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Oxygen Resuscitation does not Ameliorate Neonatal Hypoxia/Ischemia-Induced Edema

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Oxygen Resuscitation does not Ameliorate Neonatal Hypoxia/Ischemia-Induced Edema

by

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To Franco, Mariana and Guillermo.

“As for the future, your task is not to foresee it, but to enable it.”

Antoine de Saint-Exupéry

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Neonatal hypoxia/ischemia (HI) is the most common cause of developmental neurological, cognitive and behavioral deficits in children. Although cerebral edema is a common outcome after HI, the mechanisms leading to the excessive fluid accumulation are poorly understood. Hyperoxia treatment after HI (HHI) is the recommended clinical therapy for newborn resuscitation. Our objective was to evaluate edema development after HI and whether HHI treatment affected HI-induced edema. We induced HI by a permanent ligation of the left carotid artery followed by a systemic exposure to hypoxia (8% O_2) in P7 rats; a cohort of these animals was immediately treated with hyperoxia (40% or 100% O_2). Dry weight analyses and T2-MRI showed cerebral edema 1, 3, 7 and 21 days after HI in the ipsilateral cortex, and 3, 7 and 21 days in the contralateral cortex. Using a blood-brain barrier (BBB) assay we showed that HI induces BBB permeability 3 and 7 days after HI, leading to vasogenic edema in both cortices. HHI treatment failed to

prevent BBB permeability and edema development. At the molecular level, we investigated the effect of HI on AQP4, the main water channel in the brain, which has been implicated in edema development after different neuropathological conditions. Our results showed that HI significantly increased AQP4 levels 3, 7 and 21 days after HI in the ipsilateral cortex, with no effect in the contralateral cortex. Furthermore, HHI treatment did not affect HI-induced changes in AQP4, consistent with the lack of effect of HHI on edema development. Given that developmental increases of AQP4 in the brain are accompanied by significant reduction in water content, we believe that HI-induced increase in AQP4 in the ipsilateral cortex is aimed at protecting the brain, and that the lack of increase in AQP4 levels in the contralateral cortex leads to the development of edema. In agreement with our hypothesis, we showed that HI induced impaired motor coordination 21 days after the insult and HHI did not ameliorate this behavioral outcome. We conclude that HHI treatment is effective as a resuscitating therapy, but does not ameliorate HI-induced cerebral edema and impaired motor coordination.

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CHAPTER I

INTRODUCTION

The evaluation of cerebral edema after neonatal hypoxia/ischemia (HI) requires an understanding of the pathological process underlying the neonatal HI insult, and its progression in the context of the developing brain and its dysfunctional outcomes. In addition, a better understanding of the effect of the current clinical therapy of hyperoxia after HI (HHI) on neonatal HI will aid us in evaluating its potentially beneficial role in HI infant resuscitation, or its potentially harmful neurodevelopmental role.

As a consequence of the fixed nature of the brain matter enclosed in the physical bone barrier of the skull, knowledge of water and ion homeostasis in the brain is likely to be important to our understanding of the detrimental effects of pathological cerebral edema and the importance of water clearance mechanisms in the developing brain.

I.A. NEONATAL HYPOXIA/ISCHEMIA

I.A.1. Epidemiology of neonatal HI

Perinatal hypoxic-ischemic encephalopathy (HIE) is the generalized pathological outcome after neonatal HI, a major cause of neonatal morbidity and mortality, and the most common cause of developmental neurological deficits such as cerebral palsy, and delayed cognitive and behavioral deficits such as mental retardation (Boichot *et al*., 2006; Zanelli *et al*., 2008). In the United States, perinatal HIE occurs in 0.2-0.4% of term infants, and up to 60% in preterm (< 37 weeks) or very low birth weight (<1500g) infants

(Vannucci *et al*., 1999; Zanelli *et al*., 2008). Preterm birth has increased to 12-13% of US births (Allen, 2008; Centers for Disease Control and Prevention, 2009) becoming a major US health problem. In developing countries, perinatal asphyxia reports range from 4 to 9 million cases per year, with up to 1 million of HIE patients dying (Moss *et al*., 2002; Zanelli *et al*., 2008). In one particular study in China over a period of 5 years, HIE cases accounted for 8.25% of in-patients (Chang *et al*., 2007).

In severe cases of HIE, the mortality rate ranges from 50-75%, with most deaths occurring in the first week and due to multiple organ failure (Zanelli *et al*., 2008). Of the patients that survive severe HIE, up to 80% will develop serious complications, 10-20% develop moderate complications and up to 10% are healthy. In cases of moderate HIE, 30-50% of infants will develop long-term complications and 10-20% develop minor complications (Zanelli *et al*., 2008). Notably, it has been reported that in the absence of early signs of brain injury after HIE, long-term functional impairments may still be present (Zanelli *et al*., 2008).

Consequently, it is crucial to understand the pathogenesis of neonatal HIE in order to develop novel and appropriate therapies to prevent HIE-associated early and late neurological detrimental outcomes.

I.A.2. Causes and pathology of neonatal HI

Systemic hypoxemia and reduced cerebral blood flow (CBF; due to loss of CBF autoregulation or a disruption in the normal CBF) are the primary causes of HIE (Zanelli *et al*., 2008). Hypoxemia *in utero* could result from placental insufficiency, placental detachment, placental rupture or umbilical strangulation; and hypoxemia postnatally could result from respiratory insufficiency, cardiac insufficiency or both due to

complications during delivery, prematurity or low birth weight at birth (Calvert and Zhang, 2005; Sotero and Shaw, 2006; Zanelli *et al*., 2008).

The CBF of a premature infant (~28 weeks of gestation) is approximately 20% of the adult value and the CBF of a term newborn is ~40% of the adult value (Volpe, 2008). CBF is maintained by blood pressure (BP) with the approximate regulatory range being from 25 to 50 mm Hg in arterial BP, this range with actual upper and lower limits vary according to gestational or postnatal age (Blankenberg *et al*., 1997; Volpe, 2008). Due to such a small range compared to an adult, the preterm and neonatal brains are highly susceptible to changes in systemic BP (Zanelli *et al*., 2008). Furthermore, perinatal hypoxia has been shown to impair cerebrovascular autoregulation in neonates (Blankenberg *et al*., 1997; Sotero and Shaw, 2006; Volpe, 2008) allowing for the CBF to be susceptible to changes in systemic BP. In addition, immature cerebral arteries are susceptible to rapid changes in BP and CBF; changes that could induce rupture of capillaries and lead to germinal matrix hemorrhage and white matter injury, such as periventricular leukomalacia (Blankenberg *et al*., 1997; Sotero and Shaw, 2006).

The initial phase following neonatal HI is accompanied by cellular energy failure, membrane depolarization, local release of excitatory neurotransmitters such as glutamate and aspartate, edema, increase of intracellular calcium, lipid peroxidation, the production of oxygen-free radicals and decreased blood flow, all of which can lead to cell death (Calvert and Zhang, 2005; Zanelli *et al*., 2008). Specifically, cell death due to excitotoxicity is triggered in part by an impaired uptake of glutamate by glia (Ferriero, 2004). This initial phase is immediately followed by further neuronal damage, apoptosis and cerebral edema (Calvert and Zhang, 2005; Zanelli *et al*., 2008).

I.A.3. Brain development and the detrimental outcomes of neonatal HI

The human brain at birth is not a fully developed organ. The brain keeps developing for at least 20 years (Blows, 2003) and into adulthood (Casey *et al*., 2000).

At birth, the human brain is in a remarkably unfinished state. By the age of 2, the brain is 80% of the adult brain weight (Casey *et al*., 2000); and from birth to 10-12 years of age, the brain increases up to 5 times in volume and myelination continues until 35 years of age (Casey *et al*., 2000; Blows, 2003). As part of brain development, there is a significant decrease in postnatal brain water content (Yannet and Darrow, 1938; Bondareff and Pysh, 1968; Lovblad *et al*., 2003; Rivkin *et al*., 2004; Sulyok, 2006) and decrease in extracellular volume (Pixley and de Vellis, 1984; Lehmenkuller *et al*., 1993). The decrease in water content and extracellular space allows for brain growth and development. For this, the postnatal developing brain undergoes a period of active synapse formation, axonal growth, radial glia disappearance and astrocytic maturation, oligodendrocyte proliferation and myelination, and early postnatal capillary proliferation; with most of these events taking place in the first year of age (Pixley and de Vellis, 1984; Robertson *et al*., 1985; Levine, 1989; Baron et al., 1995; Hunter *et al*., 1995; Goldman *et al*., 1997; de Graaf-Peters and Hadders-Algra, 2006; Gerstner *et al*., 2006; Nishida and Okabe, 2007; Weidenfeller et al., 2007).

Due to the active postnatal development of the brain, perinatal HIE leads to detrimental outcomes that correlate with the severity of the hypoxic-ischemic insult. HIE outcomes include mental retardation, seizures and cerebral palsy (Marin-Padilla, 1999; Boichot *et al*., 2006). In addition, it has been reported that preterm infants and low birth weight infants have higher rates of cerebral palsy, intellectual disability and sensory impairment compared to term infants (Allen, 2008).

Cerebral palsy encompasses a group of non-progressive motor impairment syndromes that arise from lesions or anomalies during development and persists into adulthood (Kuban and Levinson, 1994; Annibale and Hill, 2008). Periventricular leukomalacia (PVL) is a precursor of cerebral palsy and is characterized by multifocal necrotic lesions in the cerebral white matter around the lateral ventricles, were the cortico-spinal tracts essential to muscle movement can be located (Calvert and Zhang, 2005; Annibale and Hill, 2008). Premature infants are highly susceptible to PVL because HI affects oligodendrocytes in a maturation-dependent manner, with immature oligodendrocytes more prone to cell death (Calvert and Zhang, 2005; Back *et al*., 2007). Furthermore, Robertson *et al*. (1989) showed that term infants that suffered birth asphyxia and developed severe or moderate HIE, had significant physical impairment.

Both in full term and premature infants, HIE is the most common cause of neonatal seizures (Ronen *et al*., 1999; Sheth *et al*., 1999; Volpe, 2008). In addition, seizures in the neonatal period are associated with a detrimental outcome (Sreenan *et al*., 2000). However, it has been shown experimentally that using the non-competitive NMDA antagonist MK-801 seizures associated with perinatal asphyxia can be prevented (Gunn *et al*., 1988).

With regards to intellectual disability and mental retardation, it has been reported that birth hypoxia leads to impaired cognitive and language function (Hopkins-Golightly *et al*., 2003). Specifically, children that suffered birth asphyxia at term resulting in moderate or severe HIE, had lower reading, spelling and arithmetic grade levels, receptive vocabulary scores and visual-motor integration compared to mild HIE or control groups (Robertson *et al*., 1989). Furthermore, Smith *et al*. (2008) showed that a long-term follow-up of children with mild HIE at birth have poor performance in school,

poor social skills and impaired learning abilities. Thus, mild, moderate and severe perinatal HIE lead to long-term detrimental neurological outcomes.

I.B. HYPEROXIA TREATMENT

The current clinical treatment for neonatal HI is the use of supraphysiological concentrations of oxygen (hyperoxia, HHI) for resuscitation of infants that have been asphyxiated and require assisted ventilation (Davis *et al*., 2004; Klinger *et al*., 2005; Deulofeut *et al*., 2006; Wang *et al*., 2008; Koch *et al*., 2008).

However, recent reports have shown early increases of biochemical markers of oxidative stress after HHI (Klinger *et al*., 2005). It has been shown in animal models that oxygen supplementation after asphyxia increases the formation of free oxygen radicals and decreases cerebral perfusion (Davis *et al*., 2004; Ahn *et al*., 2008; Koch *et al*., 2008).

The long-term effects of extreme hyperoxia (100% O_2) after neonatal HI remain controversial. Although, there is one experimental report that shows 100% oxygen treatment immediately after HI attenuates energy deficits by increasing ATP and phosphocreatine levels, and leads to a reduction in brain injury (Calvert and Zhang, 2007), there are several other studies that suggest that HHI maybe harmful. Hyperoxia induction of oxidative stress (Deulofeut *et al*., 2006) will particularly affect the immature brain that has high concentrations of unsaturated fatty acids, availability of redox-active iron and lacks appropriate anti-oxidant defense mechanisms (Ferriero, 2004; Calvert and Zhang, 2005; Deulofeut *et al*., 2006) and hence is vulnerable to oxidative damage. It has further been shown that oxygen therapy after HI can affect neurological outcome both in humans and in animal models (Taglialatela *et al*., 1998; Klinger *et al*., 2005; Gerstner *et al*., 2008; Koch *et al*., 2008; Shimabuku *et al*., 2005).

Experimental studies have shown that hyperoxia alone can induce cell death and oxidative stress (Taglialatela *et al*., 1998; Hoehn *et al*., 2003; Hu *et al*., 2003; Gerstner *et al*., 2008; Yis *et al*., 2008) and in particular hyperoxia after neonatal HI has been shown to worsen histological injury, cell death, induction of oxidative stress and disruption in myelination (Graulich *et al*., 2002; Mukeby *et al*., 2004; Shimabuku *et al*., 2005; Gill *et al*., 2008; Koch *et al*., 2008).

Recent reports of systematic reviews and meta-analyses have suggested that resuscitation with 100% oxygen should be prevented if possible, and resuscitation with normoxia should be preferred (Davis *et al*., 2004; Deulofeut *et al*., 2006; Rabi *et al*., 2007). However, there is still controversy in the accuracy of the difference between normoxia and 100% oxygen resuscitation based on the size of the population studied (Shah, 2005); also the reported efficacy of normoxia for resuscitation in preterm neonates (Klinger *et al*., 2005; Wang *et al*., 2008) would suggest that controlled normoxia ventilation with some oxygen supplementation during the first hours of life might be optimal. Furthermore, there are no reports on the specific effects of different oxygen doses of hyperoxia treatment after neonatal HI.

I.C. BRAIN WATER HOMEOSTASIS

Regulation of ion and water transport and excess fluid elimination are especially critical in the central nervous system (CNS) because the brain is enclosed in a rigid skull and the spinal cord is also confined to a rigid spinal column. This prevents drastic compensatory changes to brain and spinal cord swelling. Progressive swelling of the brain will lead to elevated intracranial pressure, a decrease in the cerebral blood flow, and ultimately to cerebral herniation and death (Papadopoulos and Verkman, 2007; Zador *et al*., 2009).

In the brain, water moves among different compartments (cerebrospinal fluid (CSF), blood, intracellular brain parenchyma and interstitial brain parenchyma) in response to osmotic gradients and differences in hydrostatic pressure (Papadopoulos and Verkman, 2007). Water is transported down its osmotic gradient through different mechanisms: (i) by diffusion through the cell membrane (a relatively slow process), (ii) cotransported through ion channels and transporters, (iii) and to the highest rate through water channels called aquaporins (Agre *et al*., 2002). Furthermore, there are three main routes for brain fluid elimination: (i) through the glia limitans externa into the subarachnoid space, (ii) through the glia limitans interna and ependyma into the CSF-filled ventricles, and (iii) through the blood-brain barrier (BBB) into the bloodstream. (Papadopoulos and Verkman, 2007).

Water homeostasis has been described by the Starling relationship that explains the role of hydrostatic and oncotic forces in the movement of fluid across capillary membranes. It is described as follows:

$$\boldsymbol{J_{cap} = L_{cap}\,[(\text{Hydrostatic pressure difference between plasma and tissue}) - \sigma_{protein}\,(\text{Osmotic pressure difference}) - \sigma_{salt}\,(\text{Osmotic pressure difference})]}$$

Where J_{cap} is the flow of water and salt expressed in cm^3/sec; L_{cap} is the capillary hydraulic conductivity $cm^3/sec/g$, and σ is the osmotic reflection coefficient. And in the particular case of the brain $\sigma_{protein}=1$ and $\sigma_{salt}=1$ are because the blood vessels in the brain are impermeable to salts and proteins.

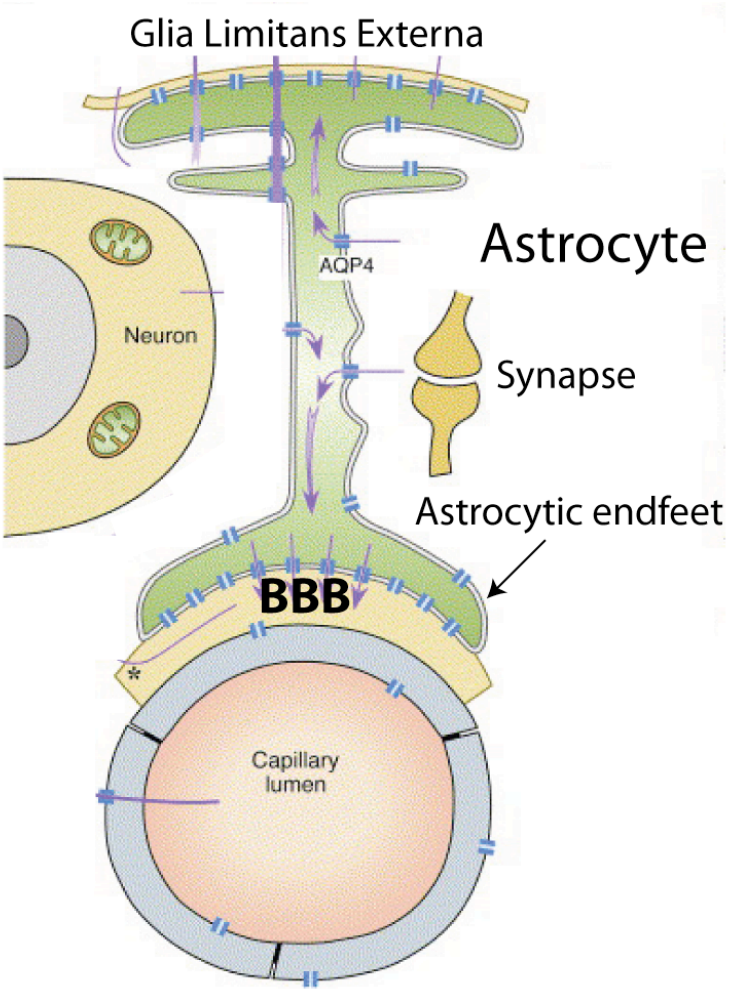
Specifically, it describes how the hydrophilic substances and ions flow with water in and out of the tissue, with the retained protein in the vascular space inducing an

osmotic driving force that will drive fluid from the tissue into the blood. However, the Starling relationship varies in the brain due to the particular characteristics of the BBB, were the tight junctions of the BBB prevent the passive passage of hydrophilic substances such as Na^+ and K^+ from the blood to the brain parenchyma. These ions and hydrophilic substances in the capillaries will add an extra variable in the movement of fluid in the brain (Kimelberg, 2004) as depicted in the previous equation.

Under normal circumstances, and following the Starling relationship from the equation, the hydrostatic pressure in plasma is higher than the intracranial pressure (ICP, equivalent to the hydrostatic pressure in brain tissue), that together with the high levels of albumin in the CSF to plasma (7000 mg/100mL compared to 20 mg/100mL, Kimelberg, 2004) and mild changes in ion concentrations in the extracellular space, will allow flow of fluid from the brain parenchyma to the blood, thus, clearing normal water produced as part of glucose metabolism. However, when the BBB gets disrupted and there is a leakage of protein to the brain parenchyma, the ratio of protein from CSF to plasma decreases, preventing normal clearance of water that will lead to edema (Kimelberg, 2004).

The key cells in the brain responsible for the maintenance of water and ion homeostasis are the astrocytes. Astrocytes are the most numerous glial cell type in the brain, and account for ~1/3 of the brain mass (Simard and Nedergaard, 2004). Astrocytic endfeet and processes are in close contact with endothelial cells (forming the BBB) and ependymal cells (lining the ventricles), in the glia limitans externa, and in peri-synaptic areas (Fig. 1), suggesting a role for astrocytes in the maintenance of the BBB and regulation of water and ion homeostasis. Furthermore, astrocytes are capable of exporting ions and organic osmolytes in response to hypo-osmotic stress as part of their regulatory volume decrease capacity (Kimelberg, 2004). Astrocytes have also been shown to play a

key role in neurotransmitter metabolism and clearance, as well as energy support for neurons (Simard and Nedergaard, 2004). For these roles, astrocytes express glutamate transporters (such as GLT1 and GLAST1), ion cotransporters and exchangers (such as the Cl^-/HCO_3^- exchanger and H^+/Na^+ antiporter), glucose transporters (such as GLUT-1) and high levels of aquaporins (Kimelberg, 2004; Simard and Nedergaard, 2004).



Modified from Amiry-Moghaddam *et al.*, Neuroscience, 2004, 129 (4): 999.

Figure 1. Due to its localization in the brain, astrocytes play a crucial role in the regulation of water and ion homeostasis, and maintenance of the blood-brain barrier.

I.C.1. Aquaporins

Water is the major component of all the cells. Approximately 70% of the mass of most living organisms is water; thus, fluid movement and balance within different compartments are needed for physiological processes (Kozono *et al*., 2002). Aquaporins form water channels that increase the membrane permeability to water and are responsible for the high rate water transport in all tissues. Due to their structure have great selectivity for water and not for protons (Agre *et al*., 2002; Kozono *et al*., 2002).

Most AQPs are homo-tetramers, where each monomer acts as a water channel. Each monomer of ~28kDa is constituted by six transmembrane helices with both carboxy- (C-) and amino- (N-) terminal domains located intracellularly (Fig. 2A; Agre *et al*., 1993; Jung *et al*., 1994; Preston *et al*., 1994; Hiroaki *et al*., 2006). The center of the pore has the narrowest diameter at 2.8 Å, big enough to allow entry to a single water molecule and allowing specific selectivity for water transport (Murata *et al*., 2000; Agre, 2006). There is an arginine residue in AQPs whose positive charge will repel protons. Furthermore, at the center of the pore, the isolated water molecule will transiently form hydrogen bonds with two highly conserved asparagines so as to move through the pore without resistance (Agre, 2006). Water molecules traverse through the pore of the channel in single file and at a fast rate due to the highly conserved hydrophobic residues lining the pathway for water transport (Murata *et al*., 2000; Agre *et al*., 2002).

Peter Agre and his group in the early 1990s identified, evaluated and described the first aquaporin later denominated as AQP1. Since then, 13 mammalian aquaporins have been reported, belonging to two classes: (i) aquaporins that facilitate the transport of water and (ii) aquaporins that are permeable to water and glycerol called aquaglyceroporins (Agre, 2006). Aquaglyceroporins have a glycine residue instead of histidine at the position 180 that allows for the pore to be 1 Å wider, and hence allow the

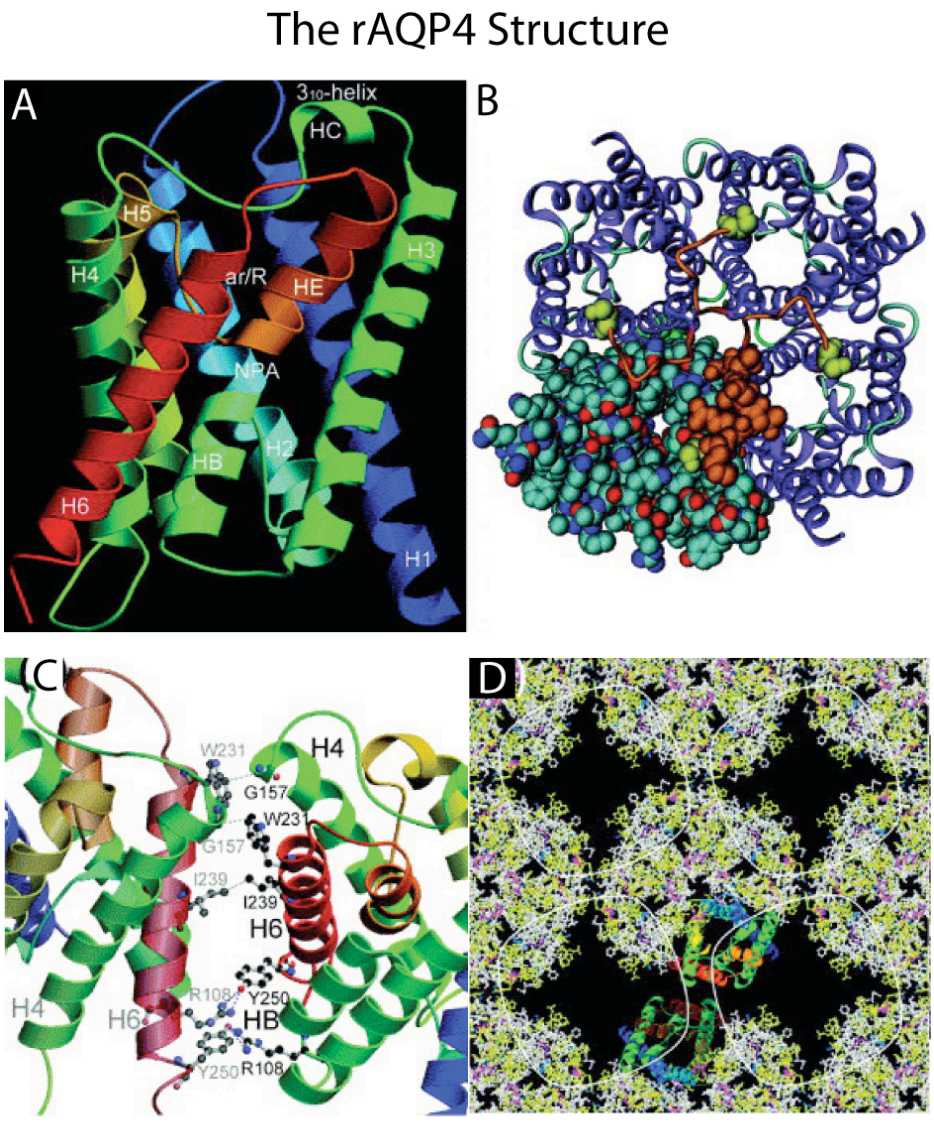
passage of glycerol (Kozono *et al*., 2002). In some cases aquaporins transport other small solutes, such as ammonia and urea across the membrane (Carbrey *et al*., 2003; Holm *et al*., 2004; Saparov *et al*., 2007).

I.C.2. Aquaporins in the brain

Brain water physiology and homeostasis is finely tuned in part by the expression of aquaporins. Aquaporin-1 (AQP1), aquaporin-4 (AQP4) and aquaporin-9 (AQP9) are expressed in the brain in different cell types and have been shown to have different functions. AQP4 is the most abundant aquaporin expressed in the brain (Neely *et al*., 1999; King *et al*., 2004) and will be described more into detail in the following section.

AQP1 is abundantly expressed in the apical membrane of the choroid plexus where it plays an important role in the formation of the CSF (Oshio *et al*., 2005). In pathology, AQP1 has been shown to play a role in the formation of CSF-filled cysts in brain tumors (Longatti *et al*., 2006); has been suggested to play a possible role in the migration of malignant astrocytes (McCoy and Sontheimer, 2007) and recently, it has been suggested to have a role in the migration of activated astrocytes and neuronal axonal elongation after spinal cord injury (SCI; Nesic *et al*., 2008). In addition, AQP1 has been shown to play a crucial role in non-pathological angiogenesis and migration of aortic endothelial cells (Saadoun *et al*., 2005).

AQP9 is an aquaglyceroporin permeable to water, glycerol, lactate and urea (Badaut *et al*., 2004; Amiry-Moghaddam *et al*., 2005). In the brain, AQP9 is expressed in astrocytes (Badaut *et al*., 2001), tanycytes (Nicchia *et al*., 2001), in endothelial cells of pial vessels (Badaut *et al*., 2001), in the mitochondria of astrocytes and midbrain dopaminergic neurons (Amiry-Moghaddam *et al*., 2005) and in catecholaminergic neurons (Badaut *et al*., 2004). Interestingly, AQP9 has been shown to increase in three



Modified from Hiroaki *et al.*, Journal of Molecular Biology, 2006, 355 (4): 628.

Figure 2. The structure of the rAQP4.

(A) Ribbon diagram of AQP4 with the 6 transmembrane helices (H1-H6). (B) AQP4 tetramer showing the tetramer-stabilizing cytoplasmic loop D (orange) and Ser180 (yellow), a known phosphorylation site, One AQP4 monomer is shown in space-filling representation. (C) Stereo view of the interactions between adjacent AQP4 tetramers in a crystalline array (it involve residues Arg108, Gly157, Trp231, Ile239, and Tyr250). (D) Crystalline AQP4 in ball-and-stick representation of AQP4 tetramers with one interacting AQP4 pair shown in ribbon representation. White outlines indicate tetramers in the adjoining layer.

scenarios: in the brain after focal ischemia (Badaut *et al*., 2001), in catecholaminergic neurons of diabetic rats (Badaut *et al*., 2008), and in the brain in response to a hyperosmotic stress with mannitol treatment (Arima *et al*., 2003). These results suggest that AQP9 could be involved in energy metabolism (Tait *et al*., 2008) and could have a role in the therapy of brain edema (Arima *et al*., 2003).

I.C.3. Aquaporin 4 (AQP4)

AQP4 is the predominant aquaporin expressed in the brain (Neely *et al*., 1999; King *et al*., 2004). It is abundantly expressed in white and grey matter astrocytes in the brain and spinal cord as well as in ependymal cells lining the cerebral ventricles (Nielsen *et al*., 1997) and endothelial cells (Amiry-Moghaddam *et al*., 2004b). However, the expression of AQP4 in brain endothelial cells has been reported to be scarce, and for this reason its expression and further function in these cells remains controversial. Specifically, AQP4 is expressed in astrocytic endfeet in direct contact with capillaries, neuropil and glia limitans (Fig 3; Rash *et al*., 1998; Amiry-Moghaddam *et al*., 2003; Rash *et al*., 2004). Due to its localization in the brain, AQP4 has a key role in the control of water flux in and out of the brain parenchyma (Fig. 3; Papadopolous and Verkman, 2007).

AQP4 expression increases with development. In the human fetus, AQP4 protein levels increase from 14 to 40 gestational weeks (Gomori *et al*., 2006). In the rat brain and cerebellum, there is a significant increase in AQP4 protein levels postnatally (Wen *et al*., 1999). This developmental increase in AQP4 has suggested a role in brain development and postnatal brain water decrease.

The *aqp4* gene encodes two mRNAs leading to two major isoforms, M1 (34kDa) and M23 (32kDa; Neely *et al*., 1999). These two isoforms have different abilities to form

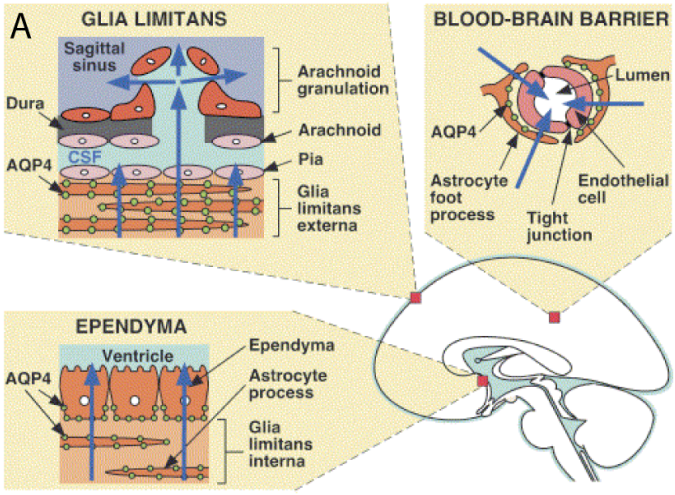
square arrays *in vitro* (Furman *et al*., 2003). Due to its arrangement into higher-order structures, AQP4 has been shown to form and be the main component of squared arrays in the plasma membrane (Rash *et al*., 1998; Rash *et al*., 2004; Verbavatz *et al*., 1997).

Using Blue-Native PAGE and immunoblotting, it has been recently shown that these squared arrays are formed by higher-order AQP4 complexes, with M23 playing a dominant role in its formation (Sorbo *et al*., 2008). Recent reports have suggested the presence of more AQP4 isoforms. Sorbo *et al*. (2008) showed the presence of a third isoform of a molecular weight ~36kDa in rat brain denominated Mz. Furthermore, Moe *et al*. (2008) have suggested that AQP4 has 6 cDNA isoforms formed by alternative splicing. Two of these correspond to M1 and M23, and AQP4e corresponds to the Mz isoform. The three of them have been shown to form tetramers. However, the other 3 isoforms described have been suggested to remain intracellularly (Moe *et al*., 2008). The role of the different AQP4 isoforms and their relative proportions in the brain before and after injury remains to be defined.

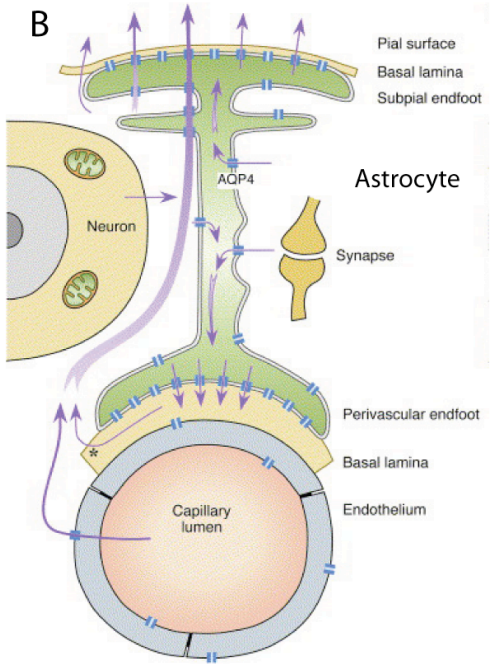
I.C.3.a. Regulation of expression of AQP4

There are several reports on the regulation of expression of AQP4 in the brain. Activation of the PKC pathway has been shown to decrease AQP4 mRNA and protein levels (Nakahama *et al*., 1999; Yamamoto *et al*., 2001). Moreover, IL-1β, through a NF-κB pathway, has been shown to induce the expression of AQP4 (Ito *et al*., 2006). The vascular endothelial growth factor (VEGF), agrin (a heparan sulfate proteoglycan of the extracellular matrix), lactic acid and testosterone have been shown to increase AQP4 protein expression (Gu *et al*., 2003; Noell *et al*., 2007; Morishima *et al*., 2008; Rite *et al*., 2008). Alternatively, dopamine has been shown to decrease AQP4 protein levels and its water permeability activity (Zelenina *et al*., 2002; Küppers *et al*., 2008).

AQP4 Localization in Astrocytes and in the Brain



Modified from Papadopoulos *et al.*, Neuroscience, 2004, 129 (4): 1011.



Modified from Amiry-Moghaddam *et al.*, Neuroscience, 2004, 129 (4): 999.

Figure 3. In the brain, AQP4 is mostly expressed in astrocytes and plays a key role in brain water homeostasis.

(A) Main routes of fluid elimination in the brain. (B) Astrocytes are the key cells responsible for water and ion homeostasis in the brain, as depicted by their location and close contacts with the BBB, neuropil and glia limitans.

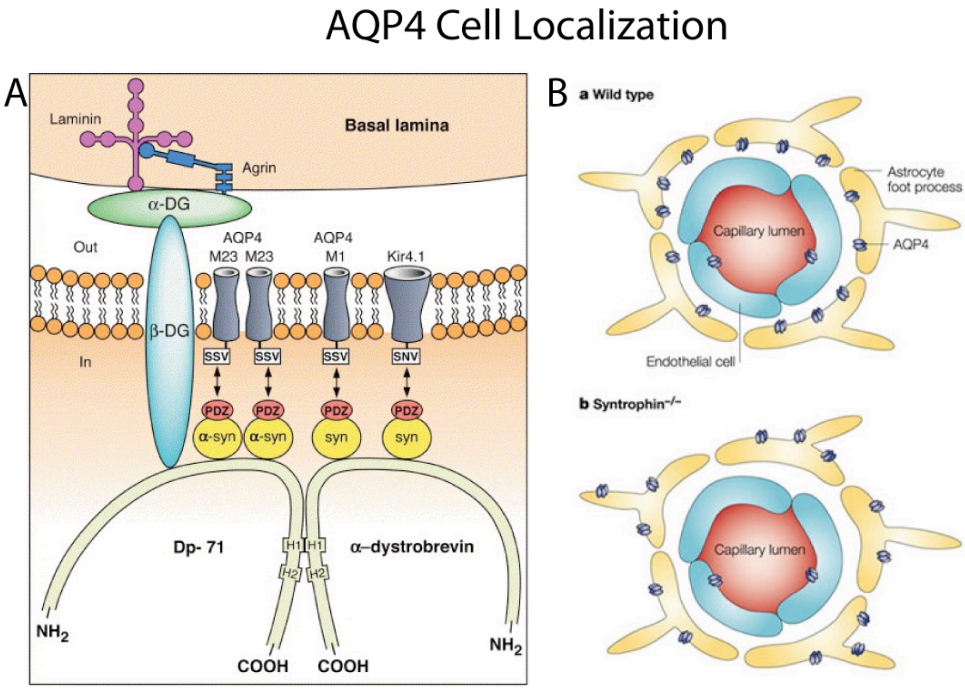
Sulphoraphane (an isothiocyanate abundantly found in broccoli) treatment after traumatic brain injury (TBI) has been shown to increase AQP4 protein levels (Zhao *et al*., 2005) and progesterone and dexamethasone treatment have been shown to regulate expression of AQP4 in a time-dependent manner and, more interestingly in a region-specific manner (Guo *et al*., 2006; Gu *et al*., 2007). Finally, it has been shown that vasopressin regulates water fluxes by activating AQP4 through PKC or PKA (Niermann *et al*., 2001; Gunnarson *et al*., 2004).

I.C.3.b. Regulation, Localization and Roles of AQP4

It has been shown that AQP4 is phosphorylated at Ser^{180} after PKC activity, and that this phosphorylation inhibits AQP4 water transport (Fig. 2B; Han *et al*., 1998; Zelenina *et al*., 2002). However, other reports have shown that glutamate induces an increase in astrocytic water permeability through a PKG pathway that induces AQP4 phosphorylation at Ser^{111} and not by increasing AQP4 protein levels (Gunnarson *et al*., 2008).

The function of AQP4 depends on the proper cellular localization to the cell membrane. Alpha syntrophin is a component of the dystrophin protein complex that encloses AQP4 to the cell membrane. Syntrophyn null mice studies have shown that AQP4 proper membrane localization in astrocytic endfeet in perivascular and subpial membranes depends on syntrophin expression (Fig. 4; Neely et al., 2001). In addition, studies in syntrophin null mice have also shown a reduced clearance of extracellular K^+ correlated with increased severity of seizures (Amiry-Moghaddam *et al*., 2003b). AQP4 and the inwardly rectifying potassium channel Kir4.1 colocalization (Nagelhus *et al*., 1999) suggests that K^+ buffering in astrocytes works in parallel with water fluxes, specifically after neuronal activity where K^+ and water are taken up by the astrocyte facing the neuropil (Amiry-Moghaddam *et al*., 2003b; Eid *et al*., 2005). Thus, these

results suggest that AQP4 plays a role in neuronal excitability (Binder *et al*., 2004; Binder *et al*., 2006). AQP4 has also been shown to play a key role in cell migration (Saddoun *et al*., 2006; Auguste *et al*., 2007), adhesion (Fig. 2C, 2D; Hiroaki *et al*., 2006) and proliferation (Küppers *et al*., 2008). Finally, it has been shown that AQP4 plays a key role in the development and maintenance of the BBB (Nico *et al*., 2001; Nicchia *et al*., 2004; Bragg *et al*., 2006; Zhou *et al*., 2008), as well as in the volume regulation of the extracellular space (Yao *et al*., 2008).



Modified from Amiry-Moghaddam *et al*., Neuroscience, 2004, 129 (4): 999.

Modified from King *et al*., Nature Reviews on Molecular Cell Biology, 2004, 5 (9): 687.

Figure 4. AQP4 localizes to the cell membrane in an α-syntrophin dependent manner.

(A) A diagram showing the presumed molecular basis for the anchorage of AQP4 to the cell membrane, through α-syntrophin and the dystrophin complex. (B) In syntrophin null mice, AQP4 not only does not locate to the astrocytic endfeet adjacent to the endothelial cells, but it is distributed in other sites of the astrocytes.

As a component of neuropathology, AQP4 has been associated with edema after spinal cord injury (Nesic *et al*., 2006), neuromyelitis optica (for a review, Argyriou and Makris, 2008; Nicchia *et al*., 2009), cancer (McCoy and Sontheimer, 2007), Alzheimer's disease (Perez *et al*., 2007), Creutzfeldt-Jakob disease (Rodriguez *et al*., 2006), amyotrophic lateral sclerosis (Nicaise *et al*., 2008) and hydrocephalia (Shen *et al*., 2006). Most importantly, AQP4 has been associated with brain edema (for a review, Papadopoulos and Verkman, 2007) after stroke (Zador *et al*., 2009), ischemia (Hirt *et al*., 2009), brain tumors (Papadopoulos *et al*., 2004), TBI (Zhao *et al*., 2005), subarachnoid hemorrhage (Badaut *et al*., 2003) and Herpes simplex virus encephalitis (Martinez Torres *et al*., 2007). In most cases, there is an increase in membrane AQP4 that has been suggested to aid in the clearance of pathology-induced water accumulation.

I.C.4. Role of AQP4 in cerebral edema

Cerebral edema is the abnormal accumulation of fluid in the brain parenchyma that is associated with an increase in brain volume (Klatzo, 1967; Kimelberg, 2004; Papadopoulos and Verkman, 2007). Cerebral edema is a serious complication after stroke, head injury, brain absess and tumor, and an important contributor to brain-injury associated morbidity and mortality (Marmarou *et al*., 1994; Graham *et al*., 1995; Papadopolous *et al*., 2002). Cerebral edema results in secondary tissue damage due to its role in compression of adjacent tissue and induction of ischemic cell death.

Brain edema has been classified by Klatzo (1967) as being of two types: cytotoxic and vasogenic. Cytotoxic edema consists of water/fluid accumulated intracellularly without a breach of the blood-brain barrier. Vasogenic edema develops due to breakdown of the BBB, allowing plasma fluid to enter the brain parenchyma (Papadopoulos *et al*.,

2002). Recently, Nase *et al*. (2008) showed *in vivo* and at the cellular level that in the early phase of cerebral edema water enters astrocytes with their volume varying depending on their proximity to blood vessels. These results further support the hypothesis that astrocytic endfeet are a major route of water flux after the development of brain edema (Amiry-Moghaddam and Ottersen, 2003c). The non-invasive technique of magnetic resonance imaging (MRI) is commonly used to determine *in vivo* brain water accumulation. Two particular techniques are used for this purpose: (i) diffusion-weighted imaging (DWI) and its quantitative measure, the apparent diffusion coefficient (ADC), which can identify the development of cytotoxic brain edema depicted as a low ADC value; (ii) T2-weighted imaging (T2WI, measures T2 relaxation time) that usually suggest vasogenic edema depicted as a hyperintensity, are subtler in the newborn because the brain is less myelinated and the water content is greater than in the older child (Triulzi *et al*., 2006; Ashwal *et al*., 2007; Obenaus *et al*., 2008).

Studies on AQP4-null mice strongly suggest that AQP4 plays a key role in the clearance of water following vasogenic edema because lack of AQP4 worsens the outcome generated by a vasogenic edema insult such as brain tumors. Furthermore, AQP4 plays a key role in the formation of cytotoxic edema, meaning that lack of AQP4 does not induce cytotoxic edema (Manley *et al*., 2000). However, the study of AQP4 and its role in different brain edema conditions is compromised by the fact that the results observed could be representative of a compensatory mechanism (specifically due to the upregulation of AQP4 postnatally). Furthermore, the development of safe molecular inhibitors of AQP4 and an AQP4 inducible KO mice, will allow a more accurate evaluation of the role of AQP4 in the development and clearance of edema.

There are multiple reports of the role of AQP4 in the pathogenesis of cerebral edema. Two reports in an animal model of adult TBI have suggested that an increase in

AQP4 helps clear out edema (Zhao et al., 2005; Guo *et al*., 2006). In addition, early upregulation of AQP4 has been shown to have a protective role in the development of edema after brain ischemia (Hirt *et al*., 2009). Contrary, it has been shown that in a model of intracerebral hemorrhage, there is an increase in AQP4 protein levels in the perihematomal areas that correlates with an increase in water content (Gu *et al*., 2007). However, due to the fact that the neonatal brain continues to develop postnatally and that AQP4 levels increase with development as well, the response of the neonatal brain to an edema-induced insult will probably differ from the adult response.

I.D. EDEMA AFTER NEONATAL HI

Up to 86% of term neonates that have severe perinatal asphyxia, develop brain edema (Boichot *et al*., 2006); and up to 80% of term HI patients diagnosed with brain edema by MRI, have poor neurological outcomes (Sargent *et al*., 2004; Chang *et al*., 2007). The development of cerebral edema is believed to start from 6 to 24 hours after neonatal HI, increase over the next 24 to 48 hours and then begin to be resolved (Zanelli *et al*., 2008). There is one particular case where a term infant suffered a cardiorespiratory arrest and 6 hours after the insult there was edema development in the brain. However, 32 hours after the HI insult, the edema was not resolved and 40 hours later the infant died (Soul *et al*., 2001). This rare case, where the exact time of the HI insult was determined and shortly after the infant was evaluated by MRI, highlights the fast development of edema, and that its persistence could lead to death.

Early after an ischemic insult, cytotoxic edema develops followed by the breach of the BBB and the development of vasogenic edema both in humans and in animal models (Mujsce *et al*., 1990; Rabinstein, 2006; Ashwal *et al*., 2007; Badaut *et al*., 2007;

Chang *et al*., 2007; Zador *et al*., 2009). The initial phase following neonatal HI is accompanied by energy failure that leads to impairment of ATP synthesis and impaired Na^+/K^+-ATPase function causing an increase in intracellular sodium. The ischemic-induced energy failure leads to anaerobic glycolysis and causes an accumulation of lactate that together with the increase in intracellular sodium draws water into the cell inducing cytotoxic edema. This initial phase is followed by neuronal damage, apoptosis and vasogenic cerebral edema (Zanelli *et al*., 2008; Zador *et al*., 2009). Vasogenic edema could occur by different mechanisms such as physical disruption of endothelial cell tight junctions or increases in matrix-metalloproteases that induce proteolysis of BBB matrix proteins. These mechanisms will lead to leakage of fluid and serum proteins through the impaired BBB into the extracellular space (Chen and Swanson, 2003; Zador *et al*., 2009).

A correlation between AQP4 and cerebral edema after neonatal hypoxia has been shown in animal models of HI and stroke. Badaut *et al*. (2007) showed that after neonatal stroke there is a direct correlation between an increase in AQP4 protein levels in the border of the lesion and amelioration of edema one and three days after the insult. Meng *et al*. (2004) showed in a neonatal model of HI that a decrease in AQP4 protein levels one day after the insult correlated with an increase in water content. These results suggest a positive role for AQP4 in clearance of edema after neonatal asphyxia.

I.E. NEONATAL HI ANIMAL MODELS

Neonatal HI can be induced adequately in rodents. As reported by Dobbing and Sands (1979), the brain maturation of a post-natal day 7 (P7) rat pup and P10 mouse pup is equivalent to a third trimester human fetus. Specifically, P7 rat pups have a histological brain similar in development to that of a 32-34 week of gestation human fetus or a

newborn infant, including a completed cortical neuronal layering, involuted germinal matrix and developing myelination (Vannucci *et al*., 1997; Vannucci *et al*., 1999).

The two most clinically relevant models of neonatal HI and stroke are the Rice-Vannucci model (RVM, Rice *et al*., 1981) and the transient filament middle cerebral artery occlusion (tfMCAO, Ashwal *et al*., 1995) respectively (for a review, Ashwal *et al*., 2001). Both models use rodent pups at P7 for rats and P10 for mice; and in a recent report aimed at comparing the progression of a HIE lesion the authors supported the use of the RVM to study neonatal hypoxic-ischemic injury (Ashwal *et al*., 2007).

For the past two decades, the RVM has been the most commonly used model to study HI injury in neonates. The RVM of neonatal HI involves a unilateral permanent ligation of the common carotid artery followed by a systemic exposure to 8% O_2 (hypoxia) for 1.5 to 2 hours in a P7 rat pup (Vannucci *et al*., 1981). The lesion is largely restricted to the cerebral hemisphere ipsilateral to the common carotid artery occlusion and mostly observed in the cerebral parietal cortex as well as in the hippocampus, striatum (basal ganglia), subcortical and periventricular white matter (Vannucci *et al*., 1999). This model results in a progressive brain injury depicted by activation of an inflammatory response, cell death (both apoptotic and necrotic) and cerebral edema (for a review, Mujsce *et al*., 1990; Ashwal *et al*., 2001). Thus, the RVM of neonatal HI possesses essential components that occur in the clinical setting to allow investigation in the role of HI on the development of edema and the further evaluation of the clinically relevant hyperoxia resuscitation.

I.F. HYPOTHESIS AND SPECIFIC AIMS

The objective of this dissertation was to evaluate the role of the clinically relevant hyperoxia treatment after neonatal HI in the development of edema. This dissertation

further sought to evaluate the time course and long lasting effects of edema in the developing brain measuring motor coordination as an outcome of the pathology. We characterized the effects of HI and HHI not only in the site of injury (ipsilateral cortex) but also on the contralateral cortex. Investigation on the contralateral cortex is aimed at evaluating the chronic response to HI and its behavioral outcome. Specifically, the aims of this dissertation were to demonstrate:

Aim 1: HI induces fluid accumulation in the brain cortex and induces impaired motor coordination.

Aim 2: Hyperoxia treatment does not improve HI-induced edema and impaired motor coordination.

CHAPTER II

MATERIALS AND METHODS

II.A. MATERIALS

All chemicals were purchased from Fisher Scientific (Waltham, MA). Trichloroacetic acid (TCA), sodium fluorescein (NaFl), albumin from bovine serum (BSA), D-mannitol, Ponceau, ethylenediaminetetraacetic acid (EDTA) and DL-dithiothreitol (DTT) were purchased from Sigma-Aldrich (Saint Louis, MO). N, N-bisacrylamide, urea and western blot molecular weight markers were purchased from Bio-rad Laboratories (Hercules, CA). Phenylmethylsulfonyl fluoride (PMSF) and Complete protease inhibitor tablets were purchased from Roche Applied Sciences (Indianapolis, IN). Surgical instruments were purchased from Roboz Surgical Instrument Company (Gaithersburg, MD) and 5-0 P3 surgical silk was purchased from Ethicon (Somerville, NJ).

II.B. SURGICAL PROTOCOL FOR NEONATAL HI AND HI WITH HHI TREATMENT

II.B.1. Animal care

All animal procedures were approved by the UTMB Animal Care and Use Committee, and complied with the recommendations in the NIH Guide for the Care and Use of Laboratory Animals. Pregnant Wistar rat dams (Charles River Laboratories, Wilmington, MA) at gestational age E17 were housed upon arrival in 12h light-dark cycle with ad libitum access to food and water. In order to minimize intrinsic animal

variability and litter effects among multiple groups, multiple dams were ordered. The day of birth was designated postnatal day 0 (P0) and on P1 the litters were culled to 10 pups and randomly mixed amongst 2 dams (having a total of 20 pups per experiment). On day P7 all pups were removed from the dam, weighed, sexed and randomly assigned to a group: sham, HI, or HI with 100% oxygen hyperoxia treatment, or HI with 40% oxygen hyperoxia treatment.

II.B.2. Surgical procedure

At P7 HI was induced as previously described by Vannucci *et al*. (1981) and modified by Grafe *et al*. (1994). Briefly, in a 37°C E-Z anesthesia chamber (Euthanex Corporation, Palmer, PA) P7 rat pups were anesthetized with 5% isofluorane balanced with 100% O_2 blood-gas grade for 5 minutes, and maintained in 2% isofluorane for the rest of the procedure (approximately 10 minutes). The left carotid artery was isolated after a mid-neckline incision, and permanently ligated by electrocauterization at two points (one rostral and one caudal). In most of the cases (>95%), the double electrocauterization cut the left common carotid; and in the cases when it was not cut, the artery was cut between the two cauterization points using microspring scissors to prevent reperfusion across cauterization points. After cauterization, the incision site was sutured with 5-0 P3 surgical silk and cleaned, and the pup was kept at normal room air (20.8% O_2) at 37°C until the effects of the anesthesia dissipated. For the surgical sham pups, after being anesthetized they received a mid-neckline incision and were immediately sutured and cleaned. The sham pups were not subjected to common carotid artery isolation to prevent minor ischemia-reperfusion events. Immediately after the last pup recovered

from the anesthesia, all the pups were returned to their dams for a recovery period of 90 minutes.

After the recovery time, all pups were removed from the dams. The sham pups were placed in a normoxia chamber at 37°C and the operated animals were placed in a humidified hypoxia chamber (8% oxygen balanced with blood-gas grade nitrogen) also at 37°C were systemic hypoxia was induced for 90 minutes. After HI induction, the pups were returned to their dam until their assigned survival time point.

II.B.3. Hyperoxia treatment

A cohort of the injured pups received extreme (100% blood-gas grade O_2) or moderate (40% O_2 balance with blood-gas grade nitrogen) hyperoxia treatment at 37°C immediately after HI induction and for a period of 120 minutes. Sham and HI-induced pups were kept at 37°C normoxia for the length of time. After the hyperoxia treatment, the pups were returned to their dam until their assigned survival time point.

II.B.4. Hyperoxia only treatment

We further evaluated the effect of Hyperoxia only on neonatal rat pups. In order to be able to directly compare our results, we treated our three groups (sham, HHI 40% and HHI 100%) as the surgical sham pups previously described. Briefly, after being anesthetized they received a mid-neckline incision and were immediately sutured and cleaned. The sham pups were not subjected to common carotid artery isolation to prevent minor ischemia-reperfusion events. Immediately after the last pup recovered from the anesthesia, all the pups were returned to their dams for a recovery period of 90 minutes.

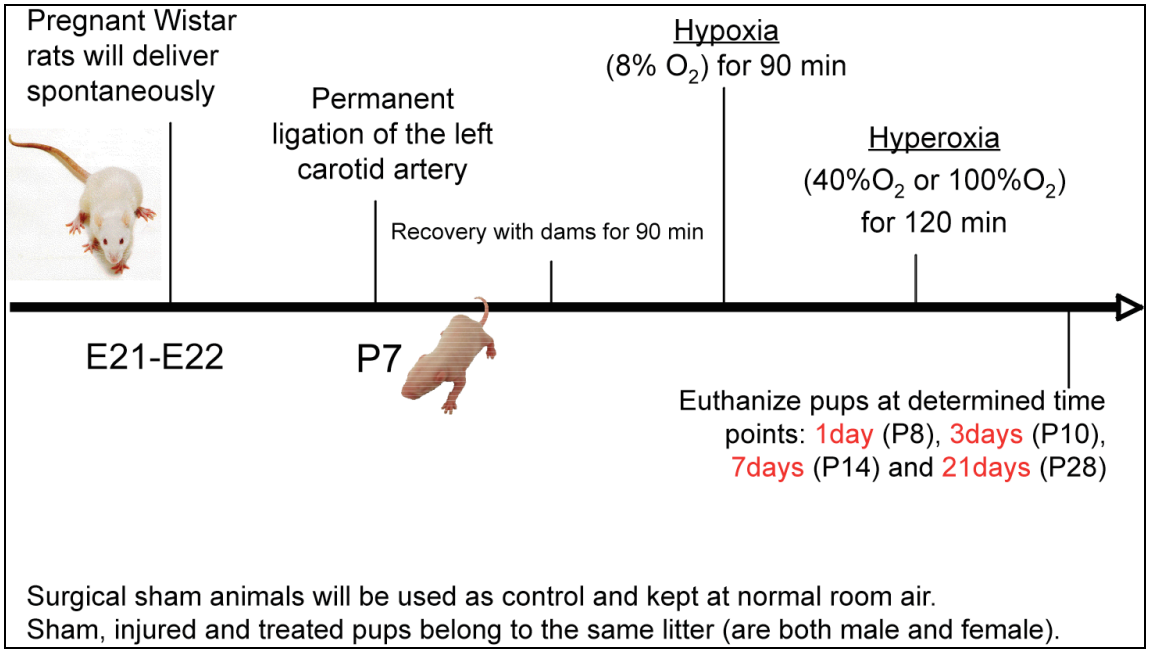


Figure 5. Schematic representation of the experimental design of the animal model for HI and Hyperoxia treatment.

After recovery, all pups were removed from the dams. The sham pups were placed in a normoxia chamber at 37°C and the Hyperoxia only treated animals were placed in a humidified hypoxia chamber with either 40% blood-gas grade O_2 or 100% blood-gas grade O_2 at 37°C for a period of 120 minutes. After the hyperoxia treatment, the pups were returned to their dam until their assigned survival time point. At the designated survival time point the pups were deeply anesthetized with isofluorane and then decapitated. The whole brains were removed, the left cortex was collected for dry weight analyses (described below) and the right cortex was collected for western blot analyses (see below), flash- frozen in liquid nitrogen and stored at -80°C until processed. Sham, HHI 40% and HHI 100% for the 1 and 7 day time points had an n=4; for the 3 day time point sham had an n=3, HHI 40% and 100% had an n=4.

II.B.5. Developmental studies in naïve Wistar rat pups

We also evaluated developmental changes in naïve rat pups at different post-natal ages: P1 (n=5), P7 (n=5), P10 (n=4), P15 (n=3), P30 (n=3) and P100 (n=4). At the designated survival time point the pups were deeply anesthetized with isofluorane and then decapitated. The whole brains were removed, the left cortex was collected for dry weight analyses (described below) and the right cortex was collected for western blot analyses (see below), flash- frozen in liquid nitrogen and stored at -80°C until processed.

II.C. DRY WEIGHT ANALYSES

Dry weight analyses were performed according to the protocol previously described by Nesic *et al*. (2006). At the designated survival time point the pups were deeply anesthetized with isofluorane and then decapitated. The whole brains were removed and both ipsilateral and contralateral cortices (from the anterior tuber cinereum to anterior of the occipital cortex – encompassing the parietal cortex) were collected from the pups at 1, 3, 7 and 21 days after the insult. Furthermore, naïve rats at different postnatal ages (P1, P7, P10, P15, P30 and P100) were deeply anesthetized with isofluorane and then decapitated. The whole brains were removed and the left cortex was collected from the pups. Immediately after, the cortices were weighed on aluminum foil (wet weight) and dried at 105°C for 24 hours. The dehydrated tissue was reweighed (dry weight) and the percent of water content was calculated using the following formula: water content (%) = [(wet weight – dry weight) / wet weight] x 100. Ipsilateral cortex: sham 1 day (n=9), HI 1 day (n=5), HHI 40% 1 day (n=5), HHI 100% 1 day (n=8); sham 3 day (n=28), HI 3 day (n=28), HHI 40% 3 day (n=14), HHI 100% 3 day (n=15); sham 7 day (n=10), HI 7 day (n=9), HHI 40% 7 day (n=6), HHI 100% 7 day (n=13); sham 21 day (n=10), HI 21 day (n=7), HHI 40% 21 day (n=7), HHI 100% 21 day (n=9).

Contralateral cortex: sham 1 day (n=13), HI 1 day (n=9), HHI 40% 1 day (n=5), HHI 100% 1 day (n=14); sham 3 day (n=16), HI 3 day (n=20), HHI 40% 3 day (n=13), HHI 100% 3 day (n=15); sham 7 day (n=10), HI 7 day (n=11), HHI 40% 7 day (n=6), HHI 100% 7 day (n=13); sham 21 day (n=10), HI 21 day (n=8), HHI 40% 21 day (n=7), HHI 100% 21 day (n=9).

II.D. BLOOD-BRAIN BARRIER PERMEABILITY

Evaluation of BBB permeability was performed using sodium fluorescein (NaFl, Sigma F6377). Three days and 7 days after the insult, and 4 hours before euthanasia, 10% of NaFl was administered to the rat pups intraperitoneally (i.p.) in a dose of 4ml/kg. Four hours after NaFl circulated in the pups, the pups were deeply anesthetized with isofluorane and perfused transcardially with cold saline solution to remove any excess dye from the blood vessels. Pups were then immediately decapitated and the whole brains removed; both ipsilateral and contralateral cortices were collected (from the anterior tuber cinereum to anterior of the occipital cortex – encompassing the parietal cortex), weighed in foil, flash-frozen in liquid nitrogen and stored at -80°C until processed. Naïve pups at postnatal age P10 (equivalent to 3 days post-HI) and P14 (equivalent to 7 days post-HI) were used as controls and divided into 3 groups: negative control (saline solution administered i.p. in a dose of 4ml/kg; n=3 for each time point), dye only (NaFl administered i.p. in a dose of 4ml/kg; n=3 for each time point), and positive control (NaFl administered i.p. in a dose of 4ml/kg together with 30uL/g of 1M D-mannitol in 0.34 M NaCl solution; n=3 for each time point).

The brain cortices were suspended in 500-600 uL of ice-cold hypotonic tris buffer (10mM Tris-HCl, 0.5M EDTA, 0.05M EGTA, 0.1M DTT) containing 1 Roche Complete

Protease Inhibitor Tablet and homogenized with a tight-fitting dounce homogenizer (Wheaton Science, Miliville, NJ). Cortices were disrupted by 30 strokes, the homogenates were vortexed for 10 seconds, and centrifuged at 2000g for 5 minutes at 4°C. The supernatant was diluted 1:20 in 20% TCA and incubated for 24 hours at 4°C. The samples were then centrifuged at 10,000g for 15 minutes at 4°C to precipitate the protein. The clear supernatants containing the NaFl dye were loaded in a 96-well microplate (Corning Incorporated, Corning, NY) together with a NaFl standard curve (ng/ml) and analyzed in a fluorometer (Polarstar Omega microplate reader, BMG Labtechologies, Durham, NC) with excitation at 485nm, and the emission measured at 520nm. The results were presented as ng of dye per gram of tissue. Ipsilateral cortex: sham 3 day (n=6), HI 3 day (n=7), HHI 100% 3 day (n=5); sham 7 day (n=4), HI 7 day (n=3), HHI 100% 7 day (n=4). Contralateral cortex: sham 3 day (n=6), HI 3 day (n=6), HHI 100% 3 day (n=6); sham 7 day (n=3), HI 7 day (n=4), HHI 100% 7 day (n=4).

II.E. MAGNETIC RESONANCE IMAGING

T2-weighted magnetic resonance images (MRI) were acquired with a 7 Tesla Bruker Biospec horizontal bore scanner (USR70/30; Bruker, Karlsruhe, Germany). A 72-mm-inner-diameter volume coil supplied by Bruker was used for radiofrequency (RF) transmission. For improved signal-to-noise ratio (SNR), a custom-designed 22 mm-outer-diameter circular surface coil was used for signal reception.

Rat pups 7 days after the insult were initially anesthetized with 5% isofluorane balanced with 100% O_2 blood-gas grade for 5 minutes, and maintained in 2% isofluorane through a custom-built tube-shaped mask for the MRI scans for less than 2 hours. Throughout the MRI scan, the animal's body temperature was kept at 36°C ± 1°C with a feedback-controlled warm air system (SA Instruments, Stony Brook, NY). The

respiration and surface body temperature were continuously monitored with a small-animal monitoring system (SA Instruments). The oxygen level and heart rate were monitored with an MRI-compatible pulse oxymeter (NONIN, Plymouth, MN). The survival rate of animals in these studies was about 95%.

Multi-slice, contiguous coronal images were acquired with dual-echo rapid acquisition and relaxation enhancement (RARE) sequence with the following parameters: TE1/TE2/TR 5 22 msec/66 msec/5,000 msec, where TE1 and TE2 are the echo times and TR is the repetition time; RARE factor = 4; slice thickness = 0.5 mm; square field of view (FOV) = 35 mm; acquisition matrix = 256 x 192, which was zero filled to 256 x 256. Each brain scan imaged between 20 and 40 slices, depending on the size of the brain. The images with the longer echo time (66 msec) were used for the evaluation of edema 7 days after neonatal HI: sham (n=3), HI (n=3) and HHI 100% (n=3).

II.F. PROTEIN EXTRACTION, ELECTROPHORESIS AND WESTERN BLOTTING

After neonatal HI and hyperoxia treatment, the pups were returned to their dam until their assigned survival time point. At the designated survival time point the pups were deeply anesthetized with isofluorane and then decapitated. The whole brains were removed and both ipsilateral and contralateral cortices were collected (from the anterior tuber cinereum to anterior of the occipital cortex – encompassing the parietal cortex) from the pups at 1, 3, 7 and 21 days after the insult, flash-frozen in liquid nitrogen and stored at -80°C until processed. Besides the cortices of shams, HI and HHI treated pups; naïve rats were deeply anesthetized with isofluorane and then decapitated at different gestation stages (P1, P7, P10, P15, P30 and P100). The whole brains were removed and the right cortex were collected from the pups, flash-frozen in liquid nitrogen and stored at -80°C until processed.

For the protein extraction, each isolated cortex was suspended in ice-cold homogenization buffer (10mM Tris Base, 300mM sucrose, 1mM EDTA, 1mM DTT, 0.5mM PMSF, pH 7.5) containing 1 Roche Complete Protease Inhibitor Tablet and homogenized with a tight-fitting dounce homogenizer (Wheaton Science, Miliville, NJ). Cortices were disrupted by 30 strokes, the homogenates were vortexed for 10 seconds, and centrifuged at 7,600 rpm for 5 minutes at 4°C. The supernatant was separated from the nuclear pellet and unbroken cells, and centrifuged at 13,000 rpm for 1hr at 4°C to pellet the crude plasma membrane. The resulting supernatant contains the cytosolic fraction, and the crude plasma membrane was resuspended in 100uL of ice-cold homogenization buffer. Protein concentrations were determined using the bicinchoninic acid (BCA) protein Assay (Pierce, Rockford, IL). Cytosolic fractions containing 50ug of protein were boiled for 10 min at 100°C with an appropriate volume of 6X sample buffer (350mM Tris–HCl, pH 6.8, 12.5% (v/v) 8M urea, 1% (v/v) 2-mercaptoethanol, 9.3% (w/v) DTT, 13% (w/v) SDS, 0.06% (w/v) Bromophenol Blue, 30% (v/v) glycerol). Cell membrane fractions containing 50ug of protein were prepared for electrophoresis separation with an appropriate volume of 6X super-denaturing sample buffer (350mM Tris–HCl, pH 6.8, 12.5% (v/v) 8M urea, 6% (v/v) 2-mercaptoethanol, 9.3% (w/v) DTT, 18% (w/v) SDS, 0.06% (w/v) Bromophenol Blue, 30% (v/v) glycerol) without being boiled. The samples were then placed on ice to cool before being loaded onto a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel for the cytoplasmic fractions, and onto a 12% SDS-polyacrylamide gel containing 24% (w/v) of urea. The samples were separated at 150V for 4hrs. Proteins were transferred from the gels onto a PVDF membrane (Millipore, Bllerica, MA) overnight at 4°C and 30V. Membranes were reversibly stained with Ponceau S to confirm the transfer of proteins, and destained in water. Membranes were then incubated for one hour at room temperature (RT, 23°C) in

blocking solution containing 4% (w/v) bovine serum albumin (BSA) (Sigma) in Tris-buffered saline (TBS, pH 7.4) with 0.2% (v/v) Tween-20. Primary and secondary antibodies were diluted in 1% (w/v) BSA in TBS-tween and 0.5% (w/v) BSA in TBS-tween respectively, and washes were done with TBS containing 0.2% (v/v) Tween-20 3x 10 minutes each. Peroxidase activity was detected using the Amersham enhanced chemiluminescence lighting system (ECL) (GE Healthcare, Piscataway, NJ). Purity of fractions (data not shown) were characterized by western blot analyses using IκBα as a marker for the cytoplasm [1:2,000], sc-847 (Santa Cruz Biothecnology, Santa Cruz, CA) and pan Cadherin as a marker for cell membrane [1:750], ab22744 (Abcam, Cambridge, MA). Antibodies: AQP4 [1:1000], AB3594 (Millipore, Billerica, MA) 1 hour at room temperature (7 day and 21 day time point) followed by an overnight incubation at 4°C (1 day and 3 day time point; naïve samples); glial fibrillary acidic protein (GFAP) [1:30,000], MAB360 (Millipore, Billerica, MA) 1 hour at room temperature; hypoxia inducible factor 1 alpha (Hif-1α) [1:1000], MAB5382 (Millipore, Billerica, MA) 2 hours at RT; and β-actin [1:10,000], A-2066 (Sigma-Aldrich, Saint Louis, MO) 1 hour at RT, used as a loading control. Sham 1 day (n=4), HI 1 day (n=5), HHI 40% 1 day (n=7), HHI 100% 1 day (n=5); sham 3 day (n=4), HI 3 day (n=5), HHI 40% 3 day (n=7), HHI 100% 3 day (n=6); sham 7 day (n=4), HI 7 day (n=5), HHI 40% 7 day (n=4), HHI 100% 7 day (n=5); sham 21 day (n=7), HI 21 day (n=5), HHI 40% 21 day (n=7), HHI 100% 21 day (n=6).

II.G. BRAIN TISSUE PREPARATION FOR IMMUNOFLUORESCENT STAINING AND CONFOCAL LASER SCANNING MICROSCOPY

Three days after the insult, the survival time point that showed significant increases in AQP4 and GFAP protein levels was selected for confocal

immunohistochemical analyses (sham, n=3; HI, n=4; and HHI 100%, n=3). At this time point the pups were deeply anesthetized with isofluorane and perfused transcardially with cold 0.9% (w/v) saline solution followed by transcardial fixation with 4% (w/v) paraformaldehyde in 0.9% (w/v) saline solution. The pups were immediately decapitated; the whole brains were removed and placed in a 4% paraformaldehyde solution overnight at 4°C. The next day, the brains were transferred to a cryoprotective solution containing 30% (w/v) sucrose and kept at 4°C until the brains sank (roughly 72 hours). The brains were cut from the anterior tuber cinereum to anterior of the occipital cortex, encompassing the parietal cortex, mounted on Tissue-Tek cryomolds (15mm x 15mm x 5mm, Sakura Finetek, Torrance, CA) using optimal cutting temperature (O.C.T.) compound (Sakura Finetek, Torrance, CA) on dry ice until frozen solid and then stored at -80°C. The brains were sectioned using a frozen microtome into 15μm coronal slices onto poly-L-lysine coated glass slides and stored at -20°C until immunohistochemical staining.

For the immunofluorescent staining, sections were post-fixed with cold 100% methanol for 5 minutes, air dry for 10 minutes and rinse 2x for 5 minutes in tris saline buffer (TBS). The sections were further permeabilized 3x for 5 minutes with TBS-triton (0.1% Triton X-100). The sections were blocked for 45 minutes with TBS-triton, 0.3% (w/v) BSA and 5% (v/v) normal goat serum (NGS). Primary and secondary antibodies were diluted in TBS-triton, 0.3% (w/v) BSA and 1% (v/v) normal goat serum (NGS). After an overnight incubation with the primary antibodies at 4°C in a humidified chamber, sections were washed in TBS 3x 10 minutes. Secondary antibodies were incubated for 2 hours at room temperature. The sections were washed in TBS 4x 10 minutes and counterstained with To-Pro-3 ([1:3000], Invitrogen, Carlsbad, CA). Antibodies: AQP4 [1:250], AB3594 (Millipore, Billerica, MA); glial fibrillary acidic

protein (GFAP) [1:200], MAB360 (Millipore, Billerica, MA); Nestin [1:200], MAB353 (Millipore, Billerica, MA); AlexaFluor 488 goat anti-mouse [1:1000] and AlexaFluor 568 goat anti-rabbit [1:1000] (Invitrogen, Carlsbad, CA).

Visualization was performed using a confocal laser scanning system (Bio-Rad Radiance 2100, K-2 system, Bio-Rad Laboratories, Tokyo, Japan). Images were collected using the 488 and 568 nm excitation lines of an Argon-Krypton laser. To avoid "bleed-through" of fluorescent signals between adjacent color channels for double immunofluorescent staining, the images were collected one channel at a time using sequential scanning. The co-localization of the two antigens was indicated in yellow. All sections were imaged using the same exposure times and parameters. Digital images were saved and processed with Adobe Photoshop (Adobe Systems Inc, San Jose, CA) for final editing.

II.H. BEHAVIORAL TESTS

We performed two behavioral tests to assess motor coordination 21 days after HI insult or HI with hyperoxia treatment (P28): bar holding test and wire mesh ascending test. At P21 the pups were weaned and randomly separated into 5 pups per cage. All behavioral training and testing was carried out between 8am and 4pm, and the animals were placed in the testing room at least 30 minutes prior to the motor evaluation in order to minimize stress-related complications. Each pup was trained 5 times per day for 3 consecutive days for each one of the tests. On the day of the testing, we performed each test with five separate trials per animal with a resting time of 5 minutes in between trials. For the bar holding test the pups spent their resting time in their home cage, and for the wire mesh ascending test the pups spent their resting time in the enclosed platform at the

top of the mesh. All pups were placed back to their original cage immediately after training and testing.

II.H.1. Bar holding test

As previously described by Tchekalarova *et al*. (2005) and slightly modified, the pups were allowed to grasp a wooden bar of 1cm in diameter and 30cm long suspended 50cm high above a padded soft surface. We measured the time spent on the bar and grasping with forelimbs with a limit of 120 seconds. The results are shown as average of the 5 recordings per animal. Sham, n=11; HI, n=23; HHI 40%, n=17; HHI 100%, n=26.

II.H.2. Wire Mesh Ascending Test

We assessed sensory-motor coordination using the wire mesh ascending test as previously described (Tchekalarova *et al*., 2005; Thonhoff *et al*., 2007). The pups were placed at the bottom of the mesh and the time necessary to ascend was measured for up to 120 seconds. The 10mm plastic mesh of 45cm high and 15cm wide was placed at an angle of 70° in contact with an enclosed platform at the top and a padded soft surface at the bottom. As a stimulus to ascend, the littermates were placed on the enclosed platform and allowed to acclimatize to the surroundings for 5 minutes before testing. The results are shown as average of the 5 recordings per animal. Sham, n=19; HI, n=25; HHI 40%, n=17; HHI 100%, n=26. This assay addresses both motor impairment and the environmental stress common to this behavioral test.

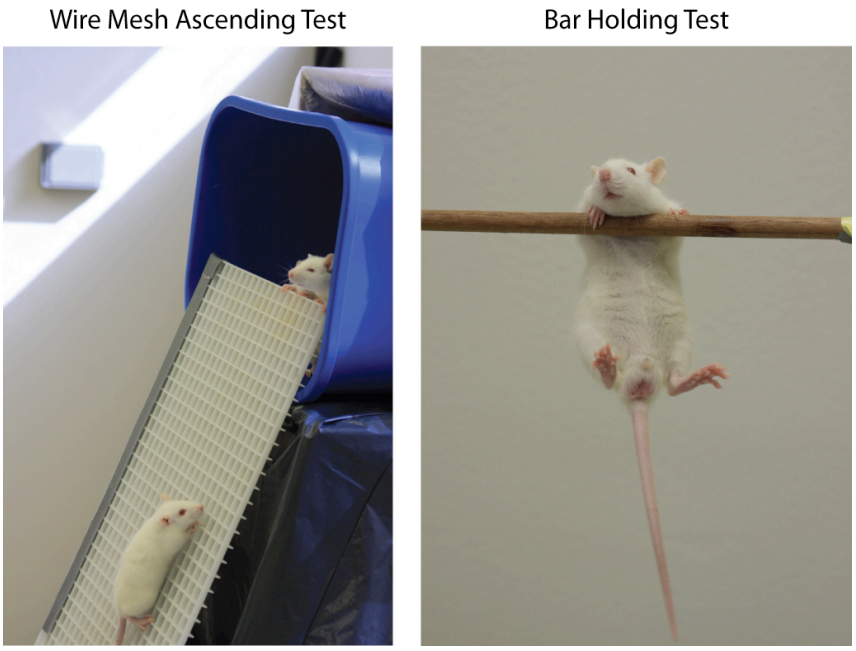


Figure 6. Representative images of the behavioral testing performed 21 days after neonatal HI.

II.I. STATISTICAL ANALYSES

Statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA). For analyses involving two groups, unpaired Student's t-test was performed with a p-value less than 0.05 considered significant. For analyses involving two or more groups, one-way analyses of variance (ANOVA) was performed using the Tukey-Kramer post-hoc test. We considered a value of $p<0.05$ significant.

CHAPTER III

HI INDUCES FLUID ACCUMULATION IN THE BRAIN CORTEX AND INDUCES MOTOR COORDINATION IMPAIRMENT

Perinatal HI occurs in 0.2-0.4% of term infants, and up to 60% in pre-term infants (Vannucci *et al*., 1999; Zanelli *et al*., 2009). The initial event after neonatal HIE is energy depletion that is followed by neuronal damage, apoptosis and cerebral edema (Zanelli *et al*., 2008). Cerebral edema is a pathological increase in the amount of fluid in the brain parenchyma that is associated with an increase in brain volume (Klatzo, 1967; Kimelberg, 2004; Papadopoulos and Verkman, 2007). It is a serious complication after stroke, head injury, brain abscess and tumor formation, and an important contributor to brain-injury associated morbidity and mortality (Marmarou *et al*., 1994; Graham *et al*., 1995; Papadopolous *et al*., 2002). However, the temporal effects on neonatal HI on the development of cerebral edema and its long-term pathological consequences have not been fully evaluated.

It has been shown that early after an ischemic insult there is development of cytotoxic edema that is followed by a breach of the BBB that leads to the development of vasogenic edema both in humans and animal models (Mujsce *et al*., 1990; Rabinstein, 2006; Ashwal *et al*., 2007; Badaut *et al*., 2007; Chang *et al*., 2007). So, in order to assess the temporal characteristics of the development of edema in the neonatal brain, we used the modified version of the Rice-Vannucci model of neonatal HI. Using dry weight analyses, we report that changes in water content are not confined only to the injury site in the ipsilateral cortex, but extended to the contralateral cortex, a novel finding. In order to further evaluate the development of edema, we evaluated the permeability of the BBB

after neonatal HI. We observed sub-acute BBB permeability changes as a cause of vasogenic edema both in the ipsilateral and contralateral cortex. Due to the important role that AQP4 plays in water transport in and out of the brain parenchyma, we evaluate changes in AQP4 protein levels after neonatal HI. Our results provided evidence for AQP4 to play a role in water clearance and development of edema after neonatal HI, as well as in the decrease of water content as part of normal brain development. Furthermore, our results showed cortical (ipsilateral and contralateral) susceptibility to hypoxia insult as evaluated by measuring changes in the protein levels of the hypoxia inducible factor-1α (Hif-1α) and on reactive astrogliosis measured by changes in glial fibrillary acidic protein (GFAP) levels. Finally, our data is consistent with a detrimental effect of HI on long-term motor coordination measured by behavioral tests.

III.A. HI INCREASES WATER CONTENT IN THE IPSILATERAL AND CONTRALATERAL CORTEX

We found significantly increased water content in the ipsilateral cortex 1 day after neonatal HI (3.75% increase, $p<0.001$, n=5) when compared to sham-treated P7 rat pups (n=9; Fig. 7A). This increase in water content persisted for 3 days (4.81%, $p<0.001$, sham n=28, HI n=28) and 7 days (6.28%, $p<0.001$, sham n=10, HI n=9). Our results support previous reports for edema development 1 to 2 days after neonatal HI (Mujsce *et al*., 1990; Meng *et al*., 2004). At 21 days after the insult, the water content markedly increased by 15.5% compared to the sham-treated ($p<0.001$; sham n=10, HI n=7). This dramatic increase in water content at three weeks after the insult indicates the formation of a cyst in the ipsilateral cortex that is abnormally depicted as a fluid accumulation in the cavity without cells/tissue. Figure 7B shows the changes in water content in injured pups compared to sham without normalization. This figure also showed in sham-treated P7 rat

pups that their values of brain water content decreased with development as previously reported (Lovblad *et al*., 2003; Rivkin *et al*., 2004; Sulyok, 2006).

We were also interested in assessing the effect of neonatal HI not only at the site of the injury (ipsilateral cortex), but also in the contralateral cortex with respect to edema development. We focused in the contralateral cortex to address the global consequences of the hypoxic insult in our model. We found that there was no significant change in water content in the contralateral cortex 1 day after HI (Fig. 8A, sham n=13, HI n=9). However, there was a significant increase in water content 3 days after the insult (0.56% increase, $p<0.001$, sham n=16, HI n=20) compared to sham. This increase persists for 7 days after HI (0.44% increase, $p<0.01$, sham n=10, HI n=11) reaching a 1.56% increase in contralateral cortex water content 21 days after the insult ($p<0.001$, sham n=10, HI n=8). Our results are in agreement with a previous report by Ashwal *et al*. (2007) where they show that the Rice-Vannucci model could result in a bilateral hemispheric injury, where the contralateral cortex experiences less injury than the ipsilateral. Furthermore, we observed a significant difference in the magnitude of increase in water content in the ipsilateral cortex (injury site) compared to the contralateral cortex (note different scales in the Y-axis of figures 7 and 8). However, small changes in water content should be evaluated carefully. It has been reported that an increase in 3-4% water content in CNS pathologies becomes lethal, where a change of 3.3% increase equates to an increase in brain volume of 31% (Mujsce *et al*., 1990). Therefore, the observed change in water content may significantly impair the development and neurophysiology in the contralateral cortex.

We further confirmed our results using non-invasive T2-weighted magnetic resonance imaging (T2), where an increase in T2 intensity reflects edema formation (Triulzi *et al*., 2006; Obenaus *et al*., 2008). Our results show that 7 days after neonatal HI

there was a clear hyperintensity in the ipsilateral cortex compared to sham animals, further supporting our results of an increase in water content 7 days after HI (6.28%, $p<0.001$) (Fig.9).

III.B. HI INDUCES BLOOD-BRAIN BARRIER PERMEABILITY

In order to address the cause for the development of edema, we performed a BBB permeability assay at 3 days and 7 days post-HI. There are two types of edema: cytotoxic edema and vasogenic edema as previously described by Igor Klatzo (1967). Cytotoxic edema consists of water/fluid accumulated intracellularly without a breach of the BBB, and is mostly located in astrocytes. Vasogenic edema develops due to a breakdown of the BBB and accumulation of fluid in the extracellular space. Neonatal HI, both in human subjects as in animal models, has been shown to induce early-on cytotoxic edema and to be followed by vasogenic edema as part of brain pathology (Rabinstein, 2006; Badaut *et al*., 2007; Zanelli *et al*., 2008).

We found that in our model of neonatal HI, there was an increase in BBB permeability ($p<0.05$) in the ipsilateral cortex (Fig. 10A) 3 days after HI (sham n=6, HI n=7) that persisted for 7 days after HI ($p<0.05$, sham n=4, HI n=3). Our ipsilateral results are consistent with the observed T2-MRI results (Fig. 9), where a hyperintensity signal usually depicts vasogenic edema (Badaut *et al*., 2007). Furthermore, our evaluation of the contralateral cortex showed that 3 days after neonatal HI the BBB is intact (sham n=6, HI n=6). However, 7 days after HI there was significant BBB breakdown ($p<0.05$, sham n=3, HI n=4) (Fig. 10B). These results address the question as to why there is persistent increase of water accumulation in the ipsilateral cortex, and the drastic increase in water content that occurs three weeks after HI in the contralateral cortex.

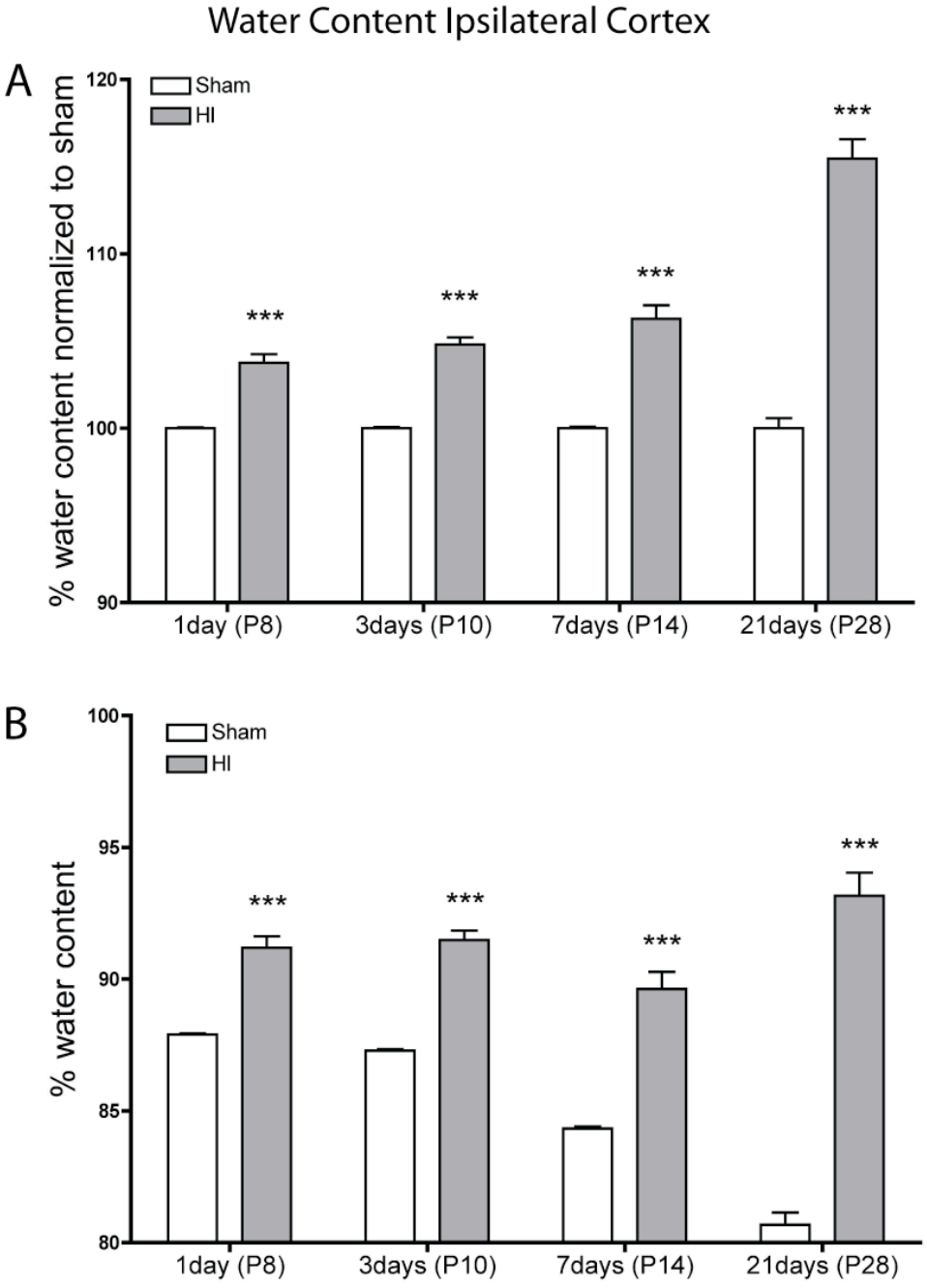


Figure 7. Neonatal HI induces an increase of water content in the ipsilateral cortex.

Ipsilateral cortex water content normalized to sham (A). HI-induced increase in water content compared to control-shams, with a decrease in sham water content normal for brain development (B). Results are presented as mean ± S.E.M. *** $p<0.001$ compared to Sham.

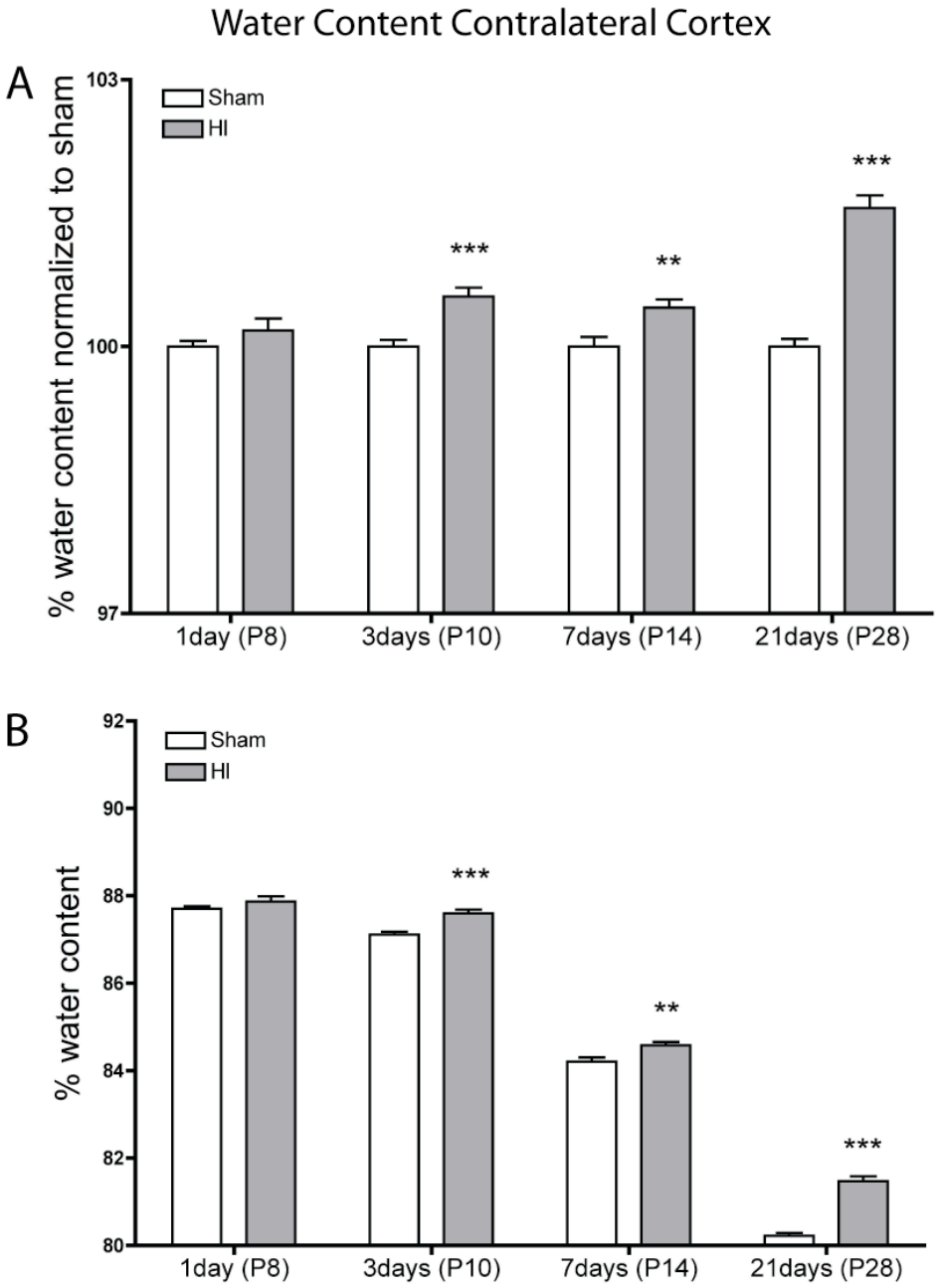


Figure 8. Neonatal HI induces an increase of the water content in the contralateral cortex.

Contralateral cortex water content normalized to sham (A). HI-induced increase in water content compared to control-shams, with a decrease in sham water content normal for brain development (B). Results are presented as mean ± S.E.M. *** $p<0.001$ compared to Sham.

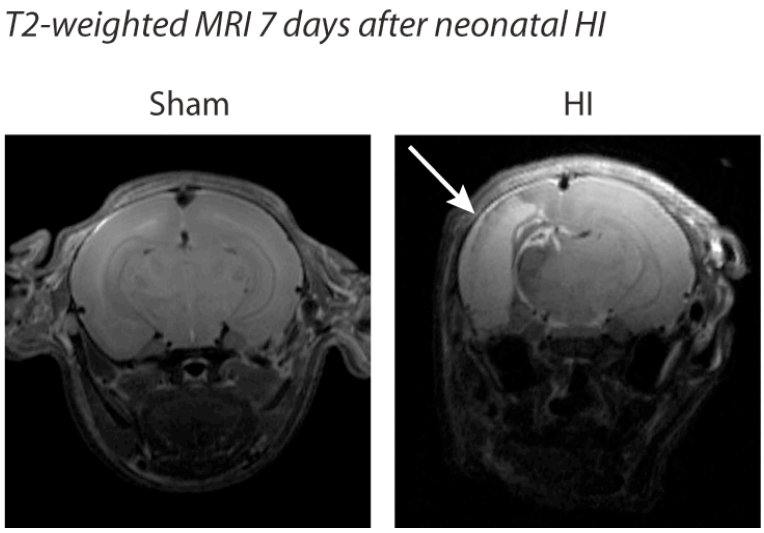


Figure 9. Neonatal HI induces edema 7 days after the insult.

Representative T2-weighted magnetic resonance images of a sham and HI-injured rat pup brains 7 days post HI. HI increases injury lesion volume within the ipsilateral cortex depicted by the hyperintensity observed (arrow) compared to sham.

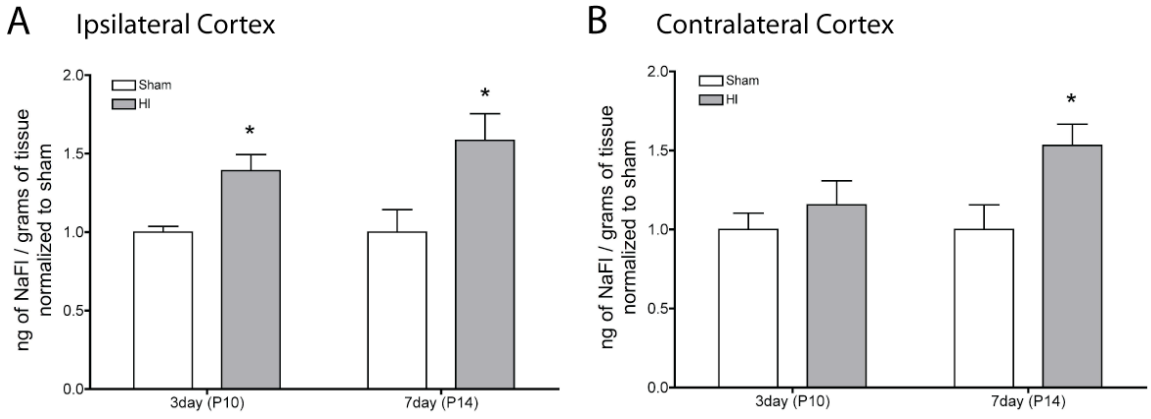


Figure 10. Neonatal HI induces blood-brain barrier permeability.

(A) Neonatal HI induces ipsilateral cortex BBB permeability 3 days and 7 days after the insult. (B) HI does not affect contralateral cortex BBB permeability 3 days after the insult, but does induce BBB permeability 7 days after compared to sham. BBB permeability expressed as ng of sodium fluorescein dye (NaFl) in gram of tissue, normalized to sham. Results are presented as mean ± S.E.M. * $p<0.05$ compared to Sham of each time course.

III.C. HI INDUCES A MOLECULAR RESPONSE TO HYPOXIA AND ASTROCYTIC ACTIVATION BOTH IN THE IPSILATERAL AND THE CONTRALATERAL CORTEX

Hypoxia-inducible factor 1 alpha (Hif-1α) is the transcription factor responsible for cellular responses to changes in oxygen levels and is responsible for the activation of the genes that encode proteins that participate in homeostatic responses to hypoxia (genes responsible for restoring blood supply, nutrients and energy production in order to maintain tissue integrity (Rius *et al*., 2008). Hence, Hif-1α protein levels are a marker of the extent of the hypoxic insult after HI. In order to assess the magnitude of the HI insult and the temporal cellular response to neonatal HI we measured Hif-1α protein levels by Western blot in the ipsilateral and contralateral cortex.

As expected, we found that in the ipsilateral cortex there was a significant increase in Hif-1α protein levels starting at 1 day after HI (33% increase, $p<0.05$, Fig. 11B) compared to sham (sham n=4, HI n=5). Three days post-HI, there was an additional increase in Hif-1α protein levels (100.4% increase, $p<0.001$, sham n=4, HI n=5), and one week after the insult Hif-1α continues to be up-regulated (48.4%, $p<0.001$, sham n=4, HI n=5), but to a lesser extent than at 3 days after HI. Interestingly, three weeks after HI Hif-1α protein levels increase again, but more significantly (119.4%, $p<0.01$, sham n=7, HI n=5). In the contralateral cortex, we found an increase in Hif-1α protein levels as early as 1 day after HI (35.1% increase, $p<0.001$, sham n=4, HI n=5) (Fig. 11C). We further found that Hif-1α up-regulation persisted, though to a lesser extent, up to 3 days after HI (11.8%, $p<0.05$, sham n=4, HI n=5), and by 7 days after the insult, the Hif-1α protein levels returned to sham levels (sham n=4, HI n=5). This result is not surprising given that our animal model is experiencing global cerebral hypoxia. However, 21 days after HI there was significant increase in Hif-1α protein levels (46.2%, $p<0.05$, sham n=7, HI n=5) in accordance with the ipsilateral results of a biphasic response to HI.

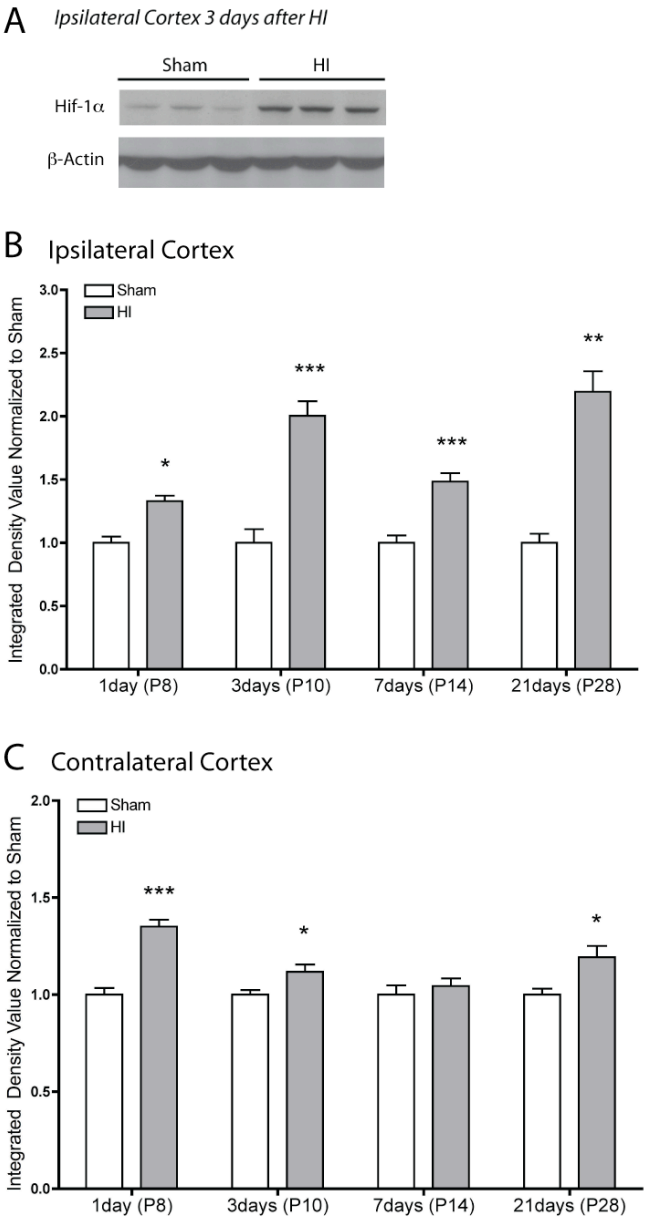


Figure 11. HI induces a hypoxic insult both in the ipsilateral and contralateral cortex.

(A) Representative Western blot for Hif-1α 3 days after neonatal HI show an increase in ipsilateral protein levels after HI (MW 120kDa). (B) Quantitative analyses of densitometric values for Hif-1α protein level temporal changes after HI in the ipsilateral cortex normalized to Sham. (C) Quantitative analyses of densitometric values for Hif-1α protein level temporal changes after HI in the contralateral cortex normalized to Sham. Results are presented as mean ± S.E.M. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to Sham.

In order to determine the extent of the injury over time after HI we looked at the amplitude of astrocytic activation at 1, 3, 7 and 21 days after injury. Reactive gliosis is an astrocytic response to CNS injury present after brain ischemia (Li *et al*., 2008), and its hallmark is an increase in expression of the glial fibrillary acidic protein (GFAP) in astrocytes. GFAP is an intermediate filament expressed in astrocytes, that together with actin filaments and microtubules are involved in the structure and function of the astrocyte's cytoskeleton (Smith *et al*., 1983). We performed western blot analyses to evaluate HI-induced GFAP changes in ipsilateral and contralateral cortices. Our results (Fig. 12B) showed an increase in GFAP protein levels in the ipsilateral cortex starting 1 day after HI (33%, $p<0.001$, sham n=4, HI n=5) that persisted for 3 (100.4% increase, $p<0.05$, sham n=4, HI n=5) and 7 days (48.4% increase, $p<0.001$, sham n=4, HI n=5). By 21 days after neonatal HI, and similarly to the changes in Hif-1α levels, there was a dramatic and still significant up-regulation of GFAP protein levels in the ipsilateral cortex (119.4% increase, $p<0.001$, sham n=7, HI n=5). We further validated our Western blot results using immunofluorescence labeling to evaluate changes in GFAP immunoreactivity in sham and HI-treated P7 rats (Fig. 13). Our results showed a clear increase in GFAP immunostaining 3 days after HI in the ipsilateral cortex, in accordance with Western blot results. We further performed a double immunofluorescence staining using GFAP (green) and nestin (red). Nestin is another type of intermediate filament and a marker of reactive astrocytes (Privat, 2003; Li *et al*., 2008).

We observed an early but not-significant increase in GFAP protein levels at 1 day post-HI in the contralateral cortex (Fig. 12C, sham n=4, HI n=5), that by 3 days became significant (94% increase, $p<0.05$, sham n=4, HI n=5). GFAP levels decreased to sham levels at 7 days (sham n=4, HI n=5) and remained unchanged (sham n=7, HI n=5) after neonatal HI.

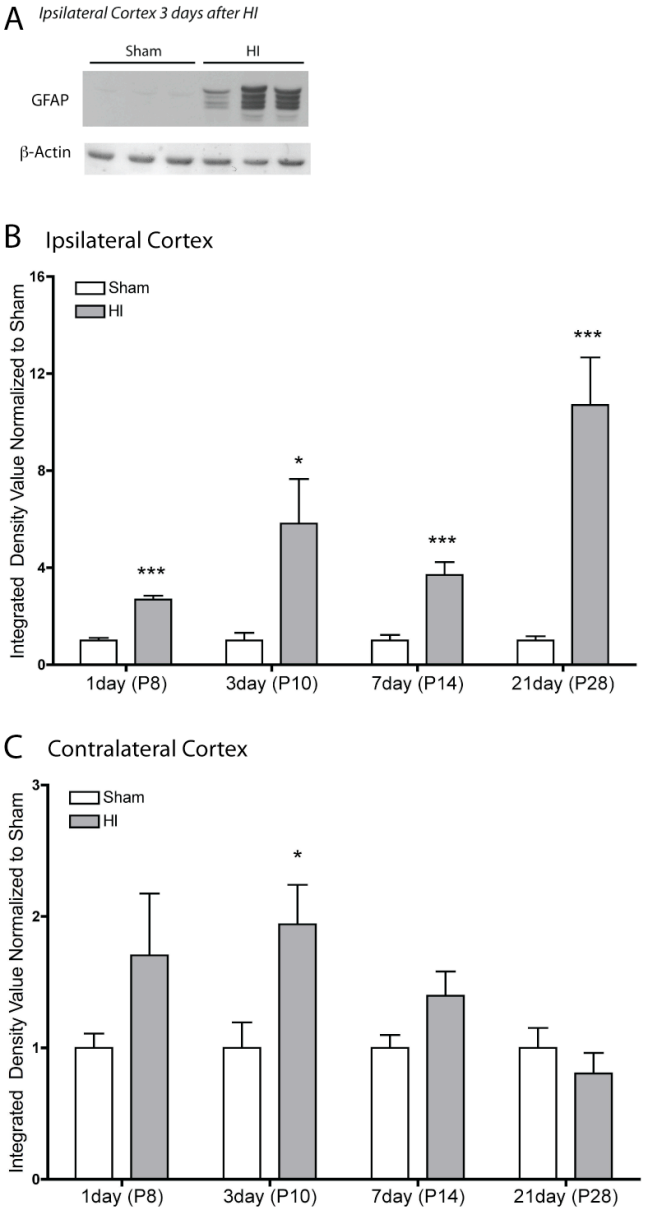


Figure 12. HI induces brain injury both in the ipsilateral and contralateral cortex measured by GFAP protein levels as a marker of astrocytic activation

(A) Representative Western blot for GFAP 3 days after neonatal HI showed an increase in ipsilateral protein levels after HI (MW 50kDa). (B) Quantitative analyses of densitometric values for GFAP protein level temporal changes after HI in the ipsilateral cortex normalized to Sham. (C) Quantitative analyses of densitometric values for GFAP protein level temporal changes after HI in the contralateral cortex normalized to Sham. Results are presented as mean ± S.E.M. * $p<0.05$ and *** $p<0.001$ compared to Sham.

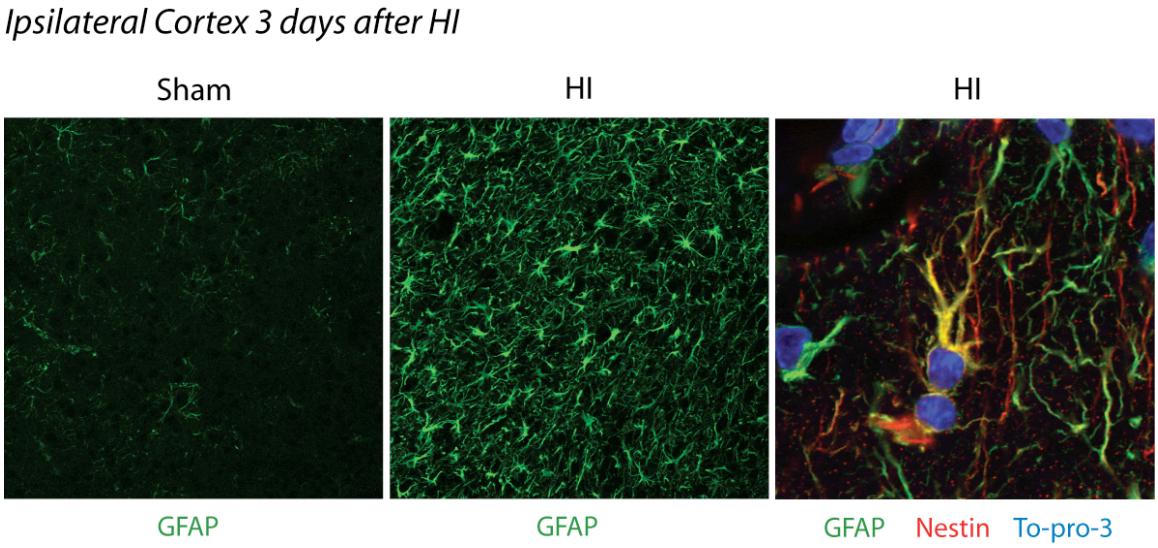


Figure 13. HI induces astrocytic activation in the ipsilateral cortex 3 days after the insult.

Representative confocal fluorescent micrographs of the ipsilateral cortex from sham and HI-injured pups. The images show reactive gliosis in the HI-injured 3 days post-HI as compared to sham. We further confirmed our results of HI-induced astrocytic activation by GFAP colocalization with Nestin (merge depicted by yellow color). GFAP only images are 20X. Dual staining with GFAP and Nestin is 60x (3X zoom). Blue is nuclear marker To-pro-3.

III.D. HI INDUCES AN UPREGULATION OF THE WATER CHANNEL AQP4 IN THE ISPILATERAL CORTEX BUT NOT IN THE CONTRALATERAL CORTEX

Due to its localization in the brain, AQP4 has a key role in the control of water flux in and out of the brain parenchyma (Papadopolous and Verkman, 2007). Furthermore, studies on AQP4-null mice strongly suggest that AQP4 plays a key role in the clearance of water following vasogenic edema, and also plays a key role in the formation of cytotoxic edema (Manley *et al*., 2000). In neonatal experimental models, Badaut *et al*. (2007) showed a direct correlation between an increase in AQP4 protein levels in the border of the lesion and amelioration of edema 1 day and 3 day after

neonatal stroke, and Meng *et al*. (2004) showed development of edema correlated with a decrease in AQP4 protein levels 1 and 24 hours after neonatal HI. These results suggest a positive role for AQP4 in clearance of edema after neonatal asphyxia. We have shown the development of vasogenic edema in our model of neonatal HI; therefore we asked whether HI induces changes in AQP4 protein levels. We measured AQP4 protein level changes after neonatal HI both in the ipsilateral and contralateral cortex using Western blot and immunohistochemical analyses.

Our western blot analyses showed 3 main AQP4 bands that correspond to the M23, M1 and Mz (~32kDa; Sorbo *et al*., 2008) isoforms (the AQP4 isoforms are described in more detail in Chapter I). We quantitated densitometry values for the three AQP4 isoforms as changes in AQP4 monomer protein levels.

We found that AQP4 protein levels increased in the ipsilateral cortex 3 days after HI (127.2%, $p<0.001$, sham n=4, HI n=5, Fig. 14A) that persisted at high levels for 7 days (377.3%, $p<0.001$, sham n=4, HI n=5) and at less pronounced level for up to 21 days (69.7%, $p<0.001$, sham n=7, HI n=5) after neonatal HI (Fig. 14B). We further confirmed our results with immunohistochemistry analyses (Fig. 15). Three days after HI, there was an increase in AQP4 immunoreactivity in the ipsilateral cortex. This increase was both in the astrocytic endfeet surrounding blood vessels and astrocytic cell bodies. Furthermore, we confirmed AQP4 immunostaining in astrocytes by double labeling of AQP4 and GFAP (Fig. 15).

However, we did not observe changes in AQP4 protein levels in the contralateral cortex at either time point evaluated compared to sham (Fig. 14C).

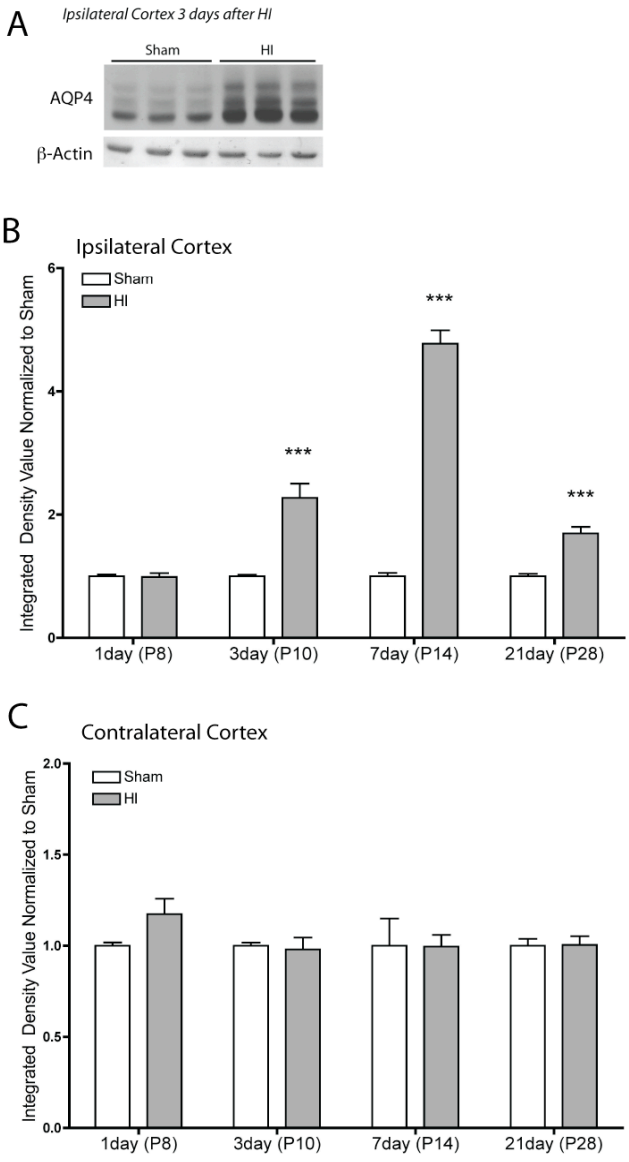


Figure 14. Neonatal HI induces an increase of AQP4 protein levels in the ipsilateral cortex but not in the contralateral cortex.

(A) Representative Western blot 3 days after neonatal HI show a increase in ipsilateral AQP4 isoforms: M23 (lower band) ~27kDa, M1 (mid-band) ~30kDa, and upper band (Mz ~32kDa). (B) Quantitative analyses of AQP4 monomer isoform protein level temporal changes after HI in the ipsilateral cortex normalized to Sham. (C) Quantitative analyses of AQP4 protein level temporal changes after HI in the contralateral cortex normalized to Sham. Results are presented as mean ± S.E.M. * $p<0.05$ and *** $p<0.001$ compared to Sham.

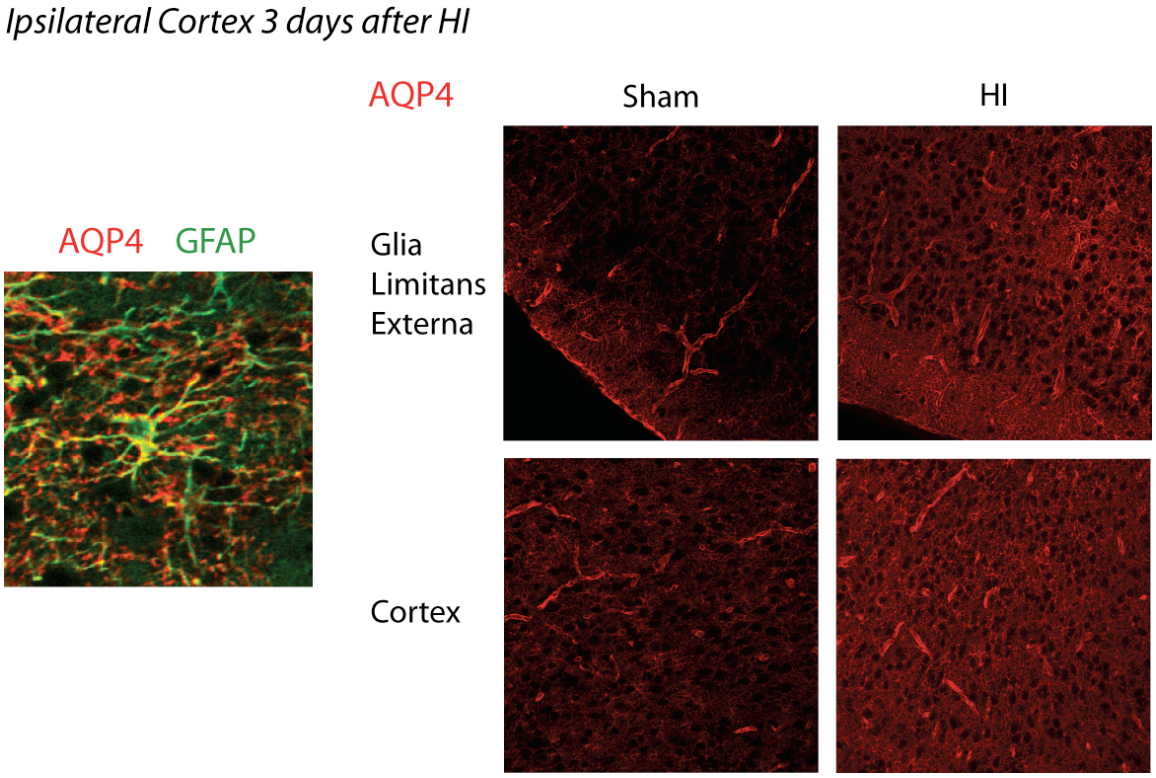


Figure 15. HI induces an increase in AQP4 in the ipsilateral cortex and glia limitans externa 3 days after the insult.

Representative confocal fluorescent micrographs of the ipsilateral glia limitans externa and ipsilateral cortex from sham and HI-injured pups. We show an increase in AQP4 immunostaining 3 days after HI compared to sham. We further confirmed that AQP4 immunostaining is limited to astrocytes by co-localization of GFAP and AQP4. AQP4 only images are 20X. Dual staining of GFAP and AQP4 is 60x (3X zoom).

III.E. DEVELOPMENTAL CHANGES IN BRAIN WATER CONTENT INVERSELY CORRELATE WITH CHANGES IN AQP4 PROTEIN LEVELS

To test the hypothesis that an increase in AQP4 protein levels after neonatal HI aids in the clearance of excess fluid in the brain, we evaluated developmental changes of water content and AQP4 protein levels in the brains of newborn rats and into adulthood.

It has been reported that postnatally there is a decrease in brain water content and in extracellular volume (Yannet and Darrow, 1938; Bondareff and Pysh, 1968; Lehmenkühler *et al*., 1993; Lovblad *et al*., 2003; Rivkin *et al*., 2004; Sulyok, 2006). In agreement with these reports, our results showed that in the brain cortex of Wistar rats even though there was no significant change in water content from P1 (88.1%, n=5) to P7 (88.2%, n=5, Fig. 16A), from P7 to P10 (87.39%, n=4) there was a significant decrease in water content (0.83%, $p<0.001$). This decrease persisted by P15 (84.42%, n=3, 2.97% less than P10, $p<0.001$), P30 (80.46%, n=3, 3.96% less than P15, $p<0.001$) and into adulthood (78.38% at P100, n=4, 2.08% less than P30, $p<0.001$). These results show an overall decrease in water content from postnatal day 1 into adulthood (P100) of 9.72%.

In addition, previous studies have shown that AQP4 protein levels, both in the rat brain and cerebellum, significantly increase during postnatal development (Wen *et al*., 1999). Furthermore, Gomori *et al*. (2006) described an increase in AQP4 brain levels in the human fetus from 14 to 40 gestational weeks. We therefore evaluated postnatal changes in AQP4 protein levels. In agreement with previous reports, we observed a temporal increase in AQP4 levels from P1 to P30 and into adulthood (P100=100%, Fig. 16B, 16C). There was a significant increase from P1 (n=5, 4.8% of adult values) to P7 (n= 5, 43.6% of adult values, $p<0.001$). These results indicate a 10-fold increase in AQP4 protein levels from P1 to P7. AQP4 protein levels continued to increase reaching 65.3% of adult values at P10 (n=4, $p<0.001$ compared to P7), 83.7% of adult values at P15 (n=3, $p<0.01$ compared to P10), and 99% of adult values at P30 (n=3, $p<0.05$ compared to P15).

Our results show that during the first weeks after birth, there was a decrease in water content in the brain cortex of Wistar rats that inversely and significantly ($p<0.05$) correlated with an increase in AQP4 protein expression with an r^2 value of 0.6953 (Fig.

17). Therefore, our results suggest that an increase in AQP4 protein levels accounts for up to 70% of the reduced developmental water clearance in the brain. Thus, these data further supports our hypothesis that HI-induced increases in AQP4 protein levels will aid in the clearance of HI-induced increase in brain fluid accumulation.

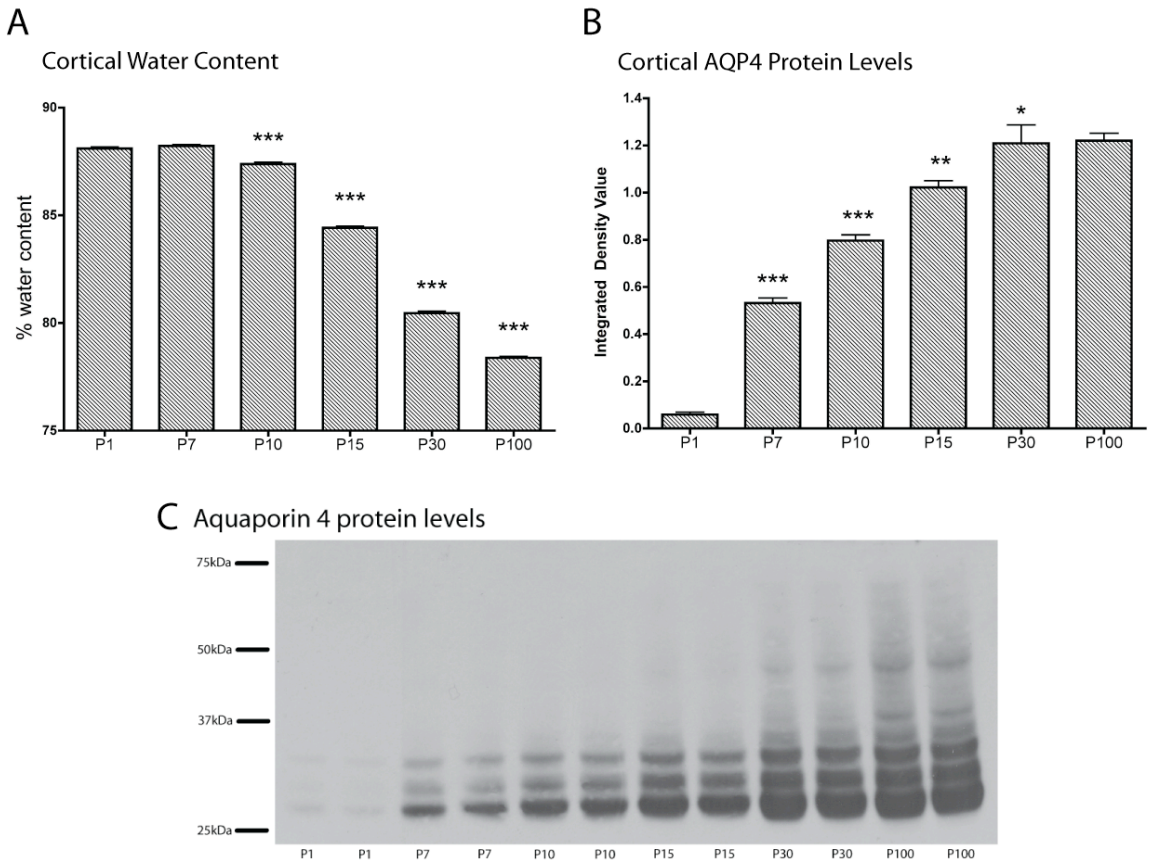


Figure 16. Developmental changes in cortical water content and AQP4 protein levels.

(A) Quantitative analyses of brain cortex water content changes with development (from P1 to P100). (B) Quantitative analyses of AQP4 protein levels during brain cortex development (from P1 to P100). Results are presented as mean ± S.E.M. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to the previous time point. (C) Representative Western blot for AQP4 protein levels during development show an increase in the 3 isoforms: M23 (lower band) ~27kDa, M1 (mid-band) ~30kDa, and upper band (Mz ~32kDa), and the appearance of a band ~50kDa starting at P30.

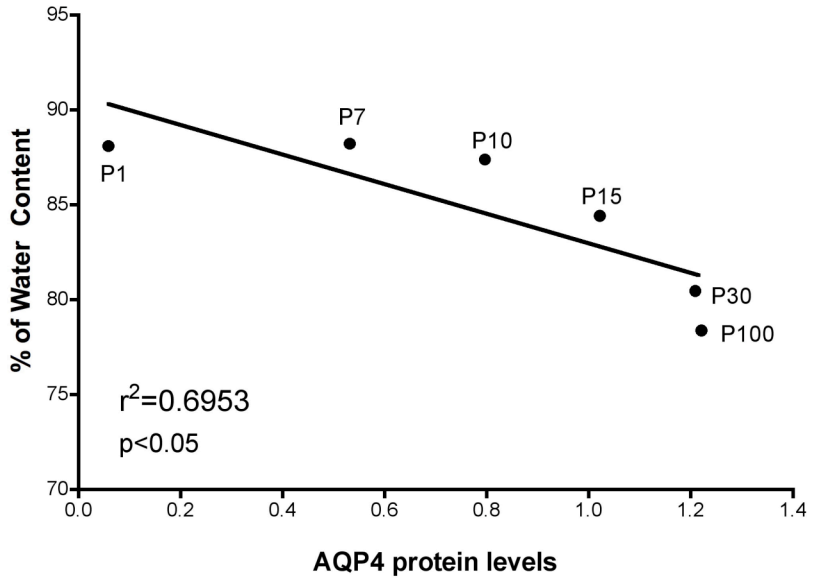


Figure 17. Inverse correlation of decreased cortical water content and increase in AQP4 protein levels during cortical brain development.

III.F. HI IMPAIRES MOTOR COORDINATION

We observed that neonatal HI induces edema, BBB permeability and astrocytic activation; all of which likely affect normal brain development. Furthermore, it has been shown that term infants as well as premature and low birth weight infants that suffer a HI insult develop cerebral palsy (Boichot *et al*., 2006; Zanelli *et al*., 2008). Cerebral palsy encompasses a group of non-progressive disorders that affect a person's ability to move, maintain balance and posture (Shelly *et al*., 2008). We therefore investigated the effect of HI on motor coordination.

Using the bar holding test (Tchekalarova *et al*., 2005), we found that 21 days after the HI insult there was impaired motor coordination. Rat pups exposed to HI were unable to hold to the elevated bar for more than 75 seconds, compared to sham pups that could

stay on the bar for the maximum allowed time of 120 seconds ($p<0.001$; Fig. 18A). We further evaluated the effect of HI on motor coordination using the wire mesh ascending test (Tchekalarova *et al*., 2005; Thonhoff *et al*., 2007). We found that the group that was exposed to HI took a longer amount of time to climb up the wire mesh compared to the sham-treated rats ($p<0.01$; Fig. 18B).

Together, these results indicate that HI significantly impairs motor skills in P7 rats. Furthermore, our behavioral analyses demonstrates the long-lasting effect of HI that is in agreement with the impaired motor coordination found in young children that suffered a HI insult at, before or after birth (Boichot *et al*., 2006; Zanelli *et al*., 2008).

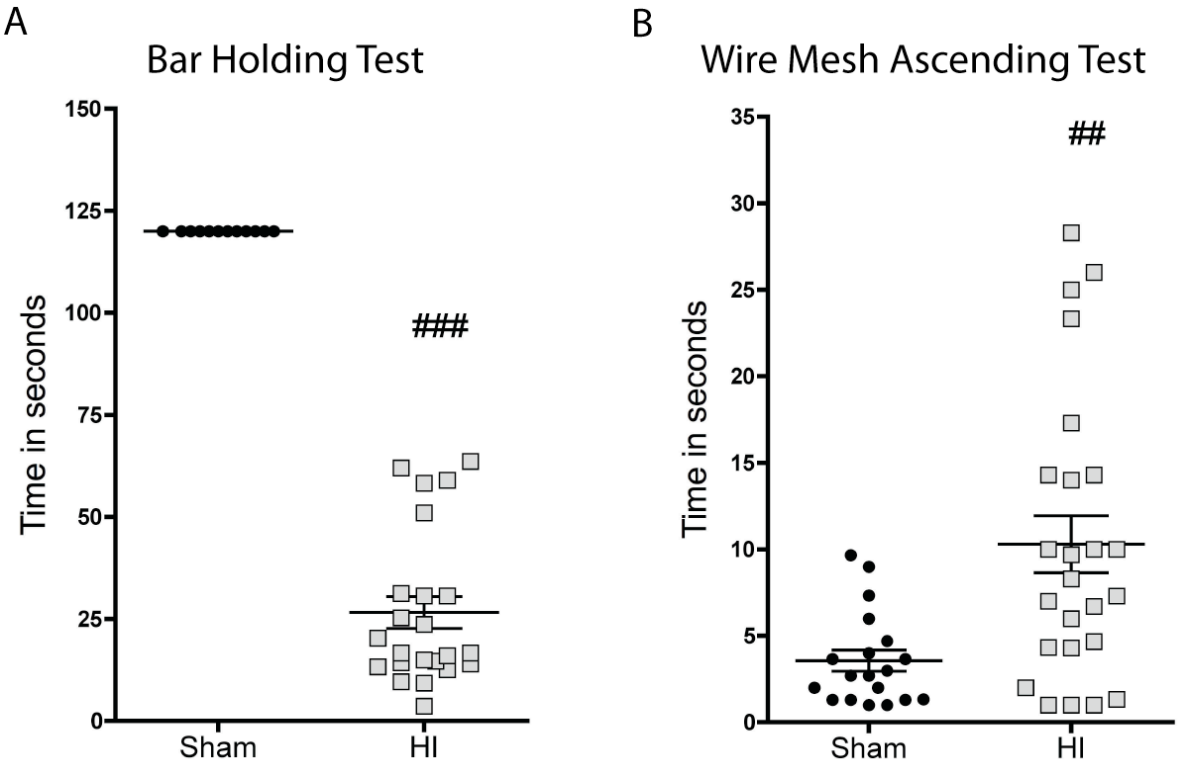


Figure 18. Neonatal HI induces long-lasting motor coordination impairment.

Bar holding test (A) and wire mesh ascending test (B). Results are presented as mean ± S.E.M. ## $p<0.01$ and ### $p<0.001$ compared to Sham.

III.G. DISCUSSION

It has been shown that early after perinatal HI, there is development of cerebral edema in neonates (Soul *et al*., 2001, Boichot *et al*., 2006; Chang *et al*., 2007; Zanelli *et al*., 2008). And it has been shown that cerebral edema is an important contributor to brain-injury associated morbidity and mortality (Marmarou *et al*., 1994; Graham *et al*., 1995; Papadopolous *et al*., 2002).

Reports on neonatal stroke and neonatal HI show an increase in edema as early as 1 day after the insult (Meng et al., 2004; Qiao *et al*., 2004; Badaut *et al*., 2007). However, there are no reports on the long-term effects of neonatal HI in the development of brain edema. Our data showed that neonatal HI induced an increase in brain water content, and that the increase is long-lasting. Specifically, we showed that HI induced edema in the ipsilateral cortex 1 day after the insult that persisted up to 7 days. However, 21 days after HI the edema previously observed in the ipsilateral cortex developed into a fluid-filled cyst. In addition, our data in the contralateral cortex showed that HI induced edema 3 days post-HI that persisted up to 21 days after the insult. However, in order to fully evaluate the causes of HI-induced cerebral edema, we evaluated the effect of HI on the permeability of the BBB. Our results showed that 3 days after HI there was a breach of the BBB in the ipsilateral cortex, that continued to be permeable 7 days after the insult. Furthermore, our results showed that HI induced a late breach of the BBB in the contralateral cortex, with the BBB becoming permeable 1 week after HI. In addition, we used the non-invasive technique of T2-MRI to evaluate the presence of cerebral edema after neonatal HI. Knowing that the presence of hyperintensity in T2-MRI usually suggests the occurrence of vasogenic edema (Triulzi *et al*., 2006; Ashwal *et al*., 2007; Obenaus *et al*., 2008), our T2-MRI results 7 days after HI not only indicated the presence of edema in the ipsilateral cortex (in agreement with our dry weight analyses) but also

confirmed our results that HI induces a breach of the BBB in the ipsilateral cortex 7 days after the insult. Therefore, our results suggest that HI induces vasogenic edema that will lead to the accumulation of excess fluid in the ipsilateral and contralateral cortices.

Due to the important role played by AQP4 in regulating brain water transport and our results showing HI-induced cerebral edema, we investigated the effect of HI on AQP4 protein levels. Badaut *et al*. (2007) has shown that there is a direct correlation between an increase in AQP4 protein levels and amelioration of edema. Moreover, Meng *et al*. (2004) showed that development of edema correlated with a decrease in AQP4 protein levels 24 hours after neonatal HI. These results support the hypothesis that AQP4 aids in the clearance of water accumulation after HI-induced edema. To test this hypothesis, we evaluated the long-term effect of neonatal HI in AQP4 protein levels and its correlation to the formation of edema 1, 3, 7 and 21 days after the insult. In the ipsilateral cortex, our results showed that HI induced an increase in water content 1 day after the insult and persisted up to 21 days post-HI. However, the increase observed in water content 1 day after HI did not correlate with changes in AQP4 levels. Three days after HI we observed an increase in AQP4 protein levels, suggesting that AQP4 is a component of the responsive mechanism to edema and not a cause. In addition, we showed that AQP4 levels in the ipsilateral cortex peaked at 7 days post lesion. This gradual increase in AQP4 levels correlated with a maintenance of increase ipsilateral cortex water content around 92%. In the contralateral cortex however, our results showed that HI did not affect AQP4 protein levels at all time points studied. This lack of increase of AQP4 protein levels correlated with an increase in water content at 3, 7 and 21 days after HI. Finally, our results with naïve rat pups suggest that an increase in AQP4 could lead to normal developmental brain water clearance. These novel results, together with the previously shown role of AQP4 in clearance of vasogenic edema (Manley *et al*.,

2000) and the reports on pharmacological up-regulation of AQP4 that leads to a decrease in cerebral edema after stroke and TBI (Zhao *et al*., 2005; Hirt *et al*., 2009), suggest that in our model, AQP4 plays a key role in clearance of the ipsilateral cortex water content and the lack of its up-regulation in the contralateral cortex, together with the development of vasogenic edema, prevents the clearance of edema.

In addition, we showed that HI induced a long-lasting hypoxic insult depicted by Hif-1α protein levels and reactive astrogliosis showed with changes in GFAP protein levels. As expected, both markers were increased throughout the time course evaluated in the ipsilateral cortex. Interestingly, we observed the development of a hypoxic insult in the contralateral cortex early after HI that disappears 1 week after HI. However, we observed that 21 days after HI there is a second induction of HI as depicted by a significant increase in Hif-1α protein levels. These results, together with the changes in AQP4 protein levels, and water content, suggest a biphasic response to HI (Fig. 19). The biphasic response suggests that there are two distinct mechanisms of HI insult that differentiates the events that occur during the first week after the injury, that correlate with the development of edema, and the second wave of the HI insult depicted by the formation of a cyst in the site of the injury (Fig. 19). Furthermore, our results support the evidence that in our neonatal animal model of HI there is a bilateral response to HI (Spiegler *et al*., 2007), and therefore, the contralateral cortex cannot be used as a control.

Finally, we evaluated the effect of HI on a clinically relevant behavioral outcome. Thus, we investigated the long-term effect of neonatal HI on motor coordination 21 days after the insult. We showed here that neonatal HI impaired motor coordination, as evaluated by the bar holding test and wire mesh ascending test.

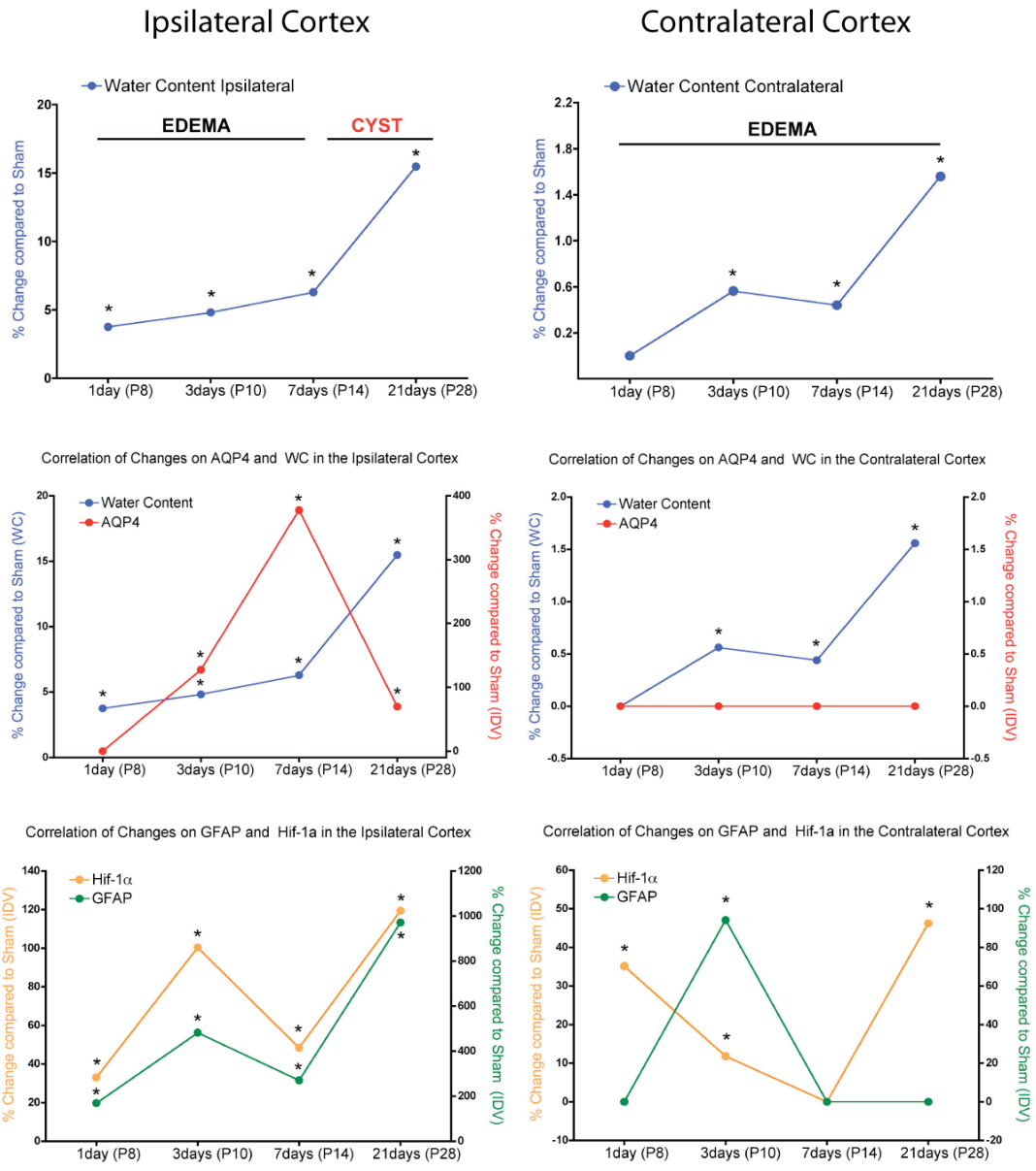


Figure 19. Summary of HI-induced increase in water content, changes in AQP4 protein levels, hypoxic insult and injury.

Water content data measured by dry weight analyses, protein levels measured with Western blot. Hif-1α protein levels depicted HI-induced hypoxic insult and GFAP protein levels depicted injury as a measure of astrocytic activation. No significant change was considered as zero (0).

Given that at birth the human brain is in an unfinished state, and that the brain continues to develop postnatally, an insult such as HI in the neonatal period could lead to detrimental effects. During the first year of age there is a significant decrease in water content and extracellular space shown to occur postnatally (Yannet and Darrow, 1938; Bondareff and Pysh, 1968; Pixley and de Vellis, 1984; Lehmenkuller *et al*., 1993; Lovblad *et al*., 2003; Rivkin *et al*., 2004; Sulyok, 2006) that is accompanied with and allows for brain growth and development when the postnatal developing brain undergoes a period of active synapse formation, axonal growth, radial glia disappearance and astrocytic maturation, oligodendrocyte proliferation and myelination, as well as early postnatal capillary proliferation; with most of these events taking place in the first year of age (Pixley and de Vellis, 1984; Robertson *et al*., 1985; Levine, 1989; Baron et al., 1995; Hunter *et al*., 1995; Goldman *et al*., 1997; de Graaf-Peters and Hadders-Algra, 2006; Nishida and Okabe, 2007; Weidenfeller et al., 2007). Therefore, it is not surprising that the consequences of neonatal HI lead to detrimental behavioral outcomes.

Specifically, our data has shown a developmental decrease in brain water content starting at P7 and progressing into adulthood, and that neonatal HI induced the development of edema. Therefore, our results cannot conclude but strongly suggest that by preventing normal water clearance in the neonatal brain, HI would be affecting brain development (Fig. 20). The persistent accumulation of excessive fluid in the brains of these P7 rat pups will therefore prevent glial cell proliferation, myelination, axonal extension and dendrite growth, that will lead to the long-term impaired motor coordination we observed (Fig. 21).

Our data is the first to address the important detrimental effect of edema in brain development and motor coordination.

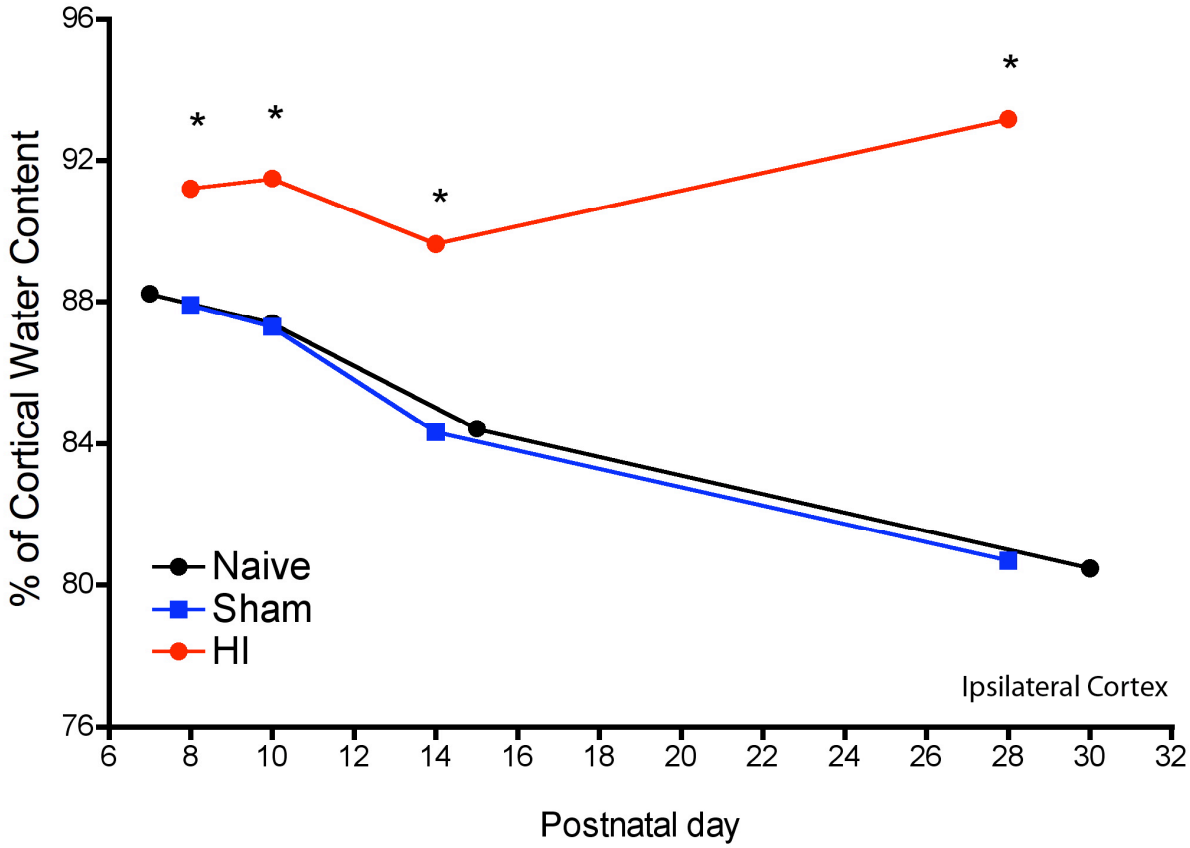


Figure 20. HI significantly increases brain water content and affects normal brain development.

Our results cannot conclude but strongly suggest that by preventing normal water clearance in the neonatal brain, HI would be affecting brain development.

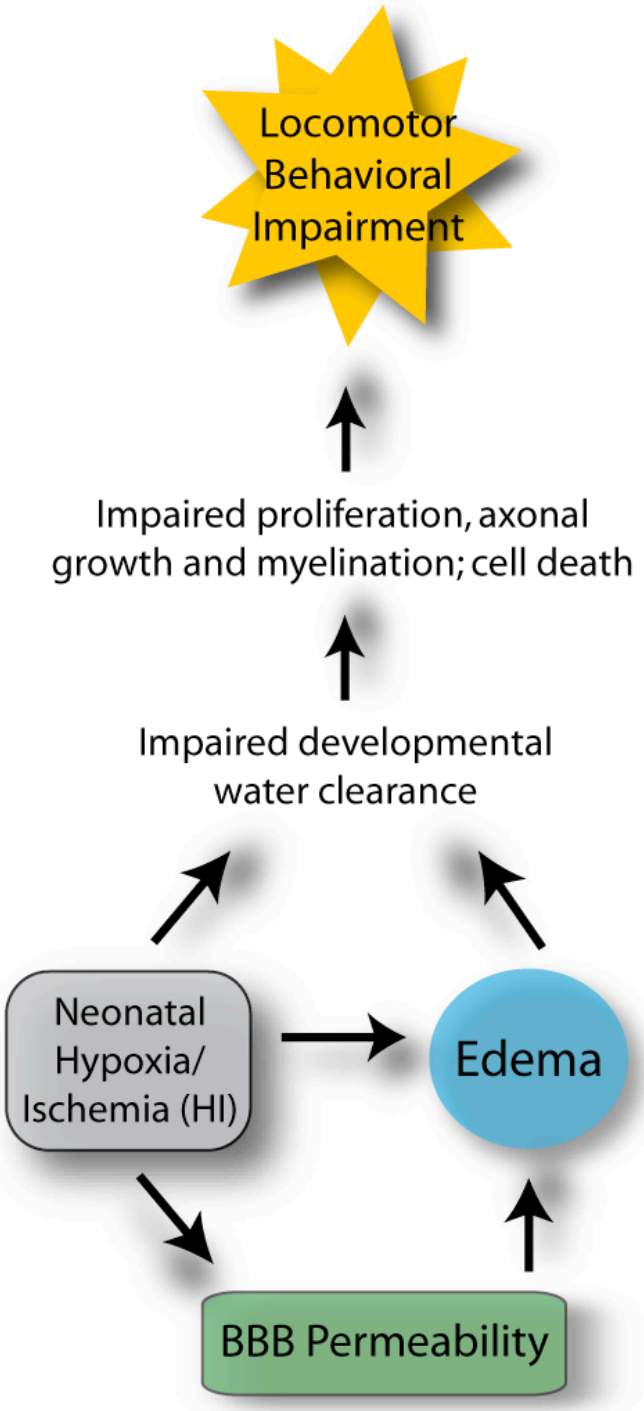


Figure 21. Schematic conclusion for Specific Aim 1 on the characterization of neonatal HI-induced edema and motor coordination.

CHAPTER IV

HYPEROXIA TREATMENT DOES NOT AMELIORATE HI-INDUCED EDEMA AND MOTOR COORDINATION IMPAIRMENT

The current clinical treatment for the resuscitation of neonates that suffered a HI insult is the use of supraphysiological concentrations of oxygen (hyperoxia, HHI; Davis *et al*., 2004; Klinger *et al*., 2005; Deulofeut *et al*., 2006; Wang *et al*., 2008; Koch *et al*., 2008). Even though the long-term effects of HHI as a treatment remain controversial (see Chapter I), there are no reports on the effects of hyperoxia treatment on the development of edema in the brain after neonatal HI.

We have shown that neonatal HI induces cerebral edema early after the insult that persists for 21 days. Our data further showed that one of the causes for the development of cerebral edema after HI, is the HI-induced breach of the BBB that lead to the development of vasogenic edema. In addition, due to its role in brain water homeostasis and its suggested role in the developmental water clearance in the brain, we showed a correlative role of increased AQP4 protein levels in clearing excess fluid accumulation in the brain after HI. Finally, we showed that HI, in part by inducing a long-lasting increase in excess fluid accumulation during brain development, leads to impaired motor coordination. Therefore, we were interested in evaluating the effect of HHI treatment after neonatal HI not only in the development of cerebral edema, but also on its long-term effect in motor coordination. Using the clinically approved resuscitating hyperoxia therapy (both moderate, 40% O_2, and extreme, 100% O_2) after neonatal HI, we provide novel evidence for the lack of HHI amelioration on (i) edema and cyst formation after HI, and (ii) long-term effect on motor coordination.

IV.A. HHI DOES NOT AMELIORATE HI-INDUCED INCREASE IN WATER CONTENT

Using dry weight analyses, we have previously shown that HI induced a significant increase in water content in the ipsilateral cortex 1 day after the insult (3.75% increase, $p<0.001$, sham n=9, HI n=5). We found that neither extreme (100% O_2, n=8) nor moderate (40% O_2, n=5) HHI prevented the HI-induced increase in water content (Fig.22A). We further showed that this increase in water content persisted for 3 ($p<0.001$, sham n=28, HI n=28) and 7 days ($p<0.001$, sham n=10, HI n=9) and we found that HHI treatment did not prevent it (3 days, HHI 40% n=14, HHI 100% n=15; 7 days, HHI 40% n=6, HHI 100% n=13). Our results have shown that HI induced an increase in water content of 15.4% in the ipsilateral cortex 21 days after the insult depicted not by edema, but by the formation of a cyst. Our results show that neither extreme nor moderate HHI treatment prevented the development of a cyst after HI (sham n=10, HI n=7, HHI 40% n=7, HHI 100% n=9). Figure 22B presents the changes in brain water content in HI-injured and oxygen-treated pups compared to sham-treated P7 rat pups without normalization, depicting the developmental decrease in brain water content in the sham-treated group.

Given that the hyperoxia treatment is systemic, we further evaluated the effects of HHI on the development of edema in the contralateral cortex. We found that neither HI (n=9) nor moderate (n=5) or extreme (n=14) HHI treatment after HI, affect brain water content 1 day after the insult compared to sham-treated P7 pups (n=13, Fig. 23A). Nevertheless, HI induced a significant increase in water content compared to sham 3 days after the insult ($p<0.001$, sham n=16, HI n=20), without a prevention or amelioration using moderate (n=13) or extreme (n=15) HHI treatment. This increase persisted 7 days

after HI ($p<0.01$, sham n=10, HI n=11) reaching a 1.56% increase in contralateral cortex water content 21 days after the insult ($p<0.001$, sham n=10, HI n=8), and HHI treatment did not prevent it (7 days, HHI 40% n=6, HHI 100% n=13; 21 days, HHI 40% n=7, HHI 100% n=9).

Interestingly, there was no significant difference between extreme (100% O_2) or moderate (40% O_2) hyperoxia treatments after HI with respect to edema or cyst formation compared to sham-treated rat pups.

We further validated our results using the non-invasive technique T2-weighted magnetic resonance imaging (T2-MRI), where an increase in T2-MRI intensity reflects edema formation. In agreement with our water content results, we found that 7 days after neonatal HI there was a clear hyperintensity in the ipsilateral cortex compared to sham animals and extreme HHI did not prevent it (Fig.24).

IV.B. HHI DOES NOT PREVENT HI-INDUCED BLOOD-BRAIN BARRIER PERMEABILITY

In human subjects and animal models, it has been shown that neonatal HI induces early-on cytotoxic edema that is followed by vasogenic edema as part of the HI-induced brain pathology (Rabinstein, 2006; Badaut *et al*., 2007; Zanelli *et al*., 2008). In agreement with these reports, we have shown that HI induced BBB permeability leads to the development of vasogenic edema. However, to date, there are no reports on the effects of HHI resuscitation after neonatal HI with respect to its effect on the stability of the BBB after a neonatal HI insult. Therefore, we evaluated the effect of extreme hyperoxia therapy 3 and 7 days after neonatal HI on the permeability of the BBB.

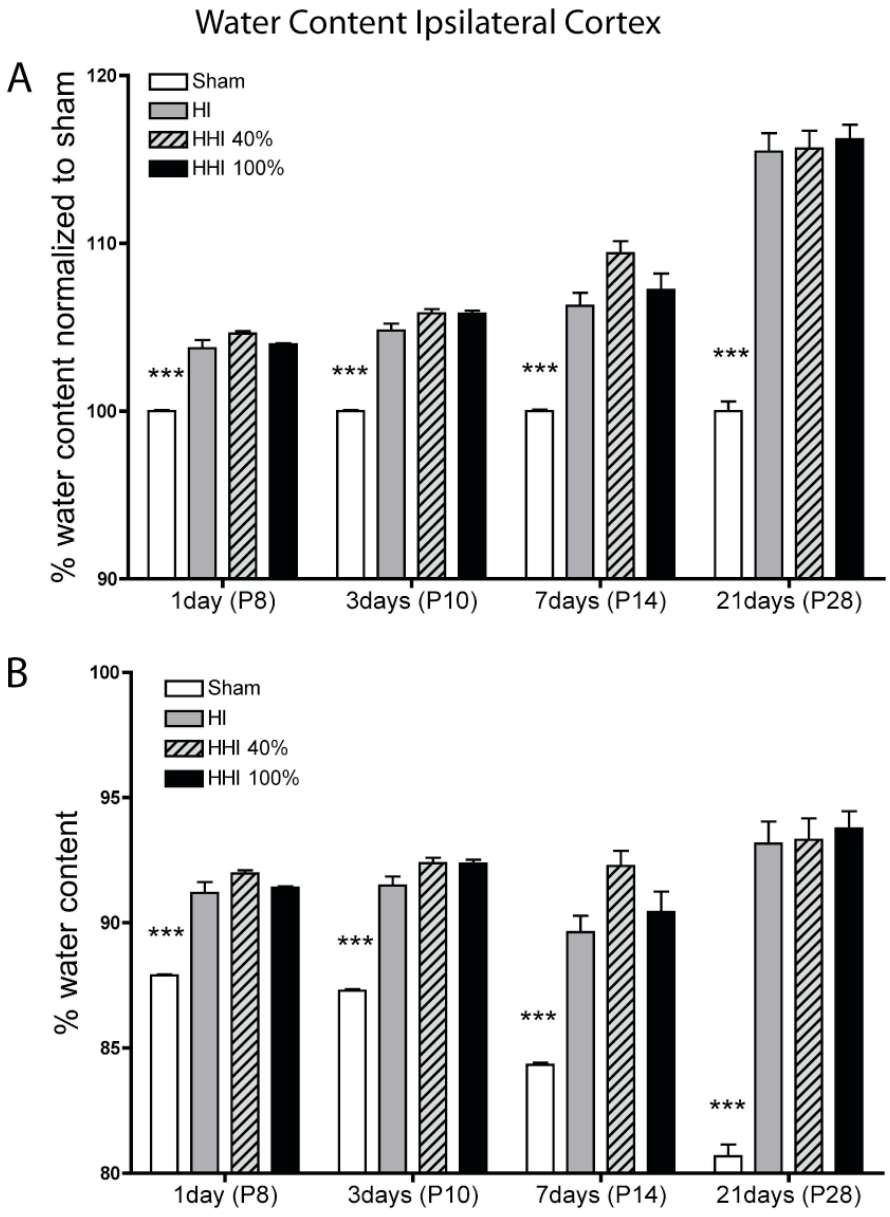


Figure 22. Hyperoxia treatment, either moderate (40% O_2) or extreme (100% O_2), does not ameliorate HI-induced edema and cyst formation in the ipsilateral cortex.

(A) Ipsilateral cortex water content normalized to sham. HI-induced increase in water content was not prevented by moderate or severe HHI. (B) Results compared to shams showing developmental decrease in sham water content with time. Results are presented as mean ± S.E.M. *** $p<0.001$ compared to HI, HHI 40% and HHI 100%.

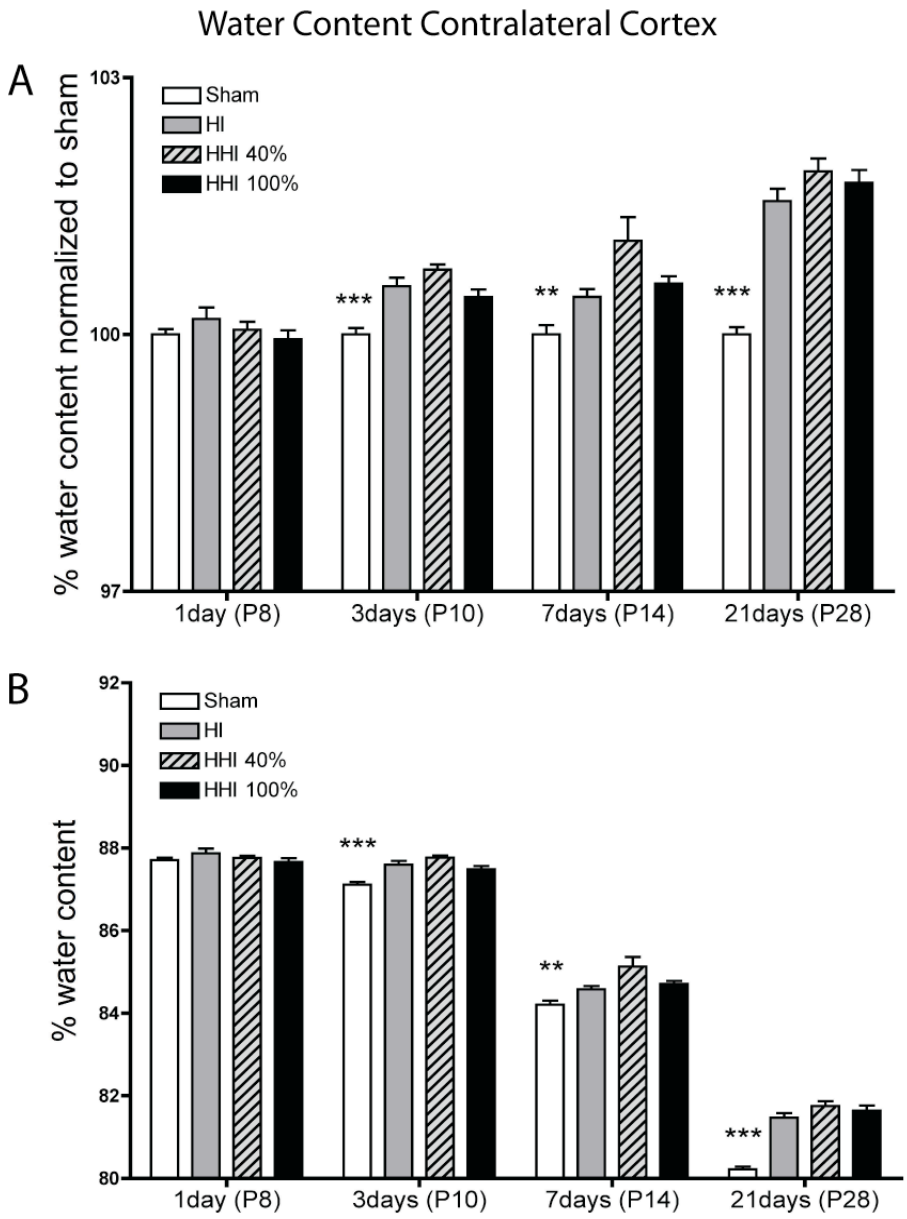


Figure 23. Hyperoxia treatment, either moderate (40% O_2) or extreme (100% O_2), does not ameliorate HI-induced edema in the contralateral cortex.

(A) Contralateral cortex water content normalized to sham. HI-induced increase in water content was not prevented by moderate or severe HHI. (B) Results compared to shams showing developmental decrease in sham water content with time. Results are presented as mean ± S.E.M. ** $p<0.01$ and *** $p<0.001$ compared to HI, HHI 40% and HHI 100%.

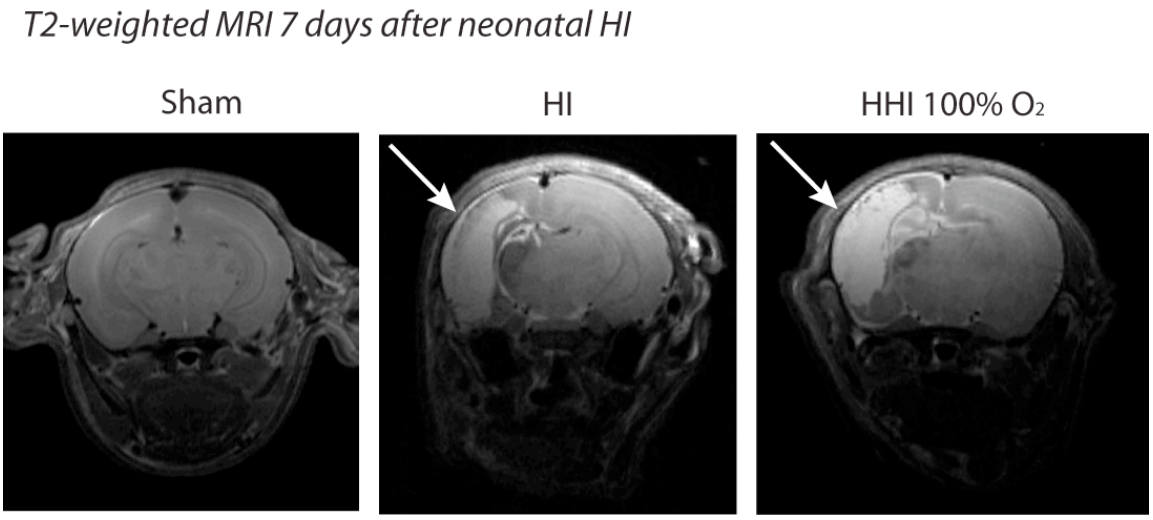


Figure 24. Extreme HHI treatment does not ameliorate neonatal HI-induced edema 7 days after the insult.

Representative T2-weighted magnetic resonance images (T2-MRI) of a sham, HI-injured, and HHI-treated rat pup brains 7 days post HI. HI increases injury lesion volume within the ipsilateral cortex depicted by the hyperintensity observed (arrows) compared to sham.

Our previous results showed that HI induced BBB permeability in the ipsilateral cortex 3 days after the insult ($p<0.05$, sham n=6, HI n=7) that persisted for 7 days ($p<0.05$, sham n=4, HI n=3). We found that extreme HHI (100% O_2) treatment did not prevent HI-induced BBB permeability at either time point in the ipsilateral cortex (Fig. 24A; $p<0.01$; 3 days, HHI 100% n=5; 7 days, HHI 100% n=4). Our ipsilateral results are in agreement with the observed increase in T2-MRI hyperintensity after HI and HI with extreme HHI treatment, and its relationship to vasogenic edema (Fig. 24).

Due to our systemic HHI treatment and the HI-induced effects observed in the contralateral cortex, we evaluated the effect of extreme HHI in the contralateral cortex after neonatal HI. Our data showed that the BBB is intact 3 days after neonatal HI (n=6) and extreme HHI treatment (n=6) in the contralateral cortex compared to sham-treated P7

pups (n=6). However, the observed breach in the BBB 7 days after HI ($p<0.05$, sham n=3, HI n=4) was not prevented by extreme HHI (Fig. 25B, $p<0.05$, n=4). Given that HHI therapy after HI does not prevent HI-induced BBB permeability, HHI will not prevent the development of edema observed in the ipsilateral and contralateral cortex after neonatal HI (Fig. 22, 23).

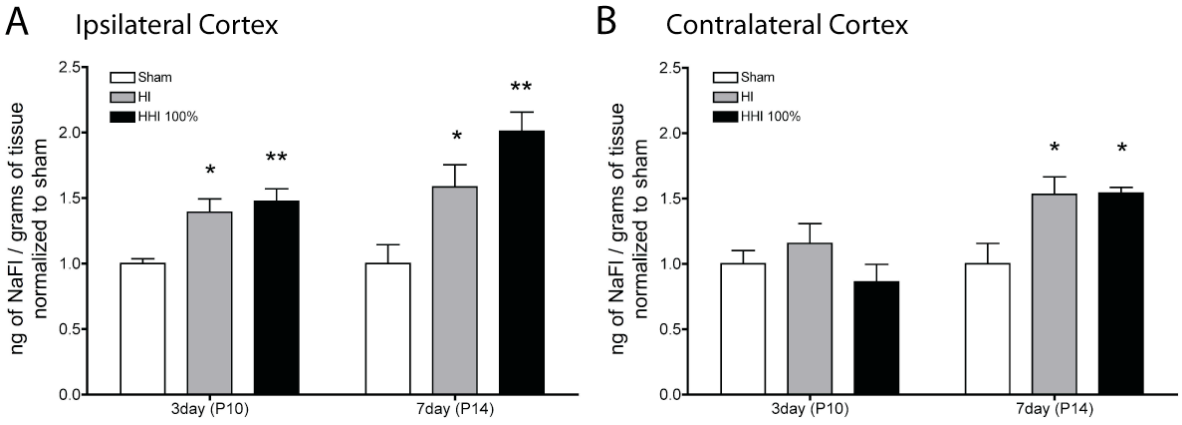


Figure 25. HHI does not prevent HI-induced blood-brain barrier permeability.

(A) Neonatal HI induces ipsilateral cortex BBB permeability 3 days and 7 days after the insult, and HHI treatment does not prevent it. (B) HI does not affect contralateral cortex BBB permeability 3 days after the insult, but does induce BBB permeability 7 days after compared to sham and HHI does not prevent it. BBB permeability expressed as ng of sodium fluorescein dye (NaFl) in gram of tissue, normalized to sham. Results are presented as mean ± S.E.M. * $p<0.05$ and ** $p<0.01$ compared to Sham.

IV.C. HHI DOES NOT AFFECT HI-INDUCED AQP4 PROTEIN LEVEL CHANGES IN THE IPSILATERAL AND CONTRALATERAL CORTEX

Given that (i) there are no reports on the effect of hyperoxia treatment in AQP4 protein levels, (ii) that AQP4 plays a key role in the control of water flux in and out of the

brain parenchyma (Papadopolous and Verkman, 2007) and (iii) that we have shown that HHI did not ameliorate HI-induced edema, we evaluated the effect of extreme (100% O_2) and moderate (40% O_2) hyperoxia treatments after neonatal HI in the ipsilateral and contralateral cortices. As previously shown, our Western blot analyses showed 3 main bands for AQP4 that correspond to the M23, M1 and Mz isoforms. We quantitated densitometry values for the three AQP4 isoforms as changes in AQP4 monomer protein levels. Our results showed that AQP4 protein levels did not change 1 day after HI (sham n=4, HI n=5) but did increase in the ipsilateral cortex 3 ($p<0.001$, sham n=4, HI n=5), 7 ($p<0.001$, sham n=4, HI n=5) and 21 days ($p<0.001$, sham n=7, HI n=5) after neonatal HI. Interestingly, neither extreme HHI (100% O_2) nor moderate (40% O_2) HHI affected the HI-induced increase in AQP4 in the ipsilateral cortex (Fig. 26B; 3 days, HHI 40% n=7, HHI 100% n=6; 7 days, HHI 40% n=4, HHI 100% n=5; 21 days, HHI 40% n=7, HHI 100% n=6). In addition, neither HI nor HHI affect AQP4 protein levels in the contralateral cortex at any of the time points evaluated compared to sham-treated P7 rats.

IV.D. HYPEROXIA ALONE DOES NOT AFFECT CORTICAL WATER CONTENT AND AQP4 PROTEIN LEVELS

Previous reports have shown that the treatment of hyperoxia without HI can lead to cell death in the developing brain (Taglialatela *et al*., 1998; Gerstner *et al*., 2006; Gerstner *et al*., 2008; Yis *et al*., 2008). These results were independent of the exposure times to hyperoxia (1 to 7 days), oxygen concentration (80% O_2 to >95% O_2), and post-natal age (P1, P3, P6, P7 and P10). Therefore, we evaluated the effect of extreme (100% O_2) or moderate (40% O_2) hyperoxia on P7 rats. Specifically, we investigated the effect of a 2 hours exposure to HHI in brain water content and AQP4 protein levels 1 day (P8), 3day (P10) and 7day (P14) after the treatment.

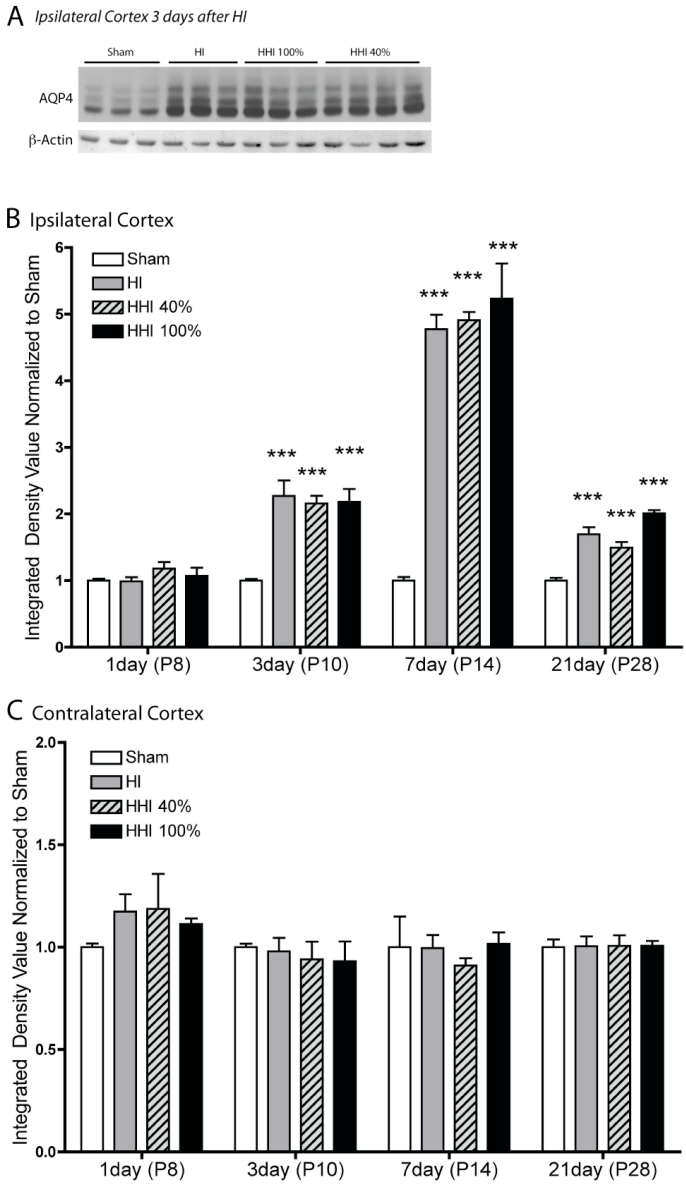


Figure 26. HHI does not prevent HI-induced increase of AQP4 protein levels in the ipsilateral cortex, and does not affect AQP4 contralateral cortex protein levels.

(A) Representative Western blot for ipsilateral AQP4 protein levels 3 days after neonatal HI and HHI treatment show an increase in protein levels after the insult. (B) Quantitative analyses of densitometric values for AQP4 protein level temporal changes after HI and HHI in the ipsilateral cortex normalized to Sham. (C) Quantitative analyses of densitometric values for AQP4 protein level temporal changes after HI and HHI in the contralateral cortex normalized to Sham. Results are presented as mean ± S.E.M. *** $p<0.001$ compared to Sham.

We found that neither extreme nor moderate HHI affected developmental decreases in brain water content nor induced edema formation (Fig. 27A, 27B) at any time point evaluated (all groups: n=4, except for sham 3 days n=3). In addition, we found no changes in AQP4 protein levels in response to extreme or moderate HHI (Fig. 27C) at any time point assayed (all groups: n=4, except for sham 3 days n=3). These results further confirmed our observations that HHI, either extreme (100% O_2) or moderate (40% O_2), did not affect HI-induced edema development or AQP4 protein changes.

IV.E. HHI DELAYED THE HI-INDUCED HYPOXIC INSULT AND ASTROCYTIC ACTIVATION IN THE IPSILATERAL CORTEX, BUT DID NOT AFFECT THE CONTRALATERAL CORTEX

Given that changes in Hif-1α protein levels are a marker of the extent of the hypoxic insult (Rius *et al*., 2008), we assessed the time course of the hypoxic response in the ipsilateral and contralateral cortex after extreme HHI treatment by Western blot analyses.

We have previously shown the expected result that HI induced a hypoxic insult in the ipsilateral cortex, as evidenced by a persistent increase in Hif-1α levels (from 1 day to 21 days post-HI). Interestingly, our data showed that extreme HHI treatment was able to delay the hypoxic response to the HI-insult in the ipsilateral cortex up to 3 days after HI (Fig. 28B; 1 day, sham n=4, HI n=5, HHI 100% n=5; 3 days, sham n=4, HI n=5, HHI 100% n=6). However, 7 and 21 days post-HI, HHI treatment did not affect the HI-induced hypoxic insult (7 days, sham n=4, HI n=5, HHI 100% n=5; 21 days, sham n=7, HI n=5, HHI 100% n=6).

In the contralateral cortex, there was an increase in Hif-1α protein levels 1, 3 and 21 days after HI, with no change observed 7 days post-HI. Interestingly, extreme HHI

treatment had no effect on preventing or delaying the hypoxic insult observed in the contralateral cortex (Fig. 28C).

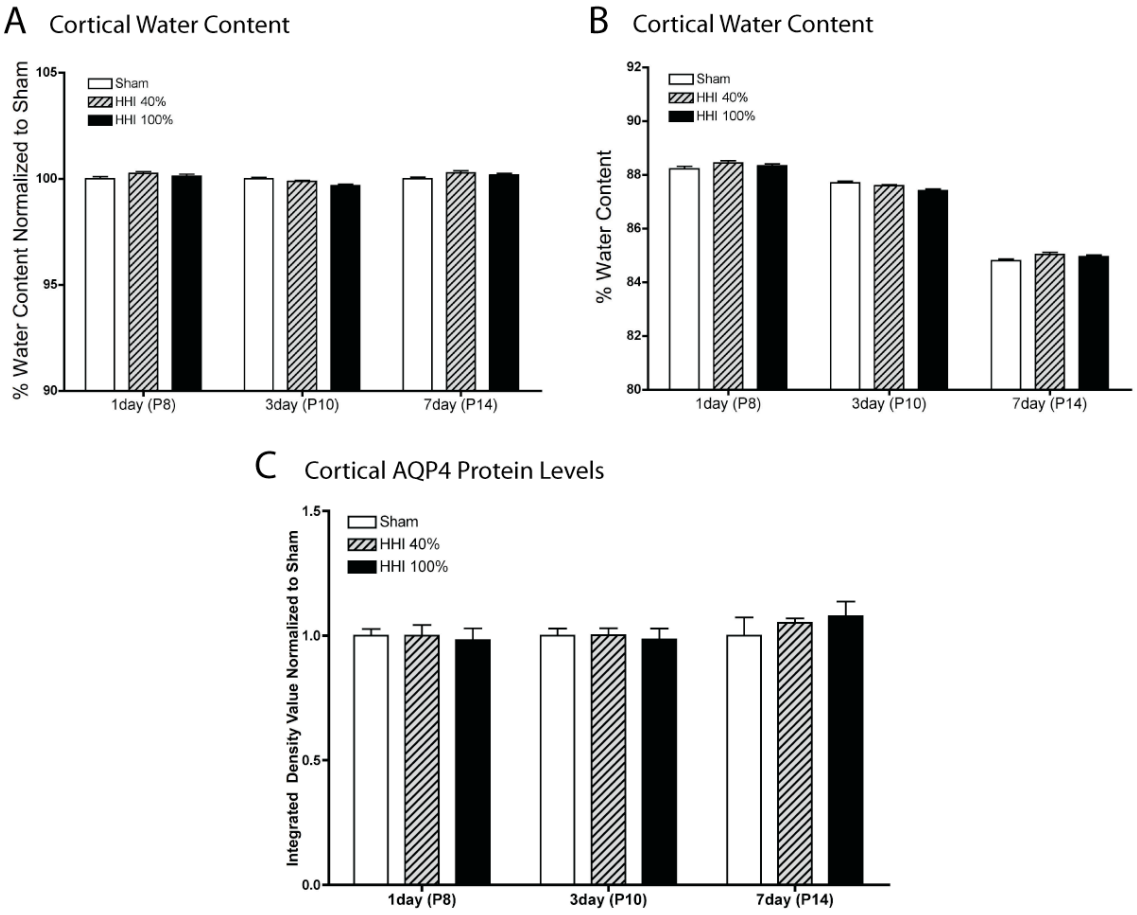


Figure 27. HHI only does not affect cortical water content or AQP4 protein levels.

(A) Quantitative analyses of brain cortex water content changes after moderate (40%) and extreme (100%) hyperoxia only treatment normalized to sham. (B) Results from (A) compared to shams, showing developmental decrease in sham water content with time. (C) Quantitative analyses of AQP4 protein levels after moderate (40%) and extreme (100%) hyperoxia only treatment normalized to sham. Results are presented as mean ± S.E.M.

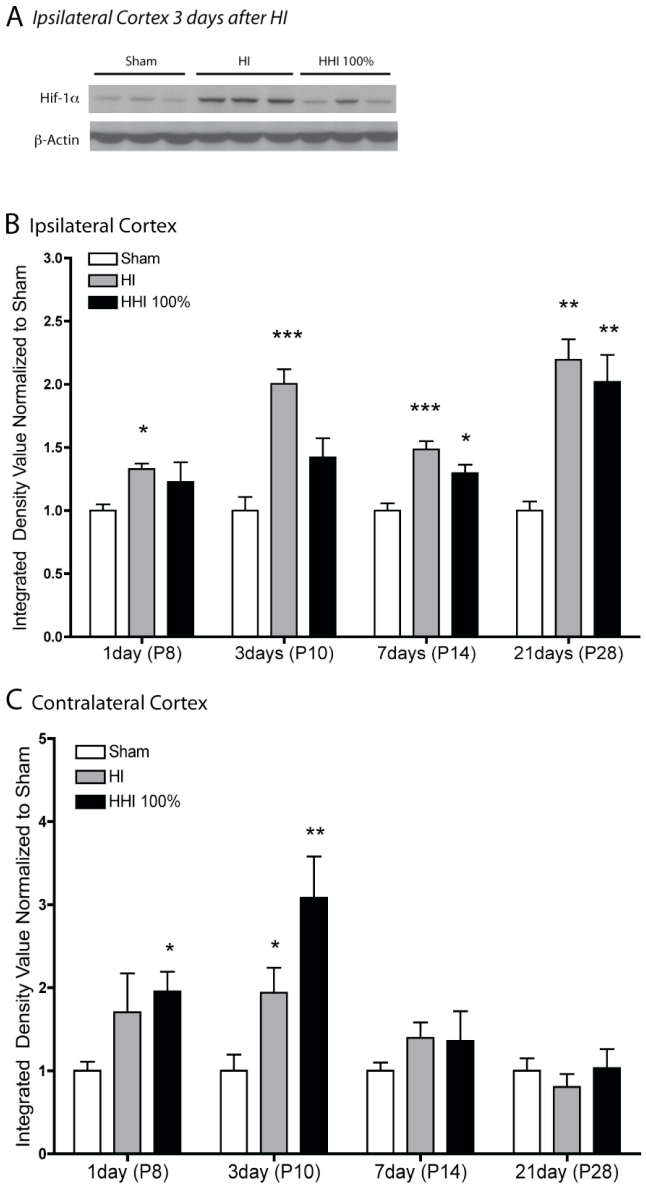


Figure 28. HHI delays the hypoxic insult in the ipsilateral cortex but not in the contralateral cortex after neonatal HI.

(A) Representative Western blot for Hif-1α 3 days after neonatal HI and HHI treatment show an increase in protein levels after the insult (MW 120kDa). (B) Quantitative analyses of densitometric values for Hif-1α protein level temporal changes after HI and HHI in the ipsilateral cortex normalized to Sham. (C) Quantitative analyses of densitometric values for Hif-1α protein level temporal changes after HI and HHI in the contralateral cortex normalized to Sham. Results are presented as mean ± S.E.M. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to Sham.

Astrocytic activation, measured by an increase in the expression of the glial fibrillary acidic protein (GFAP), can determine the extent of HI-induced injury (Li *et al*., 2008). In order to fully evaluate the effect of extreme HHI in the extent of the HI-induced injury, we examined GFAP protein levels by Western blot after HI and HI treated with HHI. We found that HI induced a significant increase in GFAP protein levels in the ipsilateral cortex 1, 3, 7 and 21 days after HI. Interestingly, we found that extreme HHI treatment was able to delay the injury response marked as a decrease in astrocytic activation 1 day after HI (Fig. 29B, sham n=4, HI n=5, HHI 100% n=5) but not at 3 (sham n=4, HI n=5, HHI 100% n=6), 7 (sham n=4, HI n=5, HHI 100% n=5) and 21 days after the insult (sham n=7, HI n=5, HHI 100% n=6).

Nevertheless, in the contralateral cortex, HHI exacerbated the early not-significant increase in GFAP protein levels observed one day post-HI (Fig. 29C, $p<0.05$, sham n=4, HI n=5, HHI 100% n=5). Furthermore, three days after the HI insult, HHI did not prevent HI-induced astrocytic activation ($p<0.05$, sham n=4, HI n=5, HHI 100% n=6). However, 7 days after HI GFAP levels decreased to sham levels and remained low 21 days after neonatal HI without any effect of HHI treatment (7 days, sham n=4, HI n=5, HHI 100% n=5; 21 days, sham n=7, HI n=5, HHI 100% n=6).

These results suggest that HHI as a treatment could be beneficial early after neonatal HI, however, the long-term effects of HI on the hypoxic insult and reactive gliosis are not affected by HHI.

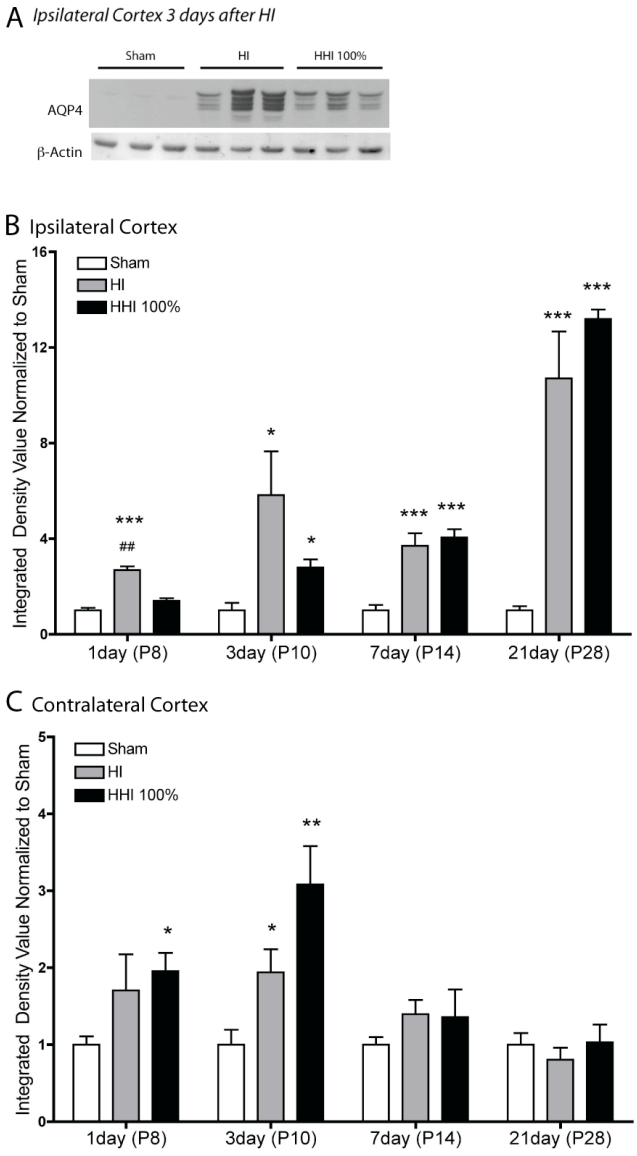


Figure 29. HHI delays astrocytic activation in the ipsilateral cortex but not in the contralateral cortex after neonatal HI.

(A) Representative Western blot for GFAP 3 days after neonatal HI and HHI treatment show an increase in protein levels after the insult (MW 50kDa). (B) Quantitative analyses of densitometric values for GFAP protein level temporal changes after HI and HHI in the ipsilateral cortex normalized to Sham. (C) Quantitative analyses of densitometric values for GFAP protein level temporal changes after HI and HHI in the contralateral cortex normalized to Sham. Results are presented as mean ± S.E.M. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to Sham, ## $p<0.01$ compared to HI One-way ANOVA.

IV.F. HHI DOES NOT AMELIORATE HI-INDUCED MOTOR COORDINATION IMPAIRMENT

We have shown that HI induced motor coordination impairment three weeks after the insult. Furthermore, with respects to edema, our results showed that hyperoxia treatment did not prevent cerebral edema and cyst formation, and even though it delayed the early hypoxic insult and reactive gliosis, HHI did not prevent the long-term changes in astrocytic activation or the outcome of the hypoxic insult after neonatal HI. Thus, we tested the hypothesis that extreme (100% O_2) or moderate (40% O_2) HHI treatment will not ameliorate the long lasting HI-induced motor coordination impairment.

Using the bar holding test (Tchekalarova *et al*., 2005), we found that 21 days after the HI insult, neither extreme nor moderate hyperoxia prevented HI-induced impaired motor coordination (Fig. 30A). Rat pups exposed to HI, or HI with HHI treatment were unable to hold to the elevated bar for more than 75 seconds, compared to sham pups that could stay on the bar for the maximum allowed time of 120 seconds ($p<0.001$).

We further evaluated the effect of HHI treatment after neonatal HI on motor coordination using the wire mesh ascending test (Fig. 30B; Tchekalarova *et al*., 2005; Thonhoff *et al*., 2007). We found that rats exposed to HI took a longer amount of time to climb up the wire mesh ($p<0.01$), and HHI treatment did not improve the HI-induced behavioral outcome.

Together, these results indicate that HHI treatment, either moderate or extreme, did not ameliorate or improve the HI-induced impairment in motor coordination.

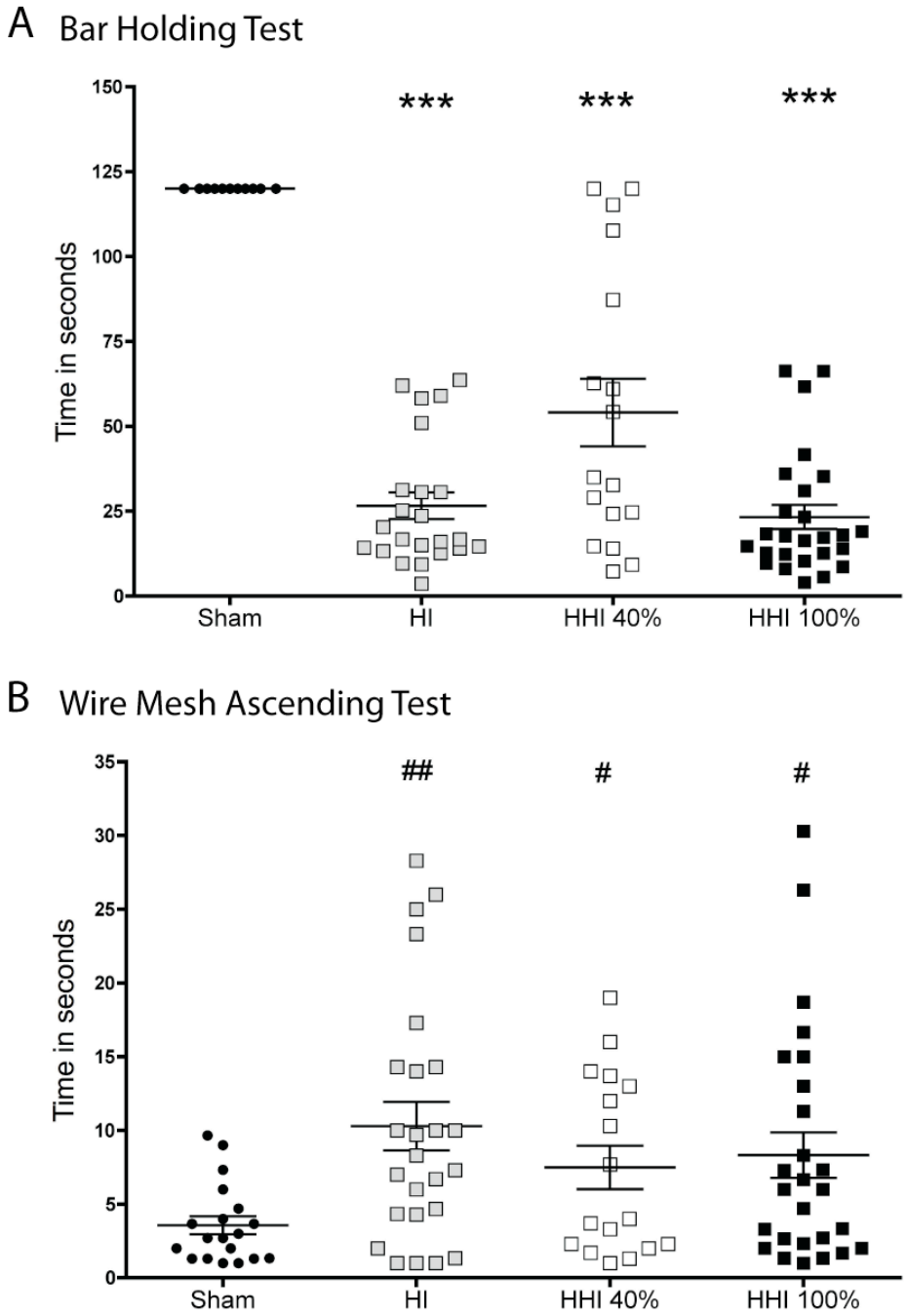


Figure 30. Moderate (40% O_2) or extreme (100% O_2) hyperoxia treatment did not ameliorate the HI-induced long-lasting motor coordination impairment.

Bar holding test (A) and wire mesh ascending test (B). Results are presented as mean ± S.E.M. *** $p<0.001$, # $p<0.05$ and ## $p<0.01$ compared to Sham.

IV.G. DISCUSSION

Even though edema has been shown to develop early in the brains of infants that suffered a Hypoxic/Ischemic event (Soul *et al*., 2001, Boichot *et al*., 2006; Chang *et al*., 2007; Zanelli *et al*., 2008), there are no reports of the effect of hyperoxia treatment on the resolution of edema. Our data shows for the first time a long lasting effect of neonatal HI on increasing brain water content, in part due to vasogenic edema, which is not ameliorated by extreme or moderate hyperoxia treatment a novel and clinically relevant finding. In addition, our results that HHI does not prevent HI-induced permeability of the BBB are in accordance with reports that free radicals disrupt the BBB (Witt *et al*., 2008). Although important for resuscitation and restoration of cerebral blood flow, hyperoxia as a treatment could lead to BBB permeability.

Our data shows that AQP4 after neonatal HI plays a key role in clearance of the ipsilateral cortex water content and the lack of its up-regulation in the contralateral cortex, together with the development of vasogenic edema, prevents the clearance of edema. HHI, either moderate or extreme, did not affect HI-induced changes in AQP4. Furthermore, we evaluated the effect of HHI only on water content and AQP4 protein levels. Our results further confirmed that HHI treatment alone does not affect brain water content and does not regulate AQP4 protein levels.

Apart from previous reports that oxygen supplementation after asphyxia increases the formation of free oxygen radicals and decreases cerebral perfusion (Davis *et al*., 2004; Ahn *et al*., 2008; Koch *et al*., 2008), we have observed that HHI after neonatal HI induced a transient delay in the ipsilateral hypoxic insult and cortical astrocytic activation, depicted by Hif-1α and GFAP protein levels. Nevertheless, this early effect of HHI treatment did not ameliorate long-lasting HI-induced impaired motor coordination.

We have previously addressed the importance of water clearance in the developing brain and the detrimental role of HI-induced edema in long-lasting impaired motor coordination. Consequently, if HHI as a treatment does not spare the developing brain of the HI insult and its detrimental effect on brain development, additional therapies should be applied to the asphyxiated infant. Specifically, due to the key role of AQP4 in clearance of vasogenic edema (Manley *et al*., 2004) and our observed delay in ipsilateral increase in AQP4 and lack of increase in the contralateral cortex, therapies targeted at increasing AQP4 protein levels could favor edema amelioration or clearance.

We conclude that Hyperoxia is an effective resuscitating treatment, but does not have positive effects in the prevention of edema, hypoxic insult, brain injury or motor coordination (Fig. 31).

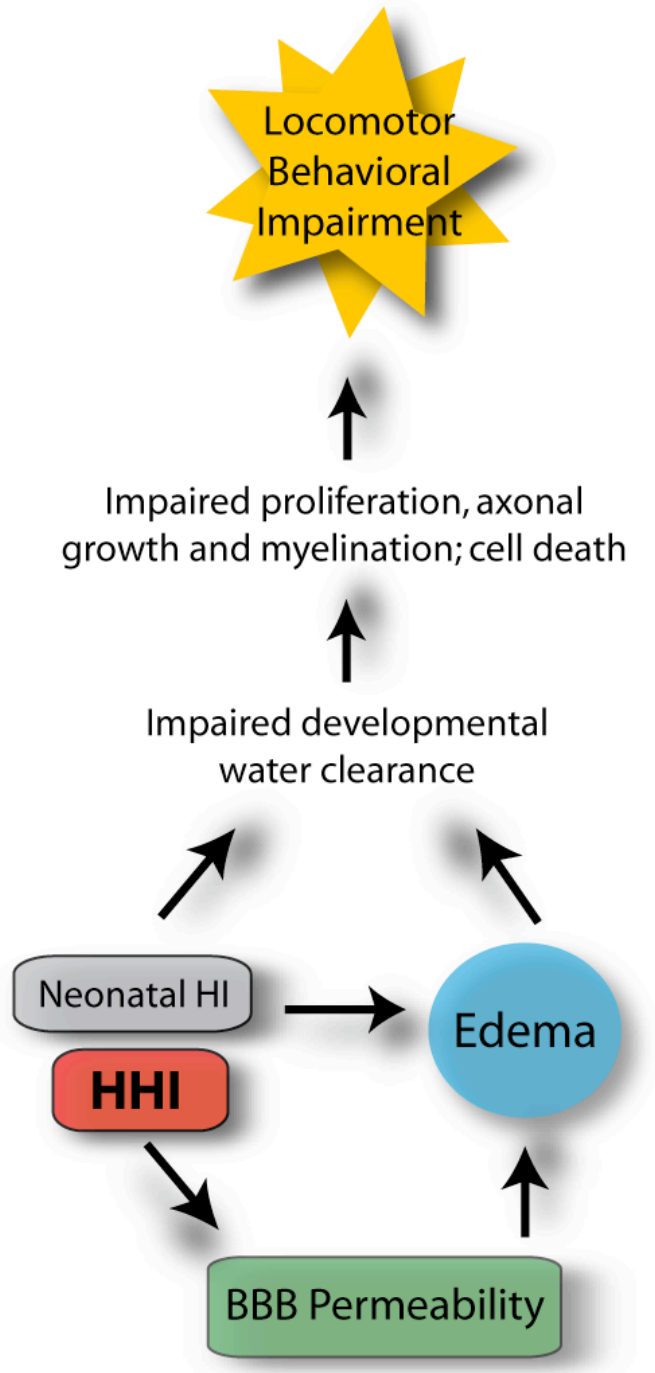


Figure 31. Schematic conclusion for Specific Aim 1 on the characterization of neonatal HI-induced edema and motor coordination.

CHAPTER V

CONCLUSION AND FUTURE DIRECTIONS

In conclusion, we report that neonatal HI induces long lasting edema development, and that the clinically relevant hyperoxia treatment does not prevent it. Both of which are novel findings. Furthermore, our results show for the first time that hyperoxia treatment after HI does not ameliorate HI-induced persistently impaired motor coordination.

To identify the causes of HI-induced cerebral edema, we investigated the effect of HI on the permeability of the BBB. We documented a significantly increased permeability of the BBB after HI suggesting a critical contribution of vasogenic edema to the excessive accumulation of fluid in the ipsilateral and contralateral cortices. The cerebral fluid accumulation after HI was further confirmed using T2-MRI measurements. Nevertheless, hyperoxia treatment after HI did not prevent HI-induced BBB permeability and HI-induced edema. Due to the importance of AQP4 in regulating water transport and edema development, we studied the effect of neonatal HI in AQP4 levels and found that HI induces significant increases of AQP4 protein levels in the ipsilateral cortex over the first three weeks after the insult. In contrast, HI did not affect AQP4 protein levels in the contralateral cortex at all time points studied. Furthermore, our results showed that HHI treatment after HI did not affect HI-induced changes on AQP4 protein levels. Given that developmental increases of AQP4 in the brain are accompanied by significant reduction in water content, we believe that HI-induced increase in AQP4 in the ipsilateral cortex is aimed at protecting the brain, and that the lack of increase in AQP4 levels in the contralateral cortex leads to the development of edema. Therefore, the persistent edema

in both ipsilateral and contralateral cortex after HI will affect brain development and consequently lead to long-term behavioral impairment. Consequently, we found that HI results in a significant dysfunction of motor coordination 21 days after HI, as presented in our results obtained in the bar holding and wire mesh ascending test. However, hyperoxia treatment did not ameliorate or prevent this behavioral outcome. Given that we found motor coordination impairment late after HI (P28), it is likely that this motor dysfunction is permanent and analogous to the motor behavioral changes observed in children who suffered HI before or after birth. We therefore hypothesize that persistent cerebral edema prevents normal brain development and thus lead to a behavioral dysfunction.

Another important finding of our study is that the contralateral cortex is also affected after HI. Thus, in agreement with other reports, strongly suggests that the contralateral cortex should not be used as a control for the changes in the ipsilateral cortex. Furthermore, these results stress the importance of a better understanding of the pathological changes in the contralateral cortex that will contribute to the overall behavioral outcome after HI.

Recent reports, together with the results presented here, strongly suggest that hyperoxia as a treatment lack long-term beneficial outcomes. Therefore, hyperoxia is useful as an acute treatment for a successful resuscitation of infants, but cannot be considered as a long-term therapeutic intervention. Thus, due to the lack of adequate neonatal treatments, alternative therapies should be used to prevent or diminish behavioral impairments in children that suffered a neonatal HI insult. Specifically, due to the fact that our results with hyperoxia treatment did not ameliorate edema after HI, additional therapies should be used to reduce persistent fluid accumulation that will improve the HI-induced behavioral impairment. Accordingly, we propose that directly targeting (i) edema with the use of hyperosomotic solutions and (ii) reducing BBB

permeability, as well as (iii) indirectly manipulating AQP4 protein levels, we can diminish the contribution of edema to the pathological outcomes after HI.

Osmotic therapy with hyperosmolar solutions, such as mannitol or hypertonic saline, is currently used in the clinics to clear edema in adults and children suffering from brain tumors or traumatic brain injury (Knapp, 2005; Rabinstein, 2006). The administration of a hyperosmotic solution will increase the intravascular tonicity (by increasing the serum osmolality) creating an osmotic force that will force movement of fluid from the edematous brain tissue into the vascular space, where it will be removed from the cranium into the circulation. Although hyperosomotic treatment has been applied to children with traumatic brain injury (TBI), it has not been used nor evaluated in neonates after a HI insult. It is important to mention that hyperosmotic treatment in neonates should be constantly monitored in order to avoid side effects such as dehydration and renal failure.

Additionally, it has been reported that adult rats injected (intravenous, intra-arterial, intra-ventricular or intra-cerebral) with mesenchymal stem cells (MSCs) derived from the bone marrow, differentiated into microglia, astrocytic-like cells, endothelial cells, and putative neurons. Most importantly, it is has been shown that an intravenous injection of MSCs facilitate restoration of the BBB and improved the functional outcome after stroke (for a review, Carroll and Borlongan, 2008; Borlongan *et al*., 2004a, Borlongan *et al*., 2004b). Moreover, Yashuhara *et al*. (2008) showed that an intravenous injection of multipotent adult progenitor cells improved neonatal HI-induced impaired motor coordination. However, there are no reports of the effect of MSCs on the restoration of the BBB after neonatal HI. We hypothesize that a therapeutical approach with MSCs injected after neonatal HI and HHI resuscitation, will prevent or restore the

HI-induced breach of the BBB, thus averting long-lasting cerebral edema and lead to a functional recovery.

There are no direct AQP4 inhibitors or activators, so indirectly we could further confirm the role of AQP4 in the development of edema in the brain after neonatal HI. In order to test this hypothesis, we could evaluate the development of edema in AQP4 KO mice or in α-syntrophin KO mice, where AQP4 is expressed but not localized into the cell membrane. However, if working with mice, first we would need to evaluate the development of cerebral edema and locomotor coordination after neonatal HI in wild-type mice. Therefore, alternative strategies should be kept in consideration. Our results suggest a role for AQP4 in the clearance of HI-induced cerebral edema. For this reason, we hypothesize that an early induction of AQP4 protein levels will aid in cerebral fluid clearance and prevent long-lasting neonatal HI-induced effects. It has been reported in an adult model of TBI and stroke that sulforaphane (an isothiocyanate abundantly found in broccoli; Zhao *et al*., 2005, is in clinical trials for cancer therapy - www.clinicaltrials.gov) and the serine protease thrombin (Hirt *et al*., 2009) respectively induce an increase in AQP4 protein levels that lead to a decrease in injury-associated edema. However, sulforaphane and thrombin have not been tested after neonatal HI. Further investigations on the role of these two compounds on AQP4 protein levels and possible prevention of cerebral edema in neonatal models of HI and stroke remain to be tested.

Therefore, our next step will be to treat the pups with mannitol after neonatal HI and evaluate its effect on brain edema clearance and later on motor impairment. In addition, we will treat the pups with mesenchymal stem cell therapy after neonatal HI. We will evaluate its effect on BBB restoration, prevention of edema and furthermore,

locomotor impairment. Finally, we will treat the pups with sulforaphane in order to increase AQP4 protein levels and help clear HI-induced cerebral edema.

Our results have evidenced that hyperoxia as a treatment does not prevent the development of edema and the impaired motor coordination observed after neonatal HI. We believe that these investigations will aid in the finding of appropriate therapies to resuscitate the infants and prevent cerebral edema as a means to minimize the brain injury caused by neonatal asphyxiation.

Abbreviations

ADC:	apparent diffusion coefficient.
AQP4:	aquaporin 4.
BBB:	blood-brain barrier.
BF:	blood flow.
CBF:	cerebral blood flow.
CNS:	central nervous system.
CSF:	cerebrospinal fluid.
DWI-MRI:	diffused weighted imaging of MRI.
GFAP:	glial fibrillary acidic protein.
HI:	hypoxia-ischemia.
HHI:	hyperoxia treatment after HI.
HIE:	hypoxia-ischemia encephalopathy.
Hif-1α:	hypoxia inducible factor 1 alpha.
ICP:	intracranial pressure.
IL-1β:	interleukin 1 beta.
MRI:	magnetic resonance imaging.
MSC:	mesenchymal stem cells.
NMDA:	N-methyl-D-aspartate.
P7:	post-natal day 7.
PKA:	protein kinase A.
PKC:	protein kinase C.
PKG:	protein kinase G.

PVL:	periventricular leukomalacia.
RVM:	Rice-Vannucci model.
SCI:	spinal cord injury.
T2-MRI:	T2-weighted magnetic resonance imaging.
TBI:	traumatic brain injury.
tfMCAO:	temporary filament middle cerebral artery occlusion.
WC:	water content.
WB:	western blot.

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