) resulted in significant long-term neuroprotection. Most importantly, the histological neuroprotection associated with inhibition of the NF-κB and JNK-pathway by using the TAT-NBD and TAT-JBD resulted in long-term motor- and cognitive improvements. Therefore the use of TAT-NBD and TAT-JBD –when timely applied- is very promising in order to combat the devastating consequences of neonatal HI. Future studies aimed to investigate the use of TAT-NBD and TAT-JBD as therapeutic agents should be conducted in larger animal models for neonatal HI that may more accurately resemble the clinical pathophysiology of HIE.

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Chapter 8

Nederlandse samenvatting

(SUMMARY IN DUTCH)

SAMENVATTING EN DISCUSSIE

Ongeveer 3-5 per 1.000 levendgeborenen krijgt rondom de geboorte te maken met een tekort aan zuurstof (hypoxie) en een tekort aan cerebrale bloedvoorziening (ischemie). Wanneer de hypoxie-ischemie (HI) lang aanhoudt ontstaat er hersenschade. De uiteindelijke gevolgen van HI kunnen zeer ernstig zijn; 30-40% sterft terwijl bij 20-40% van deze kinderen neurologische afwijkingen ontstaan. De afwijkingen zoals gevonden na HI beslaan een breed spectrum; epilepsie, cerebrale kinderverlamming, verminderd leervermogen, visuele en auditieve beperkingen evenals de ontwikkeling van gedragsstoornissen komen vaak voor. Helaas is het niet zo dat er een kant-en-klare behandeling beschikbaar is die effectief de schadelijke gevolgen van HI kan tegengaan. Er zijn wel een aantal behandelingen geformuleerd die in proefdiermodellen voor HI hebben bewezen effectief te zijn. De voorgestelde behandelingen proberen het ontstaan van schade tegen te gaan of de schade te beperken.

Het meest gebruikte diermodel voor neonatale HI is het zogenaamde 'Vannucci-Rice' diermodel. Dit is ook het diermodel dat wij in de experimenten in dit proefschrift hebben gebruikt. Van 7-dagen oude rattenpups of 9-dagen oude muizenpups wordt onder anesthesie de rechter arterie carotis afgesloten waarna de dieren in een couveuse worden geplaatst waar het zuurstofgehalte is verlaagd naar 8% (ratten) of 10% (muizen). Hierna zal –afhankelijk van voornamelijk de hypoxie duratie- unilaterale hersenschade ontstaan.

Uiteindelijk is de belangrijkste uitleesmaat voor de hoeveelheid schade na neonatale HI het functieverlies. Er is echter maar een relatief klein aantal studies gepubliceerd dat in diermodellen voor neonatale HI hier onderzoek naar heeft gedaan. De motorische en cognitieve afwijkingen in het 'Vannucci-Rice' diermodel voor neonatale HI en de functionele verbeteringen na behandeling staan echter wel centraal in dit proefschrift.

Functionele afwijkingen na neonatale HI

De functionele uitkomst gebaseerd op gedragsafwijkingen kunnen worden onderverdeeld in afwijkingen van motorische aard, cognitieve beperkingen (beperkingen in leren en geheugen) en afwijkingen die te maken hebben met emotionele stoornissen (hierbij kan bijvoorbeeld gedacht worden aan hyperagressiviteit, angstgerelateerde stoornissen, impulsiviteit en aandachtsstoornissen).

Motorische afwijkingen

Motorische afwijkingen worden vaak gezien in kinderen die rondom de geboorte HI hebben ondergaan. In diermodellen voor neonatale HI kunnen motorische afwijkingen ook worden gevonden. Motorische afwijkingen hebben wij kunnen aantonen met een

aantal verschillende testen. Zo is er de cilinder test. De dieren worden om de beurt in een plastic cilinder gezet. Doordat de cilinder een nieuwe omgeving is gaan de dieren deze onderzoeken. Dit doen de dieren door op hun achterpoten te staan en met de voorpoten tegen de cilinder aan te leunen. Van dit laatste gegeven maken wij gebruik. Dieren zonder hersenbeschadiging gebruiken beide voorpootjes zonder voorkeur. Echter, in dieren na HI is één hemisfeer (hersendeel) aangedaan en hierdoor zullen de motorische zenuwen behorende bij dit gedeelte van het brein minder goed functioneren. De motorische zenuwen van het andere voorpootje doen het nog wel goed. Door de mate van voorkeur voor het gebruik van het onaangedane voorpootje bepalen wij dan hoeveel de motorische afwijking is. Eenzelfde idee wordt gehanteerd in de sticker-verwijder test. Hierbij worden beurtelings kleine stickers op de onderzijde van de voorpoten geplakt waarna de tijd tot verwijderen wordt gemeten. Ook hier zien wij dat dieren na hersenschade beperkt zijn in het verwijderen van de sticker van het voorpootje behorende bij de beschadigde hemisfeer. Een andere test voor motorische afwijkingen is de rota-rod. Dit apparaat is eigenlijk een verhoogde elektronische tredmolen. Door de buis waarop de dieren lopen te laten versnellen kunnen we zien hoe lang de dieren het volhouden tot ze eraf vallen. Dieren die eerder van de rota-rod vallen zullen meer hersenschade hebben dan dieren met onbeschadigde hersenen of waarvan de hersenschade succesvol is behandeld.

Cognitieve afwijkingen

Voor afwijkingen op het cognitieve vlak hebben wij in onze studies gebruik gemaakt van het zogenaamde ‘modified hole board’ (mHB). Deze gedragstest was nog niet eerder gebruikt in diermodellen voor neonatale HI. Het mHB bestaat uit een plastic bord dat in het midden van een plastic box is geplaatst. Op het plastic bord staan een aantal cilinders waarvan in 3 cilinder een kleine voedselbeloning (stukje amandel) is geplaatst. Als aanwijzing voor de dieren zijn deze cilinders gemarkeerd door middel van een wit plastic ringetje om die cilinders. Er zijn een aantal parameters waarmee de cognitieve capaciteiten van de dieren getest kan worden. Zo meten we snelheid waarmee de dieren de voedselbeloningen vinden. Ook het aantal fouten dat de dieren maken tijdens de test, door in cilinders te zoeken waarin geen voedselbeloning te halen valt of in cilinders te zoeken waarin het voedsel al eerder is gevonden is een indicatie voor cognitie.

Belangrijk hierbij is de mogelijkheid gebleken om veranderingen in de originele opzet van de test toe te passen om de test voor de dieren zodoende moeilijker te maken. Zo kunnen we de cilinders met de voedselbeloning wisselen (‘cue switch’). Nog belangrijker bleek het omdraaien van de taak (‘reversal’ taak). Waar eerst de aanwijzing (witte ring) betekende dat er een voedselbeloning te vinden viel, was dat tijdens deze aanpassing nu juist niet het geval. Alleen de cilinders zonder ring bevat-

ten nu een voedselbeloning terwijl alle andere cilinders (waar geen voedsel te vinden was) wel ringetjes bezaten.

Buiten parameters indicatief voor cognitie is het voordeel van de mHB dat we een aantal parameters kunnen meten die een maat zijn voor exploratie, opwinding en angst-gerelateerd gedrag. Ook dit is belangrijk om mee te nemen in de uiteindelijke analyse. Aan de hand van de uitslag kunnen we dan namelijk bepalen in hoeverre de cognitieve afwijkingen 'puur' cognitief zijn en niet afhankelijk van andere (bovengenoemde) gedragingen van het dier.

Resultaten van dit proefschrift

In het eerste gedeelte van dit proefschrift (**hoofdstuk 2 en 3**) hebben wij ons gericht op de rol van erythropoëtine (EPO) als neuroprotectieve stof. In **hoofdstuk** 2 staat beschreven hoe EPO neuroprotectief werkt in *in vitro* (cellijnen) en *in vivo* (levende dieren) modellen voor hypoxisch-ischemische hersenschade. Meestal is beschreven dat EPO goed helpt bij het tegengaan van hersenschade door neonatale HI.

Verder is bekend dat de stof Deferoxamine (DFO) ook neuroprotectief werkt. Nu wilden wij onderzoeken of de combinatie van DFO en EPO misschien extra neuroprotectief was in vergelijking tot het gebruik van alleen DFO of alleen EPO. In **hoofdstuk 3** is beschreven dat in de hersenen van HI-behandelde dieren na EPO en DFO-EPO toediening het aantal cellen dat in apoptose (geprogrammeerde zelfdood van een cel) is gegaan verminderd is. Ondanks dat EPO en DFO-EPO behandeling het aantal cellen dat in apoptose ging kon verminderen bleek geen van de behandelingen de uiteindelijke schade te verminderen. Wellicht dat de enorme hoeveelheid schade door HI de neuroprotectie van DFO en EPO heeft belemmerd. We hebben in ieder geval kunnen vaststellen dat de combinatie van DFO en EPO niet gunstig heeft uitgepakt in de strijd tegen HI geïnduceerde hersenschade. Bovendien lijkt het er dus op dat onder bepaalde omstandigheden (zoals in **hoofdstuk 3** waarin dieren ernstige hersenschade ontwikkelden) EPO niet goed werkt. Dit idee gaat eigenlijk in tegen de huidige consensus over EPO en wij willen dan ook ervoor waarschuwen dat de verwachtingen betreffende EPO als therapeutisch middel tegen HI momenteel waarschijnlijk te hoog gespannen zijn.

Het doel in het tweede gedeelte van dit proefschrift (**hoofdstuk 4-6**) was eigenlijk tweeledig. Allereerst wilden wij vaststellen in hoeverre functionele afwijkingen (motorisch en cognitief) afhing van de hoeveelheid hersenschade na HI (**hoofdstuk 4)**. Voorts wilden wij onderzoeken of die functionele afwijkingen konden worden verminderd wanneer wij de dieren behandelden met neuroprotectieve stoffen (**hoofdstuk 5 en 6**).

In **hoofdstuk 4** hebben wij muizen die ernstige en milde hersenschade hebben gehad na HI vergeleken met elkaar en met dieren die geen schade hadden (controle die-

ren). Wij zagen in de cilinder test dat afhankelijk van de ernst van de schade dieren een voorkeur vertoonden voor het gebruik van het onaangedane voorpootje wanneer het tegen de wand van de cilinder aan staat. In de mHB (zie kopje cognitieve afwijkingen) vonden wij dat dieren die ernstige hersenschade hadden zeer veel moeite hadden om de voedselbeloningen te vinden. Meestal wisten deze dieren zelfs na 5 dagen testen (met 4 oefeningen per dag) niet de 3 voedselbeloningen te vinden binnen de 5 minuten tijdsgrens. Verrassend genoeg verschilden dieren die milde hersenschade hadden ondergaan totaal niet van dieren die geen hersenschade hadden gehad tijdens de eerste opzet van het mHB. Zo waren dieren na milde hersenschade even snel en maakten zij net zoveel fouten als dieren zonder hersenschade. Ook metingen indicatief voor exploratie, angst en opwinding verschilden niet tussen de controle dieren en de dieren met milde hersenschade. Toen de dieren zonder hersenschade en dieren met milde hersenschade werden geïntroduceerd in de 'reversal' taak bleek echter dat dieren na milde hersenschade wel degelijk cognitief beperkt waren. Deze dieren deden er immers langer over om de 3 voedselbeloningen te vinden. Ze maakten hierbij niet meer fouten door in de verkeerde cilinders te gaan kijken maar waren wel meer bezig met exploratie van het mHB in vergelijking tot dieren zonder hersenschade. Wij denken dat de dieren na milde hersenschade meer tijd nodig hebben om een 'mentaal plaatje' te maken in de nieuwe omgeving voordat ze verder konden gaan met effectief voedsel zoeken.

HI zet een aantal moleculaire routes aan waarvan de NF-κB en JNK routes zeer belangrijke zijn. Met specifieke peptides kan de activatie van de NF-κB (door het TAT-NBD-peptide) en de JNK (door het TAT-JBD-peptide) routes door HI voorkomen. Door het toedienen van de TAT-NBD en het TAT-JBD peptide vroeg na het ontstaan van de HI-schade kan een belangrijk deel van de hersenschade worden voorkomen. Toediening van het TAT-NBD-peptide liet hierbij een zeer sterke protectie zien terwijl na TAT-JBD behandeling de protectie wel significant, maar minder sterk was. Aangezien beide stoffen interfereren met de natuurlijke celdood na HI waren wij aanvankelijk bezorgd om de mogelijke gevolgen op lange termijn waaronder wellicht de vorming van tumoren. Wij hebben hier echter geen aanwijzingen voor gevonden in de hersencoupes van dieren die behandeld waren met TAT-NBD of TAT-JBD en opgeofferd 13 weken na toediening van de peptides.

Wij wilden onderzoeken of het herstel van hersenschade na TAT-NBD en TAT-JBD behandeling ook samenviel met functioneel herstel (**hoofdstuk 5 en 6**). Met de cilinder test en sticker-verwijder test hebben wij laten zien dat dit voor motorische functies inderdaad het geval is. Waar na HI onbehandelde ratten een voorkeur lieten zien in de cilinder test voor het gebruik van het onaangedane voorpootje tijdens exploratie van de cilinder was dit niet het geval voor TAT-NBD behandelde dieren. De TAT-NBD behandeling beschermde dus tegen motorische afwijkingen in de cilinder-test. In de

cilinder-test waren TAT-JBD behandelde dieren echter niet beschermd aangezien zij wel een voorkeur toonden voor het gebruik van het onaangedane voorpootje. In de sticker-verwijder test zagen wij dat zowel TAT-NBD en TAT-JBD behandelde dieren beschermd waren omdat deze dieren er minder lang over deden om de sticker van de aangedane voorpoot te verwijderen in vergelijking met onbehandelde dieren na HI. Ook op de rota-rod bleek TAT-JBD behandelde dieren het beter te doen dan niet behandelde dieren na HI aangezien deze dieren langer op de rota-rod konden blijven zonder te vallen.

Hierna werden de dieren getest in het mHB voor cognitieve functies. Tijdens de eerste opzet van het mHB waren er geen verschillen te vinden tussen de groepen dieren. Cognitieve afwijkingen kwamen echter aan het licht tijdens aanpassingen van de originele opzet. Tijdens de 'cue switch' en de 'reversal' taak bleek dat HI behandelde ratten er langer over deden om de taak te volbrengen (het vinden van alle 3 de voedselbeloningen) in vergelijking met dieren zonder hersenschade en dieren die na HI behandeld waren met TAT-NBD of TAT-JBD. Belangrijker nog was het aantal fouten dat tijdens de test gemaakt werd door te kijken in cilinders waar geen voedselbeloning te vinden was. Deze bleken tijdens de 'cue switch' maar voornamelijk tijdens de 'reversal' taak sterk toegenomen voor dieren die wel HI hadden ondergaan maar geen verdere behandeling hadden ontvangen. In de controle dieren en in dieren die na HI met TAT-NBD en TAT-JBD waren behandeld was een toename in het aantal gemaakte fouten (in vergelijking met controle dieren) niet gevonden. Zowel TAT-NBD en TAT-JBD beschermden de dieren na HI dus op motorisch en cognitief vlak. TAT-NBD en TAT-JBD peptides zijn daardoor interessante kandidaten om verder te onderzoeken als mogelijke medicatie in de kliniek tegen de gevolgen van HI. De volgende stap zou zijn om de TAT-NBD en TAT-JBD peptides te testen in grotere diermodellen voor neonatale HI die de klinische situatie waarschijnlijk beter benaderen.

Er zijn slechts een beperkt aantal gedragstesten beschreven waarin cognitie kan worden gemeten na neonatale HI. In de studies beschreven in de **hoofdstukken 4-6** is duidelijk geworden dat het mHB een welkome aanvulling kan zijn. In het bijzonder het grote aantal verschillende parameters, die indicatief zijn voor zowel cognitie als voor andere gedragingen, zorgen voor een groot onderscheidend vermogen. Wij pleiten er dan ook voor dat meer onderzoeksgroepen deze test gaan gebruiken en verwachten dat het mHB niet alleen voor het veld van de neonatale HI, maar ook voor onderzoek waarin gewerkt wordt met diermodellen voor andere hersenafwijkingen zeer waardevol zal zijn.

Tot slot willen wij er ook op wijzen dat gedragsafwijkingen na HI niet alleen motorisch of cognitief hoeven te zijn zoals hierboven uitgelegd (**Functionele afwijkingen na neonatale HI**). Zo hebben wij in een recent pilot-onderzoek gevonden (zie **hoofdstuk** 7) dat op een zogenaamde 'elevated plus maze' (een verhoogde opstelling

bestaande uit 4 armen waarvan er 2 open zijn en 2 gesloten) muizen na milde hersenschade zich meer begaven op de open armen dan in de gesloten armen terwijl dit bij controle muizen niet het geval is. Aangezien muizen van nature grote open ruimtes vermijden duidt dit resultaat erop dat de muizen na milde hersenschade waarschijnlijk minder angst kennen dan de controle dieren. Aangezien we in **hoofdstuk 4** geen verschillen zagen in algemene exploratie tussen dieren na milde hersenschade en controle dieren denken we dat de verschillen op de 'elevated plus maze' daadwerkelijk angst gerelateerd zijn. Wij zijn van mening dat het belangrijk is om buiten cognitie en motorische afwijkingen naar gedrag te kijken dat iets zegt over de emotionele status van een dier. Dit omdat sociale afwijkingen ook worden gevonden in kinderen die vroeger te maken hebben gehad met HI en omdat dit de uitkomst van gedragstesten met dieren kan beïnvloeden.

List of abbreviations

ADHD	Attention-Deficit/Hyperactivity Disorder
AMPA	α-amino-3-hydroxy-5-methyl-4-isox-azole proprionate
ANOVA	Analysis of Variance
AP1	Activator Protein 1
ART	Adhesive Removal Task
ATP	Adenosine Tri-Phosphate
BAF	boc-aspartyl-(OMe)-fluoromethyl-ketone
BBB	Blood Brain Barrier
BCCAO	Bilateral Common Carotid Artery Occlusion
BDNF	Brain Derived Neurotrophic Factor
CβR	Common Beta Receptor
CEPO	Carbamylated EPO
CRT	Cylinder Rearing Test
CS	Cue Switch
DFO	Deferoxamine
DIABLO	Direct Inhibitor of Apoptosis Binding protein with Low pI
DMSO	Di-methyl Sulph-Oxide
E(11)	Embryonal Day (11)
EEG	Electro Encephalogram
EPO	Erythropoietin
EPOR	EPO receptor
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HE	Hematoxylin-Eosin
HI	Hypoxia-Ischemia
HIE	Hypoxic-Ischemic Encephalopathy
HIF	Hypoxic-Inducible Factor
icv	Intra-Cerebro-Ventricular
IGF	Insulin-like Growth Factor
IL	interleukin
ip	intraperitoneal
iv	intravenous
JAK	Janus Kinase
JBD	JNK Binding Domain
JNK	c-jun N-terminal Kinase
LSD	Least Statistical Difference

MAPKs	Mitogen-Activated Protein Kinases
MAP2	Microtubuli-Associated Protein
MBP	Myelin Basic Protein
MCAO	Middle Cerebral Artery Occlusion
mHB	Modified Holeboard
MIP	Macrophage Inflammatory Protein
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine
NBD	NEMO Binding Domain
NEMO	NF-κB Essential Modulator
NF-κB	Nuclear Factor kappa B
NGF	Neuronal Growth Factor
NMDA	N-methyl-D-aspartate acid
OGD	Oxygen and Glucose Deficiency
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
P(7)	Postnatal day (7)
qPCR	quantitative PCR
Q-VD-OPh	quinoline-Val-Asp(Ome)-CH2-O-phenoxy
rhEPO	recombinant human EPO
rpm	rotations per minute
RT	reversal task
SAPKs	Stress Activating Protein Kinases
sc	Subcutaneous
SEM	Standard Error of Mean
Smac	Second Mitochondria-derived activator of caspase
SNc	Substantia Nigra *pars compacta*
STAT	Signal Transducer and Activator of Transcription
SVZ	Subventricular Zone
TAT	HIV-1 transactivating transcriptional activator
TH	Tyrosine Hydroxylase
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor
VTA	Ventral Tegmental Area
XIAP	X-linked Inhibitor of Apoptosis

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Een mooi proefschrift is het geworden! Dit kan ik gemakkelijk zo zeggen aangezien ik het werk gelukkig niet alleen heb hoeven doen. Ik kan terugkijken op vier mooie jaren waarin ik veel geleerd heb. Mijn allereerste dank gaat natuurlijk uit naar mijn promotoren en copromotoren die mij hebben geholpen een volwaardig onderzoeker te worden. Ook de praktische hulp van mijn overige collega's zijn van grote waarde geweest. Tot slot wil ik collega's, familie en vrienden bedanken voor de steun die ik heb gekregen op momenten waarop ik dat nodig had.

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List of publications:

***Van der Kooij MA**, Nijboer CH, Ohl F, Groenendaal F, Heijnen CJ, van Bel F, Kavelaars A*
NF-κB inhibition improves long-term motor and cognitive outcome after neonatal hypoxia-ischemia in rats. *Neurobiology of Disease, (in press),* doi:10.1016/j.nbd.2010.01.016

***Van der Kooij MA**, Ohl F, Arndt SS, Kavelaars A, van Bel F, Heijnen CJ*
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Nijboer CH, **Van der Kooij MA, van Bel F, Ohl F, Heijnen CJ, Kavelaars A*
Inhibition of the JNK/AP-1 pathway reduces neuronal death and improves behavioral outcome after neonatal hypoxia-ischemia. *Brain, Behavior and Immunity, (in press),* doi:10.1016/j.bbi.2009.09.008

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Combination of deferoxamine and erythropoietin: Therapy for hypoxia-ischemia induced brain injury in the neonatal rat? *Neuroscience Letters, 2009, 451:2; 109-113.* http://dx.doi.org/10.1016/j.neulet.2008.12.013

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Animal models concerning the role of dopamine in attention-deficit hyperactivity disorder Review. *Neuroscience & Biobehavioral Reviews, 2007, 31:4; 597-618. http://dx.doi.org/10.1016/j.neubiorev.2006.12.002*

***** Included in this thesis

Curriculum Vitae

Michael van der Kooij werd geboren op 6 februari 1981 te Den Haag en groeide op in Wassenaar. Hij behaalde het VWO diploma in 1999 aan het St. Adelbert College te Wassenaar. In 1999 werd ook begonnen met de studie Biologie aan de Universiteit Leiden. Tijdens deze studie werden een tweetal wetenschappelijke stages gelopen. Allereerst deed hij onderzoek aan de afdeling Medische Farmacologie aan de Universiteit Leiden onder begeleiding van professor Dr. M.S. Oitzl en S. Dalm.

Hierna volgde nog een stage bij het biofarmaceutisch bedrijf Solvay Pharmaceuticals te Weesp onder begeleiding van Dr. J.C. Glennon en Dr. E.M. de Lange. Tenslotte heeft hij zijn master thesis geschreven onder supervisie van professor Dr. E.R. de Kloet.

Tijdens de studie Biologie heeft hij gekozen voor een medisch aanvullingspakket en is de doctoraalstudie Medische Biologie afgerond in augustus 2005.

In januari 2006 werd begonnen met een promotieonderzoek ontstaan uit een samenwerkingsproject tussen de afdelingen Neuro-Immunologie & Developmental Origins of Disease (NIDOD) (onder supervisie van professor Dr. C.J. Heijnen en Dr. A. Kavelaars) en de afdeling Neonatologie (onder supervisie van professor Dr. F. van Bel en Dr. F. Groenendaal) in het Wilhelmina Kinderziekenhuis (WKZ), onderdeel van het Universitair Medisch Centrum Utrecht. In de afgelopen 4 jaar heeft hij gedragsonderzoek gedaan naar de neurofunctionele afwijkingen die ontstaan in diermodellen voor neonatale hypoxisch-ischemische hersenschade en het beperken hiervan door middel van therapeutische interventies. De uitkomsten van deze studies zijn beschreven in dit onderzoek.

Aan het hersenonderzoek wordt een vervolg gegeven, zo is hij per half april 2010 als post-doctoraal onderzoeker aangesteld aan de afdeling Behavioral Genetics, onderdeel van EPFL (Ecole Polytechnique Fédérale de Lausanne) te Zwitserland, onder leiding van professor Dr. C. Sandi.