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

# Apoptotic mechanisms in the neonatal brain following hypoxia-ischemia

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# Abstract


## Apoptotic mechanisms in the neonatal brain following hypoxia-ischemia



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**Introduction:** Neonatal encephalopathy is often perinatally acquired and caused by hypoxia-ischemia (HI). Brain injury develops with a delay, over 12-48 hours, after the insult. Hypothermia, an established neuroprotective treatment, saves 1 infant in 9 from neurological deficits suggesting that there is room for further improvement. HI leads to cell death through multiple pathways, including apoptosis. The aim of this thesis was to investigate different apoptotic pathways and to explore possible apoptotic targets for future pharmacological treatment after perinatal brain injury. We investigated (I) the involvement of caspase-2 alone, (II) and in combination with hypothermia, (III) the role of c-Jun N-terminal kinase (JNK), and (IV) Cyclophilin D (CypD), a regulator of the mitochondrial membrane permeability transition pore..

**Materials and methods:** Wild type (WT) C57BL/6 and transgenic mice with gene deletion of caspase-2 (I, II) and CypD (IV) were used in the ibotenate (excitotoxic)-model (I), and/or Rice-Vannucci´s HI-model (I-IV) at postnatal day 5 (I) or 9 (I-IV). The mixed lineage kinase inhibitor CEP-1347 was used to explore the role of JNK after neonatal HI (III).

**Results:** Caspase-2-deficient mice demonstrated less gray and white matter injury after both neonatal HI and an excitotoxic insult (I). Hypothermia provided additional protection in caspase-2 deficient mice (II). CEP-1347 was neuroprotective in the immature brain, by reducing apoptosis and attenuating microgliosis (III). CypD gene deficiency enhanced HI injury and Bax inhibitory peptide (BIP) reduced injury in the immature brain, whereas CypD deletion protected and BIP had no effect on brain damage in the mature mouse brain. Apoptosis was more pronounced in the immature CypD deficient mice than in WT controls, while adults showed minimal apoptotic activation.

**Conclusion:** Apoptosis has a more prominent role in the immature brain and different pathways leading to cell death after HI are at play in the immature as compared to the adult brain. This suggests that different pharmacological interventions are required in the immature and the mature brain. We suggest that caspase-2 as well as Bax dependent mitochondrial permeabilization are important neuroprotective targets in neonatal HI brain injury.


**Key words:** hypoxia-ischemia, brain, caspase-2, cyclophilin D, MLK, neonatal

# Svensk sammanfattning

Syrebrist i samband med förlossningen kan leda till obotliga hjärnskador och resultera i livslånga handikapp, till exempel cerebral pares, epilepsi och utvecklingsstörning. Tidigare forskning har visat att hjärnskadan ofta utvecklas timmar till dagar efter perioden med syrebrist. Kliniska studier har visat att hjärnskador och risken för neurologiska resttillstånd minskas genom kylbehandling. Att sådan behandling inte bara är effektiv i djurexperimentella studier utan även kliniskt innebär ett stort genombrott, men kylbehandling räddar endast ett barn av nio från att utveckla hjärnskador och för tidigt födda barn kan inte kylbehandlas. Det övergripande målet med forskningen är att hitta en behandlingsstrategi som kan användas i kombination med kylbehandling, men också ensamt till de barn där kylbehandling inte kan/får ges.

Efter syrebrist finns många olika mekanismer och vägar som leder till celldöd och hjärnskada. Genom att specifikt påverka utvalda mekanismer som är viktiga i den nyfödda hjärnan och som aktiveras vid skada, och inte de som fysiologiskt är kritiska för hjärnans utveckling, hoppas vi kunna finna nya strategier som skyddar hjärnan. Den i avhandlingen beskrivna forskningen har fokuserat på att experimentellt kartlägga och påverka de vägar som leder till programmerad celldöd, så kallad apoptos, och på de enzymer som styr celldöden. Fokus har även legat på mitokondrierna, cellernas kraftverk, som tycks bestämma om cellen skall dö genom programmerad celldöd eller överleva.

Två modeller har använts, dels hypoxi-ischemi (HI), dvs. syrebrist i kombination med minskat blodflöde, samt ibotenat-modellen där hjärnan exponeras för en signalsubstans, som i normala fall förmedlar nervsignalerna, men som i för stor mängd kan orsaka hjärnskador. Skadorna som utvecklas i dessa modeller efterliknar skadan väl hos både för tidigt födda och fullgångna barn utsatta för syrebrist i samband med förlossningen. Vi har experimentellt kartlagt vilken roll enzymet caspas-2 spelar vid utvecklingen av hjärnskador hos barn utsatta för syrebrist. Detta har vi gjort genom att slå ut caspas-2, antingen genetiskt genom att ta bort genen i möss (s.k. knock-out teknik), eller genom att blockera syntesen av enzymet med hjälp av siRNA. Caspas-2 är inte nämnvärt involverat i den fysiologiska apoptotiska processen i hjärnan, men caspas-2 finns i hög grad i den omogna hjärnan och nivån sjunker med stigande ålder. Både efter HI och i ibotenat-modellen innebär minskningen av caspas-2 minskade hjärnskador.

Skyddet ökade ytterligare om kylbehandling adderades. Vi har dessutom funnit att kylbehandling är effektivt i en HI modell på nyfödd mus. Gängse behandling idag för syrebrist efter en förlossning är kylbehandling, vilket gör att varje möjlig ny terapi i framtiden troligen kommer att kombineras med kylning. Denna experimentella modell kan således vara ett sätt att pröva olika farmakologiska terapier i kombination med kylbehandling.

I HI-modellen har vi dessutom använt substansen CEP-1347, som genomgått kliniska prövningar hos vuxna för behandling av Parkinson med nedslående resultat, men som har mycket få biverkningar. Vi fann att hos nyfödda råttor skyddar CEP-1347 hjärnan från skador efter syrebrist, vilket gör att den kan vara värd att undersöka närmare i detta sammanhang. Den påverkar inte inflammationen som uppstår i hjärnan efter HI skada, utan minskar apoptosen, genom att påverka c-Jun N-terminal kinase (JNK), ett enzym som är viktigt i den apoptotiska skadeprocessen.

I mitokondrierna finns en port, en så kallad por, varigenom apoptosen styrs. Cyclophilin D (CypD) är ett protein delaktigt i öppnandet av denna por. Återigen användes transgena möss för att klarlägga rollen för detta protein efter HI skada. Här kunde vi påvisa att hjärnskademekanismerna är olika beroende på hjärnans ålder. Där en behandling som kan skydda vuxna, i vårt fall borttagande av CypD, istället ökar skadan hos de nyfödda djuren. Istället visade sig en annan por, som regleras av proteinet Bax, vara viktig hos nyfödda. Att hämma Bax kan vara en sätt att skydda den omogna hjärnan mot skador efter syrebrist.

Sammanfattningsvis ter sig apoptotiska processer vara mycket viktiga i den omogna hjärnan, betydligt viktigare än i den vuxna hjärnan i samband med hjärnskador. Hypotesen är att en substans som hämmar caspas-2 och/eller påverkar mitokondriernas poröppning har möjlighet att bli en användbar terapi i samband med hjärnskador hos nyfödda utsatta för syrebrist. Först måste man dock närmare granska vad det är som aktiverar caspas-2 och mitokondriernas poröppning och hur man bäst kan hämma detta utan att störa andra viktiga funktioner i den växande hjärnan. Forskningen som presenteras i denna avhandling har visat att mekanismerna för hjärnskador är olika i den omogna och i den vuxna hjärnan. Det är alltså viktigt att poängtera att detta kan innebära att en del läkemedel, som utvecklas för behandling av hjärnskador hos vuxna, förmodligen inte kan användas till nyfödda barn. Slutligen är det värt att notera att det finns många möjligheter att påverka apoptosen efter HI. Det är möjligt att en kombinationsbehandling, alternativt skräddarsydd behandling kan bli aktuell i framtiden.

# List of original papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I **Genetic inhibition of caspase-2 reduces hypoxic-ischemic and excitotoxic neonatal brain injury**

Carlsson Y*, Schwendimann L*, Vontell R, Rousset CI, Wang X, Lebon S, Charriaut-Marlangue C, Supramaniam V, Hagberg H**, Gressens P**, Jacotot E**.

*Ann Neurol. 2011 Mar 28. doi: 10.1002/ana.22431*

II **Combined effect of hypothermia and caspase-2 gene deficiency on neonatal hypoxic-ischemic brain injury**

Carlsson Y, Wang X, Schwendimann L, Rousset CI, Jacotot E, Gressens P, Thoresen M, Mallard C, Hagberg H.

*Submitted*

III **Role of mixed lineage kinase inhibition in neonatal hypoxia-ischemia**

Carlsson Y, Leverin AL, Hedtjärn M, Wang X, Mallard C, Hagberg H.

*Dev Neurosci. 2009;31(5):420-6*

IV **Developmental shift of cyclophilin D contribution to hypoxic-ischemic brain injury**

Wang X, Carlsson Y*, Basso E*, Zhu C, Rousset CI, Rasola A, Johansson BR, Blomgren K, Mallard C, Bernardi P, Forte MA, Hagberg H.

*J Neurosci. 2009;29(8):2588-96*

*Both authors contributed equally to this article; **shared senior authorship

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# Abbreviations

| | |
|---|---|
| **AIF** | apoptosis inducing factor |
| **AMPA** | α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid |
| **AP-1** | activator protein-1 |
| **Apaf-1** | apoptotic protease activating factor-1 |
| **ATF-2** | activating transcription factor-2 |
| **ATP** | adenosine triphosphat |
| **Bax** | pro-apoptotic Bcl-2 associated X protein |
| **Bcl-2** | b-cell lymphoma-2 |
| **BIP** | bax inhibiting peptide |
| **$Ca^{2+}$** | calcium |
| **CARD** | caspase recruitment domain |
| **Casp2** | caspase-2 |
| **$Casp2^{-/-}$** | homozygous casp2 knock-out |
| **$Casp2^{+/-}$** | heterozygote casp2 mice |
| **CP** | cerebral palsy |
| **CR3** | complement receptor 3 |
| **CsA** | cyclosporin A |
| **CypD** | cyclophilin D |
| **$CypD^{-/-}$** | cyclophilin D knock-out |
| **Cyt C** | cytochrome C |
| **DIABLO** | direct inhibitor of apoptosis-binding protein with low pl |
| **DISC** | death-inducing signalling complex |
| **EAA** | excitotory amino acids |
| **Figure** | Fig. |
| **GABA** | gamma-aminobutyric acid |
| **GAD** | glutamate decarboxylase |
| **Heterozygote** | het |
| **HI** | hypoxia-ischemia or hypoxic-ischemic |
| **HIE** | hypoxic-ischemic encephalopathy |
| **$H_2O_2$** | hydrogen peroxide |
| **IAP** | inhibitors of apoptosis |
| **i.c.v** | intracerebroventricularly |
| **i.p**. | intraperitoneally |
| **JNK/SAPK** | c-Jun N-terminal kinase or Stress-activated protein kinase |
| **IL** | interleukin |
| **MAP-2** | microtubulus associated protein-2 |
| **MAPK** | mitogen-activated protein kinase |
| **MBP** | myelin basic protein |
| **MCP-1** | monocyte chemoattractant protein-1 |
| **MHC I** | major histocompatibility complex I antigen |
| **MLK** | mixed lineage kinase |
| **MPT** | (mitochondrial) membrane permeability transition |
| **MRI** | magnetic resonance imaging |
| **NE** | neonatal encephalopathy |

| | |
|---|---|
| **NF** | neuronal filament |
| **NMDA** | N-methyl-D-aspartate |
| **NNT** | number needed to treat |
| **NO** | nitric oxide |
| **NOS** | nitric oxide synthase |
| **Olig-2** | oligodendrocyte transcription factor-2 |
| **PCR** | polymerase chain reaction |
| **PIDD** | p53-induced protein with a death domain |
| **PND** | postnatal day |
| **PTP** | permeability transition pore |
| **PVL** | periventricular leukomalacia |
| **RAIDD** | receptor-interacting protein (RIP)-associated ICH-1/CED-3-homologous protein with a death domain |
| **ROS** | reactive oxygen species |
| **SEM** | standard error of the mean |
| **siRNA** | small interfering RNA |
| **Smac** | second mitochondria-derived activator of caspase |
| **TNF-α** | tumour necrosis factor-α |
| **TUNEL** | terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling |
| **WT** | wild-type |
| **XIAP** | X-linked inhibitor of apoptosis |

# Introduction

Asphyxia originates from the greek word for pulselessness. Today however, the term asphyxia is generally used for a condition with severly deficient gas exchange leading to hypercapnia and hypoxemia and ultimately severe lactic acidosis [Fatemi, et al., 2009]. Asphyxia can arise during pregnancy, known as chronic asphyxia, and can be caused by an underlying disease in the mother or the placenta. Asphyxia can also arise during delivery, so called perinatal asphyxia, due to for example placental abruption, rupture of the uterus, umbilical cord compression or maternal hypotension.

Neonatal encephalopathy (NE) is a clinically defined syndrome of disturbed neurological function in the earliest days of life in the term infant, manifested by difficulty with initiating and maintaining respiration, depression of tone and reflexes, subnormal level of consciousness and often seizures. Also preterm babies can present with this, but the signs are often more subtle [du Plessis and Volpe, 2002]. Several different etiologies, such as metabolic disease, infection, drug exposure, nervous system malformation and neonatal stroke are possible causes of neonatal encephalopathy and should be excluded, but generally the term NE refers to hypoxic-ischemic encephalopathy (HIE) resulting from perinatal asphyxia.

Although questioned [Nelson and Chang, 2008], som recent studies have shown that the majority of children with neonatal encephalopathy, seizures or both, but without specific syndromes or congenital heart defects, had evidence of perinatally acquired insults and that the majority of cerebral palsy (CP) both in preterm and in term seem to arise during the perinatal period [Jacobsson, et al., 2002; Cowan, et al., 2003; Himmelmann, et al., 2010]. The overall CP prevalence in Sweden between the years of 1999-2002 was 2.18/1000 live births [Himmelmann, et al., 2010].

Perinatal asphyxia at term often results in a dyskinetic cerebral palsy, which is associated with multiple handicaps with a larger impairment load, such as only 16% walking without aids, 60% being bound to a wheelchair and 50% suffering from epilepsy, than other forms of CP and hence a larger need of aid from society

[Himmelmann, et al., 2009]. The prognosis for HIE in preterm is also depressing; Logitharajah et. al [Logitharajah, et al., 2009] rapported recently a 2-year outcome where one third had died, nearly a quarter had developed a severe form of CP and only one third of the infants had normal outcomes.

In the last ten years a significant increase in prevalence in children born at term and with dyskinetic CP has been reported, in spite of decreasing neonatal mortality [Himmelmann, et al.; Sellier, et al.]. The cause behind this is unknown.

# Perinatal brain injury

## *Clinical background*

The preterm white matter is more susceptable to hypoxic-ischemic (HI) injury, because of the vulnerability of immature oligendrocytes prior to myelination [Dammann, et al., 2001; Leviton and Gressens, 2007; Logitharajah, et al., 2009]. This gives rise to the pathological diagnosis periventricular leukomalacia (PVL), which shows a reduction in myelination leading to a lack of white matter. PVL represents the typical response of the preterm brain to an insult, either for example HI or damage caused by cytokines associated with infection [Rennie, et al., 2007]. Children born at term with HIE injury often have cortical/subcortical lesions as well as basal ganglia lesions [Fatemi, et al., 2009; Himmelmann, et al., 2010]. Lesions in the basal ganglia, thalamus and the internal capsule are predictive of CP [Rutherford, et al., 2005]. These different patterns of injury might however not be as distinctly separated as previously thought. MRI studies have lately questioned this, showing a high degree of basal ganglia damage also in preterm infants [Logitharajah, et al., 2009].

## *Therapeutic window*

HIE is not a single event but rather an evolving process [Ferriero, 2004]. MRI studies show progression of lesion size over the first few days after injury [Cowan, et al., 2003]. After the initial decrease in oxidative energy metabolism during HI, at least

partial recovery occurs [Wyatt, et al., 1989; Yager, et al., 1992; du Plessis and Volpe, 2002], before a secondary decline follows approximately 6-15 hours later. Hence, many neurons and other cells seem to "commit" to die or survive over a period of days to weeks [Beilharz, et al., 1995; Ferriero, 2002; Gunn and Thoresen, 2006]. Those infants who did not show even a transient recovery suffer from a very high mortality and neurodevelopmental outcome has been shown to be closely associated with the degree of secondary energy failure after 24 to 48 hours [Roth, et al., 1997]. This biphasic pattern creates a "window of opportunity", where intervention might be able to affect the outcome later in life (figure 1 (Fig.)).

Fig.1

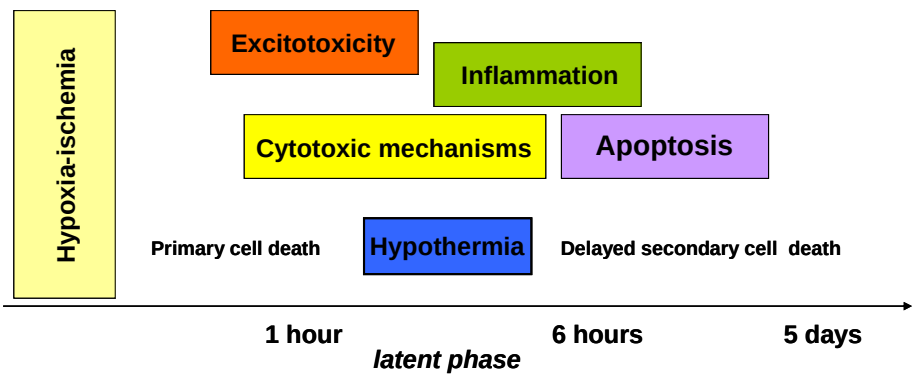


Fig.1 Cell death
Cell death occurs in a two-phased manner, where neuroprotective intervention during the latent phase, before delayed cell death starts, can be effective.

## *Mechanisms – primary injury*

During the initial phase of HI there is rapid depletion of adenosine-triphosphate (ATP) leading to disruption of ATP-dependent processes, such as failure of the $Na^+/K^+$-pump and depolarization of the cell membrane [Wyatt, et al., 1989; Yager, et al., 1992; Dirnagl, et al., 1999]. The depolarization of neurons and glia also leads to the release of excitatory amino acids into the extracellular space. Reuptake mechanisms, which are ATP-dependent, become compromised leading to accumulation of glutamate to excitotoxic levels and the overactivation of N-methyl-D-aspartate (NMDA) receptors increases intracellular calcium ($Ca^{2+}$) levels

[Vannucci and Hagberg, 2004]. The increased level of intracellular $Ca^{2+}$ causes influx of $Ca^{2+}$ into cell mitochondria, thereby uncoupling oxidative phosphorylation, triggering ATP hydrolysis and causes mitochondrial swelling or if only a limited amount of mitochondria is involved evokes the release of pro-apoptotic proteins into the cytosol [Dirnagl, et al., 1999; Puka-Sundvall, et al., 2000].

### *Mechanisms – secondary injury*

The primary insult is often followed by at least a partial restoration of cell energy metabolism [Wyatt, et al., 1989; Yager, et al., 1992], before a secondary decline of energy failure follows. Exactly what triggers this secondary decline is unknown; however this secondary phase is characterized by excessive entry of $Ca^{2+}$ into cells, induction of free radicals such as reactive oxygen species (ROS) and nitric oxide (NO), another wave of excitotoxic amino acids (EAA) release, inflammatory reactions and apoptosis [McRae, et al., 1995; Bona, et al., 1999; Blomgren and Hagberg, 2006; Northington, et al., 2011b].

## Cell Death – Apoptosis and Necrosis

The form of cell death depends on the severity of injury and on the brain area involved. Necrosis predominates in more severe cases and in an earlier phase and in the forebrain, whereas apoptosis occurs in areas with milder ischemic injury, often days after the insult, localized at the border of the insult and are especially centered to the thalamus and brainstem but also in other areas [Beilharz, et al., 1995; Hu, et al., 2000; Northington, et al., 2001; Puyal, et al., 2009]. Necrosis is associated with swelling of the cytoplasm and organelles and leakage of cytoplasmic contents into the extracellular space leads to a secondary inflammatory response [Vannucci and Hagberg, 2004]. Apoptosis, on the contrary, is highly regulated and an energy requiring process whereby the cell commits to suicide [Orrenius, et al., 2003]. Several studies have shown an important role of apoptosis in HI injury in the neonatal brain as opposed to the adult brain [Beilharz, et al., 1995; du Plessis and Volpe, 2002; Johnston, et al., 2002; Zhu, et al., 2005; Zhu, et al., 2007b]. The

newborn brain is primed to respond to various insults with the activation of apoptotic cascades due to the importance of programmed cell death in the normal development of the central nervous system. Pro-apoptotic proteins are highly expressed in the developing brain [Zhu, et al., 2005; Wang, et al., 2009]. In addition to necrosis and apoptosis, neurons in rodents subjected to neonatal HI are showing morphology intermediate between that of classic apoptosis and necrosis [Leist and Jaattela, 2001; Northington, et al., 2007]. The nuclei of such cells have large, irregularly shaped chromatin clumps, similar to apoptotic neurons, but the cytoplasm shows changes similar to necrotic neurons. This morphology coincides with mitochondrial energy failure and activation of apoptotic pathways after neonatal HI. Mitochondrial energy failure likely prevents execution of a full apoptotic phenotype and it is presumed that mitochondrial failure may interrupt apoptotic cascades initiated by injury to the immature rodent brain, resulting in the hybrid phenotype of neuronal cell death [Northington, et al., 2007; Northington, et al., 2011b]. Lately it is becoming clear that necrotic cell death can be as controlled and programmed as caspase-dependent cell death. This so-called necroptosis has been shown in the delayed phase of neonatal brain injury [Northington, et al., 2011a].

## Apoptotic pathways

Multiple apoptotic pathways have been shown to be involved in neonatal HI cell death [Vannucci and Hagberg, 2004; Northington, et al., 2011b]. There are at least two broad pathways that lead to apoptosis, an "intrinsic" and an "extrinsic" pathway. More than 30 proteins in the family of B-cell lymphoma-2 (Bcl-2), which regulates apoptosis, have been described. They can be divided into two groups: anti- and pro-apoptotic members [Orrenius, et al., 2003]. In the normal state a fine balance is maintained between the pro-apoptotic proteins such as Bak, Bid and Bax (pro-apoptotic Bcl-2 associated X protein), with their BH-3 death domain and the anti-apoptotic proteins such as Bcl-2 and Bcl-XL.

## *Caspases*

**C**ysteine-dependent **asp**artate-directed prote**ases** (caspases) are a group of intracellular proteases preserved through evolution [Siegel, 2006]. They are essential for the execution step in apoptosis and they also mediate inflammation and apoptosis in the neonatal brain [Johnston, et al., 2002]. In humans, as well as in mouse, 14 members have been identified so far [Riedl and Yigong, 2004]. All executioner caspases are pro-enzymes, which can be cleaved and activated by other caspases, in a cascade-like manner. The caspase protein contains three domains, an amino-terminal prodomain, a large subunit (~20kDa) and a small subunit (~10kDa). Caspases can be classified as initiator or upstream caspases, such as caspase-2, -8, -9, or as effector or downstream caspases, such as caspase-3, -6, and -7, as defined by their place in the cascade. A third group of caspases is involved in mediating inflammatory reactions, for example caspase-1 activates interleukin (IL)-18 and IL-1β. Caspases are inhibited by the inhibitor of apoptosis protein (IAP) family. X-linked inhibitor of apoptosis (XIAP) for example inhibits caspase-3, -7 and -9. Caspases have been found to be critically important during brain development and lacking for example caspase-3 and caspase-9 results in severe malformation of the nervous system [Kuida, et al., 1996; Hakem, et al., 1998; Kuida, et al., 1998; Zheng, et al., 2000]. Studies have shown that when one caspase is being knocked out, others tend to increase compensatorily [Troy and Salvesen, 2002].

## *Caspase-2*

Caspase-2 (Casp2) is an initiator caspase, a key enzyme in the route to destruction. Casp2 is developmentally regulated and can initiate mitochondrial outer membrane permeabilization [Enoksson, et al., 2004]. Casp2 activity has been shown to increase after HI in immature as opposed to adult mice [Wang, et al., 2009]. Casp2 plays a role in stress-induced apoptosis and can be induced by DNA damage through ultraviolet radiation, trophic factor withdrawal and cytokine deprivation [Robertson, et al., 2002; Zhivotovsky and Orrenius, 2005]. Like other caspases, pro-casp2 contains a long pro-domain, closely related to the structure of caspase-9, both containing a caspase-recruitment domain (CARD), important for its ability to induce

cell death [Paroni, et al., 2002]. Pro-casp2 also contains two subunits, but the cleavage specificity is more related to the effectors caspase-3 and -7. Hence casp2 is unique having features of both initiator and effector caspases. It can be spliced into two different mRNAs; Caspase-2L a pro-apoptotic enzyme and Caspase-2S which have antagonistic effects on cell death. The expression of casp2 varies with developmental stage [Wang, et al., 2009] and is the only pro-caspase present constitutively in the nucleus. Pro-casp2 is thought to associate with receptor-interacting protein (RIP)-associated ICH-1/CED-3-homologous protein with a death domain (RAIDD), and together with p53-inducible death domain-containing protein (PIDD) forming the PIDDosome complex [Zhivotovsky and Orrenius, 2005; Baptiste-Okoh, et al., 2008; Vakifahmetoglu-Norberg and Zhivotovsky, 2010]. Casp2 is also known to be able to dimerize by itself, hence activation do not always require cleavage [Orrenius, et al., 2003]. Casp2 can cleave Bid, but depletion of Bid does not stop casp2 from releasing cytochrome C (Cyt C) and Smac (second mitochondria-derived activator of caspase) also known as DIABLO (direct inhibitor of apoptosis-binding protein with low pl) from the mitochondria [Guo, et al., 2002]. Casp2 can in addition cleave and interact with Bax [Kumar and Vaux, 2002; Chauvier, et al., 2005], but is also able to induce the release of Cyt C independently [Robertson, et al., 2004b]. Casp2 can also induce the release of apoptosis inducing factor (AIF). AIF translocates to the nucleus and induces caspase-independent apoptosis by causing chromatin condensation and large-scale DNA fragmentation in the nucleus [Guo, et al., 2002]. Casp2 has also been shown to be involved in c-Jun N-terminal kinase (or Stress-activated protein kinase; JNK/SAPK) as well as mitogen-activated protein kinase (MAPK) p38 activation [Dirsch, et al., 2004]. Casp2 is activated prior to Caspase-9 and -3 [Dirsch, et al., 2004]. In contrast to this picture of being a key caspase, casp2 knock-out mice develop normally, besides excess numbers of germ cells in the ovaries and an enhanced cell death in facial motor neurons [Bergeron, et al., 1998; Zhivotovsky and Orrenius, 2005]. Caspase inhibition has been shown to promote survival and functional outcome in a variety of neurological disease models [Rideout and Stefanis, 2001; Orrenius, et al., 2003; Northington, et al., 2005].

## *Intrinsic pathway*

DNA damage, infection and the presence of free radicals can trigger the intrinsic apoptotic pathway [Rudel, et al., 2010]. Bax and/or Bak are activated. Bax translocates to the mitochondria, where Bak is already present in the outer mitochondrial membrane and both can through independent oligomerization in the outer mitochondrial membrane create a pore, through which multiple proteins including Cyt C, Smac/Diablo, endonuclease G, Omi/HtrA2 and AIF can escape. Cyt C activates the apoptosome complex [Kroemer and Martin, 2005; Siegel, 2006] with apoptosis activating factor-1 (Apaf-1) and pro-caspase-9 in the presence of dATP. Smac/DIABLO release to the cytosol contributes to apoptosis by interacting with endogenous IAPs, thereby enhancing caspase-3 and -9 activities, as does Omi/HtrA2. This results in the activation of pro-caspase-9, which is followed by conversion of procaspase-3 to active caspase-3 [Benjelloun, et al., 2003]. Caspase-3 activation results in proteolysis of essential cellular proteins, including cytoskeleton proteins and kinases, and is required for DNA fragmentation and the morphological features associated with apoptotic cell death [Jänicke, et al., 1998] (Fig.2).

The intrinsic pathway might also operate through caspase-independent mechanisms such as through AIF referred to above. The translocation of AIF from the mitochondria to the nucleus is triggered by the release of molecular signals from the nucleus like poly(ADP-ribose) monomers. The AIF translocation to the nucleus after HI depends on the formation of an AIF-cyclophilin A complex [Zhu, et al., 2007a] in the cytosol. Movement of AIF into the nucleus leads to chromatinolysis and is greater in the immature brain than in the adult [Zhu, et al., 2003] (fig.2).

## *Extrinsic pathway*

A number of cell surface receptors respond, through cross-linking of death receptors, to cytokine (inflammatory) stimulation, resulting in activation of cell death signalling programs, known as the extrinsic apoptotic pathway. Among those, Fas death is one of the most extensively studied. Fas receptors activate caspase-8, which cleaves Bid. Bid induces the translocation, oligomerization and insertion of Bax and/or Bak into

the mitochondrial outer membrane leading to mitochondrial permeabilization [Fatemi, et al., 2009; Rudel, et al., 2010]. Caspase-8 can also, through activation of the death-inducing signalling complex (DISC) activate caspase-3 directly [Siegel, 2006]. Caspase-8 and the DISC complex is also known to be able to activate casp2 [Vakifahmetoglu-Norberg and Zhivotovsky, 2010]. HI activates Fas death receptor signalling in the neonatal brain [Vannucci and Hagberg, 2004]. The intrinsic apoptosis cascade can also be activated following Fas death receptor signalling and functions to amplify Fas-mediated cell death (fig.2).

## *The mitogen-activated protein kinases*

MAPKs are serine/threonine kinases. They regulate a diverse array of functions, such as neuronal survival, cell growth and proliferation as well as apoptosis, all depending on the stimuli and cell-type involved in the activation [Abe, et al., 2000; Saporito, et al., 2002; Bogoyevitch, et al., 2004]. They are activated by MAPK kinases (MAPKK), which can be induced by growth factors or cytokines as well as cell-stressors. Three main pathways can be discerned: firstly the extracellular signal-regulated protein kinases (ERKs), mainly involved in proliferation and cell survival, secondly p38 MAPK regulating mainly inflammatory cytokines and thirdly JNK. JNK is a mediator of stress-induced apoptosis, shown to be activated by growth factors and cytokines. Three isoforms exist, where JNK1 is constitutively expressed and JNK3 is stress-induced and the isoform that exists in the brain. JNK is activated by MKK4 and MKK7, which are two MAPKs that act directly on JNK. Mixed lineage kinase (MLK) 3 can activate MKK4/7, but MLK3 can also activate p38. An interaction between casp2 and JNK has been observed in some cells and JNK inhibition has been shown to partially inhibit casp2 processing [Zhivotovsky and Orrenius, 2005], suggesting that JNK contributes to casp2 dependent apoptotic signalling upstream of mitochondria [Dirsch, et al., 2004]. The loss of JNK1 and JNK2 in combination has been shown to be embryonically lethal [Bogoyevitch, et al., 2004], while $JNK3^{-/-}$ are protected both from adult [Kuan, et al., 2003; Bogoyevitch, et al., 2004] and neonatal HI damage [Pirianov, et al., 2007]. JNK is activated upstream of mitochondria and JNK inhibition reduces Cyt C release as well as caspase-9 and -2 release and subsequent DNA fragmentation, probably via Bim, a

Bcl-2 family protein [Bogoyevitch, et al., 2004; Dirsch, et al., 2004; Gao, et al., 2005; Pirianov, et al., 2007]. JNK is activated by ROS induction and N-acetylcysteine treatment inhibits JNK [Dirsch, et al., 2004]. JNK phosphorylates c-jun [Pirianov, et al., 2007; Nijboer, et al., 2010], but other targets exist as well such as activating transcription factor-2 (ATF-2). ATF-2 is expressed in neurons and involved in neuronal migration, however over-expression results in cell death [Pearson, et al., 2005]. Activator protein-1 (AP-1) is a transcription factor, which is a heterodimeric protein, composed of the proteins c-Fos, c-Jun and ATF-2. AP-1 is activated by JNK [Vexler, et al., 2006]. JNK can also cleave Bid, resulting in jBid releasing Smac/Diablo from the mitochondria [Deng, et al., 2003].

## *Mitochondrial permeabilization*

Mitochondrial dysfunction plays a central role in the delayed mechanisms of brain cell injury [Hagberg, et al., 2009]. As mentioned before, mitochondrial respiration is markedly decreased in a biphasic pattern, with an initial decrease immediately after HI which then recovers to almost normal levels at 3 hours followed by a secondary diminution 8-24 hours after HI [Gilland, et al., 1998]. Mitochondrial permeabilization plays an important role as an event that marks the point of no return in multiple pathways to cell death [Hagberg, et al., 2009]. Several apoptotic pathways converge upon the mitochondria and can induce mitochondrial permeabilization. In the acute phase of brain cell injury $Ca^{2+}$ is accumulating intracellularly due to lack of ATP. As a consequence mitochondria try to regulate the intracellular environment and increase their uptake, resulting in an increase in the permeability of the inner mitochondrial membrane, loss of membrane potential ($\Delta\Psi$), mitochondrial swelling and rupture of the outer mitochondrial membrane referred to as mitochondrial membrane permeability transition (MPT). Hence, an increase in intracellular $Ca^{2+}$ can cause MPT [Robertson, et al., 2004a]. The opening of the permeability transition pore (PTP) in the inner mitochondrial membrane is crucial for MPT. The exact composition of the PTP and its regulation is still not completely known. Cyclophilin D (CypD), a mitochondrial member of the cyclophilin family with enzymatic capacity and a crucial role in protein folding, is believed to be a main regulator of MPT in the adult brain [Orrenius, et al., 2003; Tsujimoto, et al., 2006].

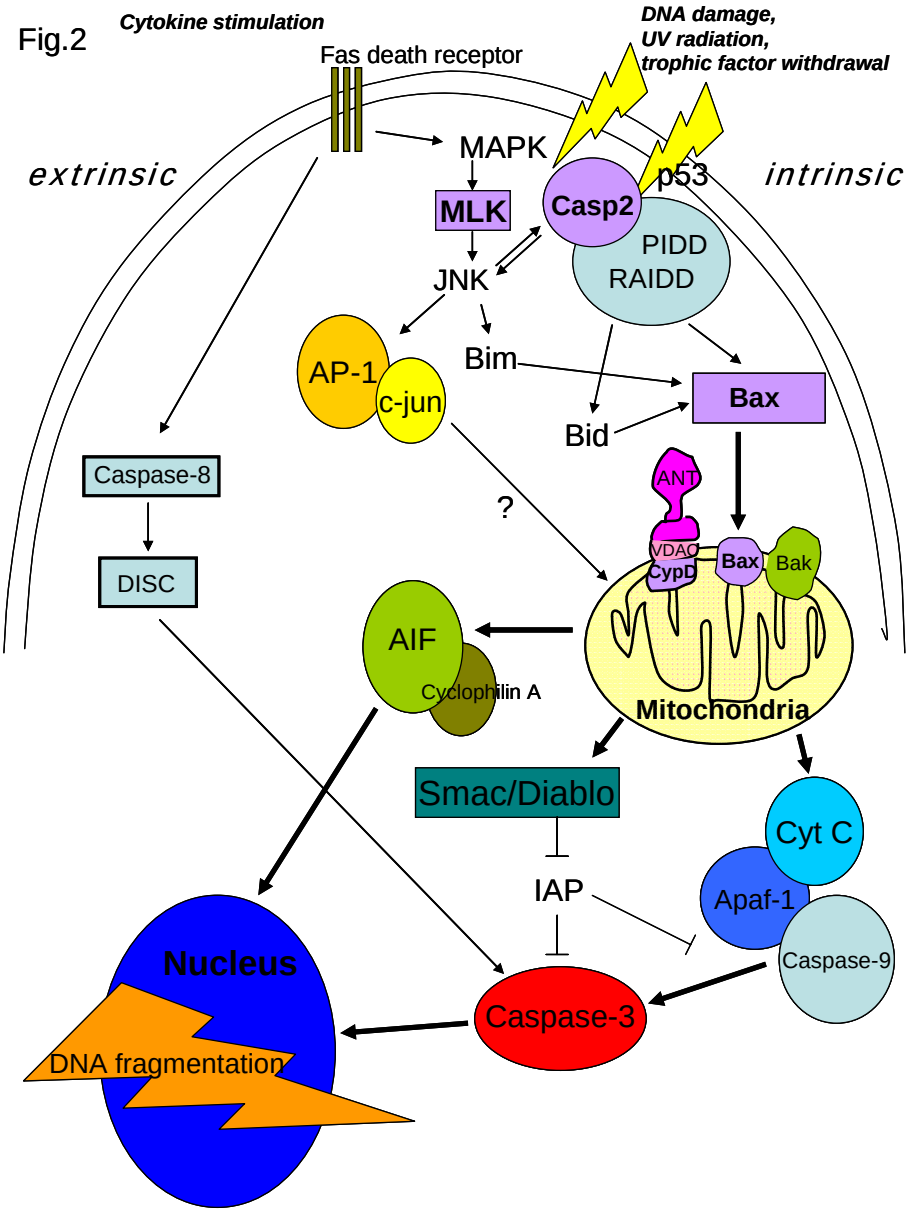


**Fig.2 Apoptosis pathway**
There are at least two broad pathways leading to apoptosis, an extrinsic and an intrinsic pathway. In both pathways, signalling results in the activation of a family of cysteine proteases, named caspases which act in a proteolytic cascade to dismantle and remove the dying cell.

CypD resides in the mitochondrial matrix, but associates with the inner mitochondrial membrane during the MPT. VDAC and ANT are two other proteins thought to be part of the PTP together with CypD, however this has been questioned lately and they might only have regulatory functions [Tsujimoto, et al., 2006; Rasola, et al., 2010]. MPT is inhibited by Cyclosporine A (CsA), a known CypD inhibitor. However, in the developing brain, Bax seems to play a more prominent role for mitochondrial permeabilization primarily of the outer mitochondrial membrane [Hagberg, et al., 2009]. HI in the neonatal brain has been shown to induce membrane permeabilization and lead to the release of pro-apoptotic proteins, such as Cyt C, AIF, Smac/DIABLO and caspase-9, from the mitochondrial intermembrane space into the cytoplasm [Wang, et al., 2004; Tsujimoto, et al., 2006]. Inhibition of both AIF and caspases are protective indicating that inhibiting the result of mitochondrial permeabilization is neuroprotective [Zhu, et al., 2007b].

# Inflammation

Fetomaternal infection is known to increase the risk of CP [Jacobsson, et al., 2002]. The inflammatory response and cytokine production, which follows an infection, seem to render the brain more vulnerable to later HI, even when the infection is distant from the brain [Eklind, et al., 2001]. Cytokines are a group of soluble proteins and peptides which act at low concentrations and play an important role in cell to cell communication and in modulating the functional activities of individual cells. Cytokines, such as IL-6 and chemokines such as monocyte chemoattractant protein-1 (MCP-1) are ubiquitous signalling molecules that help regulate growth, development and acute responses such as fever and inflammation [Dammann and O'Shea, 2008]. Inflammatory cytokines are associated with neonatal HIE and the cytokine level of IL-6 and IL-8 are associated with the degree of HIE [Sävman, et al., 1998]. IL-1β and IL-6 are elevated in newborns who have evidence of perinatal brain damage as compared to controls [Dammann and O'Shea, 2008]. After experimental neonatal HI an up-regulation of many inflammatory genes, including IL-6, associated with cellular activation of microglia in the injured hemisphere occurs [Bona, et al., 1999]. IL-6 is neuroprotective after excitotoxic injury in rat hippocampus [Pizzi, et al.,

2004]. IL-1β triggers the recruitment of neutrophils and is increased 3-6 hours after neonatal HI [Hagberg, et al., 1996]. It plays a role in worsening HI injury after infection and antagonism protects the neonatal brain [Hagberg, et al., 1996; Vexler and Yenari, 2009]. IL-1β is often used as a marker of inflammation after HI [Hagberg, et al., 1996]. MCP-1 is a chemokine produced locally in the brain [Fox, et al., 2005], released from microglial cells, regulating monocyte accumulation. MCP-1 increases 4 hours after neonatal HI and remains elevated until 48 hours after HI [Ivacko, et al., 1997]. JNK is involved in the induction of MCP-1 production [Zhou, et al., 2007]. It appears, therefore, that inflammatory processes may either potentiate HI-induced injury or exert a neuroprotective effect, all depending on context [Nijboer, et al., 2008; Vexler and Yenari, 2009].

## *Microglia*

Microglial cells are resident macrophages in the brain and the main cell type providing immunosurveillance in the brain. Microglial aggregation has been observed in human infants after HI insults. Microglia change their phenotype depending on activation and they are activated and seen within hours after HI in the immature brain; coinciding with DNA degradation. They reach maximal levels after 2-3 days and the number of activated microglia remain elevated up to 14 days after an HI insult [Beilharz, et al., 1995; McRae, et al., 1995; Bona, et al., 1999]. Although microglia have been suggested to play an important role in removing cellular debris and stimulating tissue regeneration it has also been suggested that microglia play a role in inflammatory and injurious processes after HI injury [Hagberg, et al., 1996; Hedtjarn, et al., 2002; Svedin, et al., 2007]. Microglia may contribute to secondary brain injury through the production of pro-inflammatory cytokines, ROS, NO, complement factors, and excitotoxic neurotransmitters [Fatemi, et al., 2009]. Overall microglia activation is associated with increased cell loss [Barrett, et al., 2007]. It appears that the way they are activated plays a crucial role if they are harmful or not [Schwartz, et al., 2006]. Caspase-8 as well as caspase-3 and -7 have been shown to be able to regulate microglia activation [Burguillos, et al., 2011].

# Excitotoxicity

Excitotoxicity, which refers to excessive glutamatergic activation leading to cell injury and death, is an important mechanism of injury in the neonatal brain [Johnston, et al., 2002; Vannucci and Hagberg, 2004]. Glutamate is the predominant excitatory amino acid neurotransmitter in the brain. There are three major groups of glutamate receptors within the post-synaptic membrane; NMDA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainic acid. The glutamate transporter is dependent on a sodium gradient created by $Na^+/K^+$ ATPase that is powered by anaerobic glucose metabolism, and impaired delivery of glucose to the brain by ischemia and/or hypoglycemia impairs glutamate removal from the synapses. Glutamate has been shown to accumulate in the brain in asphyxiated neonatal lambs [Hagberg, et al., 1987] as well as in cerebrospinal fluid in asphyxiated human infants [Hagberg, et al., 1993] and this coincide with an increase in intracellular $Ca^{2+}$ and pro-apoptotic pathways via caspase-3 [Vannucci and Hagberg, 2004]. The excitatory neuronal circuits, important for synaptic plasticity, as well as the expression of specific glutamate receptor subtypes in excitatory synapses, changes during development in the perinatal brain, and these changes can be related to the changing patterns of pathology at different gestational ages [Grafe, 1994; Johnston, 1995; Blomgren and Hagberg, 2006]. The distribution and molecular characteristics of NMDA-type glutamate receptors appear to be an especially important determinant of the pattern of neuronal injury in the perinatal brain [Vannucci and Hagberg, 2004; Johnston, 2005; Kaindl, et al., 2009]. The immature NMDA receptor channels open more easily and flux more $Ca^{2+}$ than their adult counterparts. NMDA receptors probably mediate much of the injury to neurons in structures such as cerebral cortex, basal ganglia, hippocampus and thalamus associated with HI injury in animal models [Johnston, 1995]. Topiramate is an AMPA antagonist [Liu, et al., 2004] showing promising treatment effects in combination with hypothermia. The immature brain can withstand longer periods of energy deprivation than the adult brain because of its low energy requirement [Barrett, et al., 2007], yet when a critical threshold of energy deprivation is reached,

excitotoxic injury is enhanced because of the developmentally enhanced excitatory pathways [Johnston, 2005].

## Free radicals

The neonatal brain has a high rate of oxygen consumption, high availability of iron for the catalytic formation of free radicals and low concentration of antioxidants, making it susceptible to damage [Blomgren and Hagberg, 2006]. Oxidative stress-regulated release of pro-apoptotic factors from mitochondria appears to play an important role in the immature brain [Blomgren and Hagberg, 2006]. Mature oligodendrocytes carry increased antioxidant enzymes compared with the oligodendrocyte precursors present in the immature brain, which may partly explain the susceptibility of premature infants to white matter damage [Lafemina, et al., 2006]. After HI excess $Ca^{2+}$ influx via glutamate receptors leads to severe oxidative stress and excitotoxic cell death itself also involves direct activation of neuronal nitric oxide synthase (nNOS) and the generation of nitric oxide (NO). This leads to mitochondrial dysfunction and increased formation of ROS, oxidative damage and cell death [Vannucci and Hagberg, 2004; Fatemi, et al., 2009]. There is an accumulation of hydrogen peroxide ($H_2O_2$) after HI in neonatal mice because of the low capacity of glutathione peroxidase in the immature brain [Lafemina, et al., 2006]. Accumulation of $H_2O_2$ is damaging to the immature brain, due to the high levels of free iron [Vannucci and Hagberg, 2004]. Most data suggest that oxidative stress contributes to the post-ischemic impairment of mitochondrial respiration [Fatemi, et al., 2009] and may initiate mitochondrial permeabilization, which eventually allows the release of mitochondrial intermembrane proteins with the potential to execute apoptosis. Consistent with the notion that excessive NO in the neonate may be detrimental; Ferriero et al have shown that neuronal NOS (nNOS) knock-out mice are protected from neonatal HI-induced brain damage [Ferriero, et al., 1996]. The free radical scavenging agent N-acetylcysteine is able to cross the placenta and has been shown to be neuroprotective in neonatal rats [Wang, et al., 2007; Fatemi, et al., 2009].

# Neuroprotective strategies

The ultimate goal for HIE treatment is improving long-term motor and cognitive outcomes as well as decreasing mortality. The theory of a therapeutic window was proven when treatment after HIE with hypothermia showed an effect decreasing mortality as well as survival free of any sensorimotor disability at the 18-24 months follow-up [Edwards, et al., 2010; Jacobs, et al., 2011]. Further supportive information comes from MRI studies that suggested that both head cooling and total body cooling were associated with a reduced incidence of basal ganglia/thalamic brain lesions [Logitharajah, et al., 2009] predictive of CP [Rutherford, et al., 2005]. Moderate (32-34 ºC) and prolonged (24-72 h) therapeutic hypothermia has now become standard of care for neonatal HI brain injury [Perlman, et al., 2010]. Hypothermia studies [Jacobs, et al., 2011] show that in the control groups approximately 60% dies or develop major disability [Gluckman, et al., 2005; Shankaran, et al., 2005; Azzopardi, et al., 2009]. The ICE trial, recently published, shows an absolute risk reduction by 15% [Gunn and Thoresen, 2006; Jacobs, et al., 2011] and the clinical hypothermia trials have a number needed to treat (NNT) of 9, suggesting that further improvement may be feasible with add-on treatments [Edwards, et al., 2010]. We also still lack a neuroprotective treatment option for preterm infants, who are not eligible for hypothermia treatment. A few studies indicate that hypothermia might be safe for at least the near-term preterm [Bennet, et al., 2007], but studies have also shown hypothermia being risky for preterm babies [Barrett, et al., 2007]. Hypothermia has also failed to show effect in severly affected children [Gluckman, et al., 2005; Azzopardi, et al., 2009]. Pharmacological treatment with both xenon, erythropoietin and topiramate have shown additional effect in combination with hypothermia and for xenon this effect was additive, which supports the hypothesis that neuroprotection offered by hypothermia can be further improved [Liu, et al., 2004; Hobbs, et al., 2008; Cilio and Ferriero, 2010]. Another drawback of hypothermia is that treatment needs to be started within 6 hours. In animal studies certain drugs have shown a neuroprotective effect with a wider therapeutic window [Medja, et al., 2006].

# Mechanisms of hypothermia

Possible side-effects of hypothermia include sinusbradycardia, prolonged QT-interval without arrythmia, pulmonary hypertension, scalp-edema in the case of selective head-cooling and overt bleeding due to increased thrombocytopenia. Several larger clinical trials have however reported that moderate hypothermia in the neonatal setting is safe [Gunn and Thoresen, 2006; Edwards, et al., 2010].

Although mild to moderate, hypothermia still has a remarkable neuroprotective effect against ischemic brain injury. This effect is likely attributed to its broad inhibitory actions on a variety of harmful cellular processes induced by HI. Hypothermia reduces cerebral metabolism by about 5% for every degree of temperature reduction, which delays the onset of anoxic cell depolarization [Gunn, et al., 1997]. However the protective effect from hypothermia is still there although controlling depolarization, implicating that the critical effect of hypothermia lies somewhere else [Gunn and Thoresen, 2006]. Hypothermia also seems to decrease the levels of extracellular EAA and NO [Thoresen, et al., 1997]. Among the proposed key mechanisms underlying hypothermic neuroprotection is the inhibition of intracellular signalling events that initiate the cell death cascade [Edwards, et al., 1995]. Hypothermia increases neuronal survival in the basal ganglia and suppresses the activation of caspase-3 [Barrett, et al., 2007] and has also been shown to suppress microglial activation [Wagner, et al., 1999]. Hypothermia reduces inflammation triggered by ischemia [Silverstein, et al., 1997], reducing the expression of TNF-α, IL-1β and IL-18 and increases the anti-inflammatory cytokine IL-10 [Wagner, et al., 1999; Azzopardi and Edwards, 2007]. In summary, it is likely that hypothermia influences multiple pathways that contribute to neuroprotection.

# Aims of this thesis

The overall objective was to investigate different apoptotic pathways after excitotoxic and HI perinatal brain injury and to explore possible targets for future pharmacological treatment after HIE.

Specific objectives were

Paper I to evaluate the hypothesis that casp2 may be an operative target during perinatal brain injury.

Paper II to explore the neuroprotective efficacy of hypothermia in combination with casp2 gene deficiency after HI in neonatal mice.

Paper III to evaluate the cerebroprotective potency as well as the effects on apoptotic and inflammatory markers of a MLK-inhibitor, CEP-1347, after HI in neonatal rats.

Paper IV to examine the contribution of MPT in immature brain injury through genetic deletion of its positive regulator CypD and to assess the contribution of Bax dependent permeabilization in the immature versus the adult brain.

# Methodological considerations

This thesis is based on studies *in vivo* and *in vitro*. Detailed descriptions of the methods are given in each individual paper. Additional methodological considerations are discussed in this section.

## Genetically modified mice (I, II, IV)

Mice are very useful as experimental animals due to their easy maintenance and short breeding time. Genetically modified mice have led to great advances in research, allowing researchers to explore the role of genes in normal development and physiology as well as in disease states. The mice are inbred for at least 20 generations, making them as genetically alike as possible and hence improving the possibilities to explore the reaction to different events or substances. The most commonly used laboratory mouse is the C57BL/6. It is intermediately sensitive to HI as compared to 129Sv and CD1, two other commonly used strains. The damage induced by HI in C57BL/6 mice is increasing, when increasing the duration of HI, in contrast to the strain 129Sv, who show approximately the same amount of injury irrespective of HI duration [Sheldon, et al., 1998]. Mixed breeding involving heterozygote (het) x het mating (or homocygote x het) , allows comparison of all three genotypes (wild type (WT), het, and homozygote) within each litter, which is an optimal design considering the litter to litter variability. In those cases when the experimental conditions doesn't allow the mixed litter design (e.g. hypothermia experiments in the present study) homozygous WT are compared with homozygous knock-out litters. This usually require a higher number of animals in each group due to higher variability and the animals have to be back-crossed to make sure that the WT mice are genetically alike the knock-out mice in all other respects but the gene deleted.

# Age of brain (I-IV)

It is difficult to translate brain developmental age from rodents to humans and the comparison depends on the parameter being studied. Rats and mice differ from humans as regards to rodent brain being lissencephalic rather than gyrencephalic and the limited proportion of white matter in rodents. Romijn et al. [Romijn, et al., 1991] compared 4 different markers for brain age in rodents and human (numerical synapse formation, development of glutamate decarboxylase (GAD) activity, which is the key enzyme for the synthesis of the main inhibitory neurotransmitter gamma-aminobutyric acid (GABA), the development of choline acetyltransferase, the key enzyme for the synthesis of neurotransmitter acetylcholine and finally development of electrical activity) and came to the conclusion that rat postnatal day (PND) 10-14 is equivalent to the human neonate at term. Most data suggest that the maturity of the mouse brain is very similar to the rat brain at least with respect to white matter and examining the oligodendrocyte lineage progression PND7 rodent white matter is similar to that of human between 30 and 36 gestational weeks and by P14 mature oligodendrocytes are so abundant in the rat and mouse that it would be equivalent to the full-term infant of 40 gestational weeks [Craig, et al., 2003]. The maturity of the subventricular zone in mice at PND10 is equivalent to that of human at term [Brazel, et al., 2004]. In summary PND9 (or 10 depending on if the birthday is counted as zero or 1) in mice corresponds to near-term or term depending on what you are studying, while a PND7 rodent might be considered slightly more immature corresponding to 32-34 weeks of gestation [Hagberg, et al., 1997; Hagberg, et al., 2002].

# HI model (I-IV)

The most widely used model of neonatal HI is the Rice-Vanucci model in PND7 old rats [Rice, et al., 1981; Hagberg, et al., 1997], later modified to mice as well [Ditelberg, et al., 1996; Hedtjarn, et al., 2002], resulting in neuronal injury in 92% of the animals. The method to produce HI brain damage in immature rodents consists of a permanent unilateral common carotid artery ligation followed by a period in a hypoxic environment. Neither ligation nor hypoxia alone induces any injury due to

extensive collaterals in most rodent strains. During hypoxia cerebral blood flow is reduced by 40–60% in the hemisphere ipsilateral to the ligation [Vannucci and Hagberg, 2004] and this together with a compensatory vasodilatation of the vessels, leads to the fact that the collaterals no longer can support the ligated hemisphere fully, leading to partial focal brain ischemia. Cerebral blood flow is then restored to control values immediately upon return to normoxic conditions [Vannucci and Hagberg, 2004]. The injury is primarily seen in areas supplied by the middle cerebral artery; cerebral cortex, hippocampus, thalamus and striatum, hence including periventricular white matter, ipsilateral to the side of the carotid artery [Rice, et al., 1981; Towfighi, et al., 1991; Vannucci and Hagberg, 2004]. The cerebellum and brain stem are not damaged. Damage distribution between rats and mice seems to differ to some extent. Mice have more damage in hippocampus and less in cerebral cortex compared to rats [Brywe, et al., 2005] (II), which might be due to differences in vascular anatomy. No morphological damage is detected in the contralateral hemisphere [Grafe, 1994; Towfighi, et al., 1994] giving the advantage of using it as an "internal" control. Studies have also shown that sham-operated animals (5 min of anesthesia and cervical incision and suture) are comparable to naive controls, including no differences in energy and glycolytic metabolites and apoptosis in the brain [Grafe, 1994]. However, ligation only has in one mouse strain (CD1) produced brain injury [Comi, et al., 2009]. This does not seem to be the case for C57BL/6, who reacts very differently to HI in comparison to CD1 mice [Sheldon, et al., 1998], but we are currently investigating this further.

Another major advantage with the model is that it allows inclusion of a sufficient number of animals for dose-response evaluation of neuroprotective agents as well as allowing long-term evaluation of brain injury and functional impairments. It also shares several important features with birth asphyxia in the human neonate such as the combination of hypoxia and ischemia followed by reperfusion and changes in cellular energy metabolism after HI, however a disadvantage and the difference from birth asphyxia in human neonates is its unilateral distribution of damage and the lack of multi-organ dysfunction [Hagberg, et al., 1997]. Another draw-back is the great variability in the model between animals within and between litters [Grafe, 1994;

Hagberg, et al., 1997; Sheldon, et al., 1998]. The commonly used anesthetic isoflurane has been shown to decrease injury in our model after prolonged exposure [Chen, et al., 2011]. Hence we try to limit operation time and thus the exposure to isoflurane to a maximum of five minutes per mouse. The 50 minute duration of hypoxia was chosen based on titration to produce consistent moderate brain damage and without increasing the mortality (usually <10% in most of our studies) [Hedtjarn, et al., 2002; Hagberg, et al., 2004].

## Ibotenate model (I)

An intracerebral injection of ibotenate (excitotoxic alkaloid acting on NMDA receptors) induces focal lesions in 5-day-old mice [Marret, et al., 1995]. This produces a white matter lesion, mimicking the injury observed in preterm neonates. The development of the lesion can be blocked by treatment with a NMDA receptor antagonist. Neuronal cortical cell death induced by ibotenate mimics gray matter lesions observed in some human term or near-term neonates and the sensitivity of the developing brain to HI damage parallels sensitivity to NMDA neurotoxicity [Johnston, 2005]. Microglia activated by ibotenate release soluble factors such as cytokines, nitric oxide, free radicals and glutamate. Glutamate has been shown to cause white matter damage linking inflammation to excitotoxicity [Johnston, 2005]

## Hypothermia treatment (II)

During hypothermia a chamber, formerly used for human neonates, was used and each animal was placed in individual boxes, preventing them from heating each other. Those boxes are placed in the middle of the chamber, so that all animals should be heated to the same degree. One animal in each chamber (hypothermia as well as normothermia) was used as sentinel for measurement of core temperature using a rectal probe and one probe was also placed in each chamber to monitor the environmental temperature. The temperature was controlled with these probes and a temperature reading was obtained every second (Software Daisy lab 10.0, Physitemp Instruments, Inc., Clifton, New Jersey). The mouse pups quickly lost temperature during every moment of handling, hence the low starting temperature in paper II.

However, the drop in temperature was very short-lasting and did not differ between the experimental groups. The pups were put, both as regards to HI as well as hypothermia, into a pre-heated chamber set at a temperature to keep the pups at about the correct goal temperature and as quickly as they lose temperature they are also heated. This quick temperature loss/warming is probably due to the low body volume of mice.

## siRNA (I)

Small interfering RNA molecules are short sequences of double-stranded RNA (19-27bp in length), which suppress expression of target genes by inducing the breakdown of the cognate mRNA and hence hinders it to be used for translation of a protein. According to the rules that have been established to recognize on-target effects and mitigate off-target effects of siRNA [Cullen, 2006; Jackson, et al., 2006], low quantities of three different siRNA were used (si2a, si2b, si2c) targeting the same gene (mouse casp2). As each siRNA has unique off-target spectrum, but the same intended target, observing the same phenotype (neuroprotection) with multiple individual siRNA that contain distinct seed sequences increase confidence that the phenotype results from silencing of the intended target. In vitro 95% of Casp2 was blocked. In vivo in the ibotenate model 50% of mRNA was blocked. However this lower percentage is uncertain, since the penetration abilities of the siRNAs are not known and has to be put in relation to the sample analysed for mRNA.

## Gray matter injury evaluation (I-IV)

### *MAP-2*

Microtubule associated protein -2 (MAP-2) is part of the cytoskeleton in intact neurons (dendrites and soma) and proximal axons. In the immature neonatal brain, loss of MAP-2 staining corresponds well to areas of brain injury and hence infarction in gray matter. The loss in staining is gradual over 12-24 hours after HI and parallels the secondary loss of glucose metabolism in the tissue and accompanies activation of caspase-3 [Gilland, et al., 1998; Puka-Sundvall, et al., 2000]. Complete loss of MAP-

2 occurs in dead and irreversible injured cells, but it is possible that a partial temporary decrease can be seen in surviving cells.

### *Total tissue loss*

The volume of tissue loss was calculated according to the Cavaliere Principle, which states that the volume of an irregularly shaped object can be estimated from a set of two-dimensional slices throughout the object, provided that they are parallel, separated by a known distance and begin randomly within the object. In the formula of $V=\sum Apt$, V is the total volume expressed as $mm^3$ , $\sum A$ is the sum of the areas measured, P is the inverse of the sections sampling fraction, and t is the section thickness (5-9 μm) [Hedtjarn, et al., 2002]. This measures preferably the sum of infarction and total tissue volume loss.

### *Neuropathological score*

Neuropathological score could be used as a complement to total tissue loss, but is also a tool to evaluate injury in each brain region. It does however not include an evaluation of the white matter injury, which is done separately [Hagberg, et al., 2004].

## Subcortical white matter injury (I-II)

### *NF, MBP and Olig-2*

Neurofilament (NF) is the major intermediate filament of neurons and plays an important role in the conductivity of impulses down the axon [Miller, et al., 2002]. Myelin basic protein (MBP) is an indicator for developing oligodendrocytes and myelin, and loss of MBP can be an indicator of disruption in the myelination process [Kinney and Back, 1998; Back, et al., 2002]. Studies have shown that acute oligodendroglial injury can be quantified in a sensitive manner by measurements of MBP immunostaining, confirmed by Hedtjärn et. al [Hedtjarn, et al., 2005], as well as oligodendrocyte transcription factor-2 (Olig-2), a marker of oligodendrocytes throughout their lineage [Liu, et al., 2002] [Billiards, et al., 2008].

# Markers for apoptosis (I, III-IV)

## *TUNEL, Caspase-3 and AIF*

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) is a nuclear labelling used as a measure of DNA cleavage and hence as evidence for apoptosis being involved in neuronal death. TUNEL labels random DNA fragmentation, that could have other causes but apoptosis [West, et al., 2006]. TUNEL has however been shown to correlate well with the expression of caspase-3 and the loss of MAP-2 [Zhu, et al., 2000]. Positive TUNEL labelling begins already at 6 hours post HI, increases with a maximum reached at 18-24 hours [Zhu, et al., 2000].

Caspase-3 has been identified as one of the key executors of apoptotic cell death and caspase-3 activation is required for DNA fragmentation and the morphological features associated with apoptotic cell death [Jänicke, et al., 1998].

AIF is a marker of the caspase-independent apoptotic pathway and is translocated to the nucleus upon activation. This leads to large-scale DNA fragmentation and chromatin condensation [Zhu, et al., 2007b]. AIF redistribution is shown to occur in areas with neuronal damage and displays a close correlation with TUNEL labelling [Zhu, et al., 2003].

# Markers for microglia (III)

## *Isolectin, OX-18 and OX-42*

Microglial cells were identified by *Griffonia simplicifolia* isolectin-$B_4$, which is a glycoprotein that has high specificity to α-D-galactosyl residues which can be found on the surface on microglial cells and on cerebral capillaries [Streit and Kreutzberg, 1987; Streit, 1990]. Isolectin stains both resting inactive and activated microglia. To evaluate the acute microglia response OX-42 (complement receptor 3, CR3) and OX-18 (major histocompatibility complex I antigen, MHC I) immunoreactivity was used. According to McRae et al. [McRae, et al., 1995], the expression of OX-18 and OX-

42 on microglia occurs early and these are sensitive markers of microglial activation after HI.

## Gender (I-IV)

Studies show a gender difference in CP prevalence, where there is a female majority among children born at term, while a male predominance in children born preterm [Himmelmann, et al., 2010]. This observation is consistent with earlier data showing that the cognitive and motor outcome of brain injury is worse in male than in female in low birth weight infants [Johnston and Hagberg, 2007]. A gender difference has also been shown in animal models. Sex-related differences in cell death pathways are present in neonatal mice and rats in vivo [Zhu, et al., 2006b; Nijboer, et al., 2007]. Although no difference in injury after HI were present, the apoptotic pathways differs between sexes [Zhu, et al., 2006b], with greater translocation of AIF from mitochondria to nuclei in males and greater activation of caspase-3 in females. A greater influence of the excitotoxic pathway in immature males has also been shown [Johnston and Hagberg, 2007]. This agrees with that XY neurons are more sensitive to oxidative stress and glutamate excitotoxicity, while XX neurons are more sensitive to etoposide and staurosporine, agents that activate caspase-dependent apoptosis [Du, et al., 2004]. The important conclusion to draw is that gender can influence degree of damage, mechanism of injury and thereby the efficacy of neuroprotective agents.

## Statistics (I-IV)

Data was tested for normal distribution using GraphPad Prism Version 4 or 5. The Student's t-test was used in cases of normal distribution when two experimental groups were compared and the paired t-test was used for comparisons between uninjured and injured hemispheres. To compare multiple groups, a two-way-ANOVA was used, followed by Bonferroni post hoc test. Data that was not normally distributed was analysed using non-parametric tests (Mann–Whitney U test and Kruskall Wallis, followed by Dunn´s correction). Results are given as mean ± standard error of the mean (SEM). Statistical differences were considered significant when $p<0.05$. Poweranalysis was performed for each experiment and based on 80%

power, significance level 0.05 and variability in the model according to our previous studies.

# Summary of Results

## Involvement of Casp2 in neonatal brain injury (I, II)

Casp2 expression is high in both the neonatal mouse and rat brain with progressive decrease from the second week of life.

### *Role of Casp2 in HI brain injury*

Casp2-deficient 9-day-old mice (casp2$^{-/-}$ mice) presented a significant reduction of infarct volume when compared to WT mice (31.6%, p=0.025; I, Fig.2A). Brain injury in WT mice was more pronounced in hippocampus than in striatum, cerebral cortex and thalamus (II, Fig.1B). Brain damage was significantly reduced in both casp2 heterozygote (casp2$^{+/-}$) and casp2$^{-/-}$ mice compared with WT mice in hippocampus, thalamus and cerebral cortex. In the striatum brain injury was lower in casp2$^{-/-}$ mice as compared to both WT and casp2$^{+/-}$ mice (II, Fig.1B). The decrease in injury was not sex dependent and casp2$^{-/-}$ mice also presented reduced NF degradation in the subcortical white matter (NF loss: 39% in casp2$^{-/-}$ versus 51% in WT, p=0.046; I, Fig.2C).

These data was confirmed once more and with an even stronger protective effect in casp2$^{-/-}$ mice, when exploring the effect of a novel caspase-9 inhibitor. The infarction volume was reduced by 59% from 22.7 ± 3.1 in WT mice to 9.3 ± 2.2 in casp2$^{-/-}$ mice (p=0.0003, Fig.3A). The area of infarction measured as lack of MAP-2 immunoreactivity, was significantly reduced in 8 out of 9 brain levels in casp2$^{-/-}$, with the largest difference at the level of hippocampus (Fig.3B).

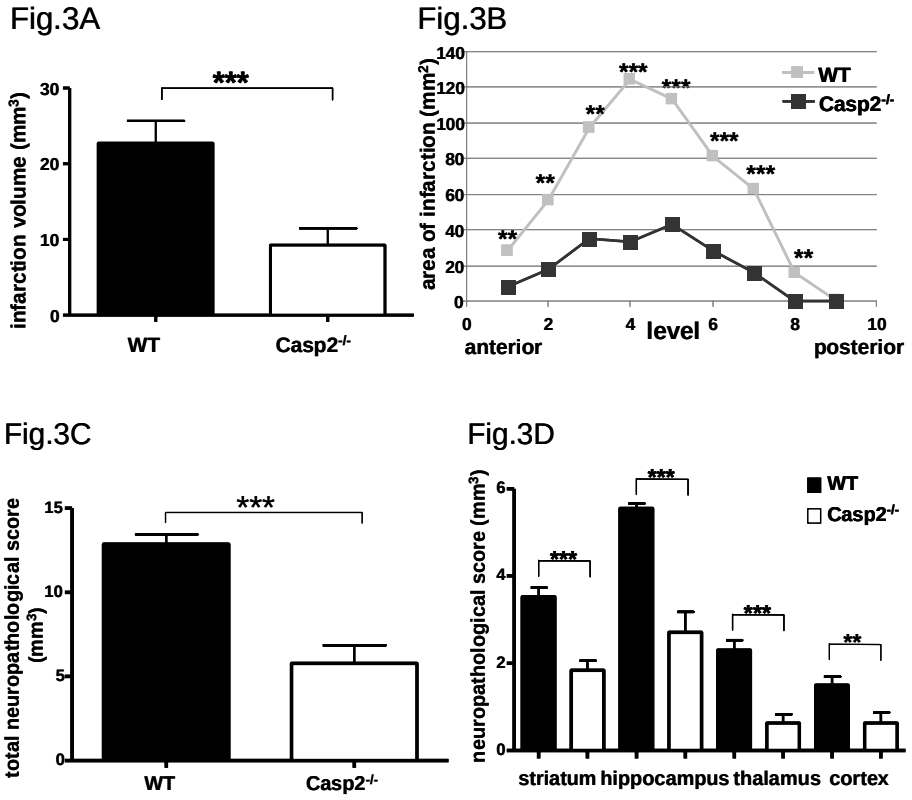


**Fig.3 Gray matter injury evaluation**
Infarction volume calculated according to the Cavalieri Principle from the area loss of MAP-2 immunoreactivity in the ipsilateral hemisphere (A). Infarction size, expressed as loss of MAP-2 immunoreactivity in the ipsilateral hemisphere, was quantified in 9 coronal sections numbered from anterior to posterior levels, significantly different for level 1 through 8. (B). Neuropathological scoring of HI brain injury in the striatum (0–6), hippocampus (0–6), thalamus (0–6) and cerebral cortex (0–4) is shown (D) as well as the total injury score (0–22) for WT and casp2$^{-/-}$ mice (C). A reduction of brain injury in casp2$^{-/-}$ mice compared with WT mice is shown. Data were analyzed with two-Way ANOVA and values are expressed as means ± SEM. Results from Bonferroni post hoc test are given in the figure; ***p<0.001; **p<0.01.

The neuropathology score was substantially lower in casp2$^{-/-}$ mice than in WT mice for all regions evaluated; being most marked in the thalamus (73%), followed by cerebral cortex (58%), hippocampus (51%) and striatum (48%) (Fig.3D). The total neuropathology score was significantly reduced (p<0.0001) in casp2$^{-/-}$ mice (5.8 ± 1.0) as opposed to WT mice (12.9 ±0.6) (Fig.3C). Casp2$^{-/-}$ animals showed, in agreement with our previous findings a decrease in both NF staining (p=0.0073,

Fig.4A), as well as MBP (p=0.0008, Fig.4B) after HI. There was a significant loss of NF and MBP immunoreactivity in WT animals subjected to HI indicating white matter injury affecting both axons and myelin. The deficit in NF staining was significantly reduced, by 50%, in casp2$^{-/-}$ mice (9.2 ±2.4) compared with WT mice 18.3 ±2.1 and the loss of MBP was decreased by 44% in casp2$^{-/-}$ mice (23.1 ± 4.2) versus WT mice 41.1 ± 2.8 (Fig.4A-B). The neuroprotection offered by casp2 gene deficiency was not sex dependent (data not shown).

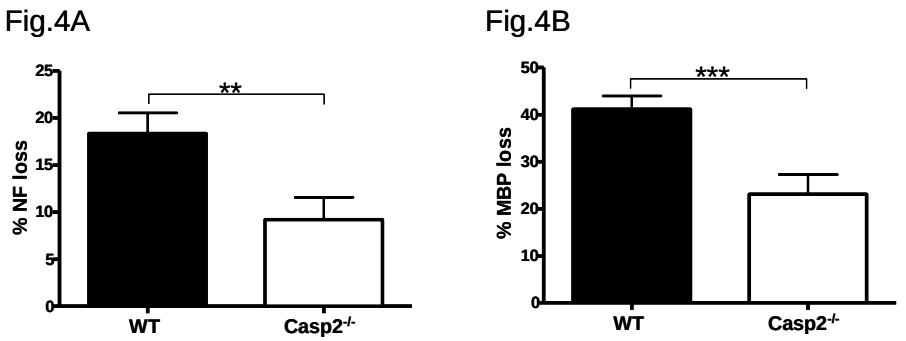


**Fig.4 White matter injury evaluation**
Percentage tissue loss of NF (A) or MBP (B) staining in the ipsilateral hemisphere in relation to the contralateral hemisphere. Injury was evaluated 3 days post HI. Casp2$^{-/-}$ mice are protected after HI as compared to WT mice. Data were analyzed with two-Way ANOVA and values are expressed as means ± SEM. Results from Bonferroni post hoc test are given in the figure; ***p<0.001; **p<0.01.

## *Role of Casp2 in the ibotenate-model of brain injury*

Ibotenate induces focal lesions in 5-day-old mice; however homozygous casp2 gene deficiency conferred a 41% reduction of lesion volume of cortical and a 59% reduction in white matter injury as compared to WT animals (I, Fig.2E). Similar to casp2$^{-/-}$ but with a less marked effect, casp2$^{+/-}$ neonates showed reduced lesion volumes of cortical (23%) and white matter (28%) injury (I, Fig.2E). Immunohistochemistry of casp2$^{-/-}$ mice, both after HI as well as after ibotenate, showed a decrease in the number of cleaved caspase-3 positive cells as compared to WT mice; hence caspase-3 activation appeared to be, at least in part, casp2-dependent (I, Fig.2G-I).

## *Role of Casp2 siRNA in brain injury*

In order to further evaluate the involvement of casp2 in perinatal brain damage casp2 -specific siRNA was used to target casp2. Through intraparenchymal administration of three casp2-specific siRNA a more than 50% protection in both gray and white matter of the neonatal brain against excitotoxic insult was achieved, whereas no significant differences were observed between non-treated animals and non-targeting controls (I, Fig.3A-B).

## *Casp2 and hypothermia*

The administration of 5 hours of hypothermia directly after HI provided additional protection in mice with casp2 gene deletion (II, Fig.3). Regional score demonstrated that hypothermia enhanced the protective effect of casp2 gene deletion in hippocampus and thalamus. Such an additive effect was not found in the other two regions; striatum was mostly protected by casp2 gene deficiency, whereas the cerebral cortex was mostly rescued by hypothermia (II, Fig.4A-D). The infarction volume confirmed the reduction in injury after hypothermia in WT and casp2$^{-/-}$ mice as assessed by neuropathological score ($p<0.0001$, Fig.5).

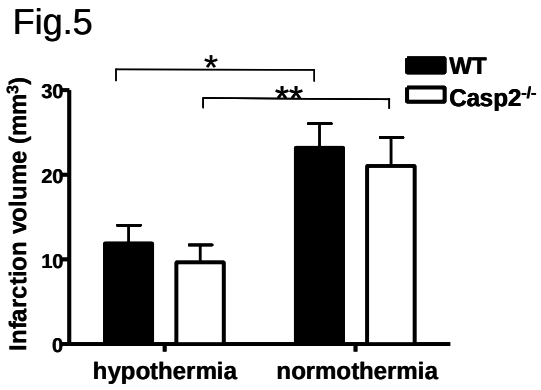


**Fig.5 Brain injury evaluation after hypothermia**
The infarction volume was significantly reduced in WT and casp2$^{-/-}$ mice after hypothermia ($p<0.0001$). Data were analyzed with two-Way ANOVA and values are expressed as means ± SEM. Results from Bonferroni post hoc test are given in the figure; **$p<0.01$; * $p\leq0.05$.

White matter injury, assessed on the basis of MBP and NF immunoreactivity, was reduced by hypothermia independently of casp2 genotype (two-way ANOVA; MBP: genotype $p=0.948$, temperature $p<0.0001$; NF: genotype $p=0.764$, temperature $p=0.0003$, II, Fig.5). This finding was supported by the number of Olig-2 positive

oligodendroglial cells in the subcortical white matter that was rescued by hypothermia (temperature p=0.017) but not by casp2$^{-/-}$ (genotype p=0.925). The decrease in injury was not sex dependent.

Hypothermia treatment, delayed by 2 hours, reduced overall injury (p=0.049), as well as cerebrocortical (p=0.001) and thalamic (p=0.03) injury (Fig.6A). Delayed hypothermia offered additive protection with casp2 gene deletion in the hippocampus (p=0.004, Fig.6B), but not in the other regions.

Fig.6A

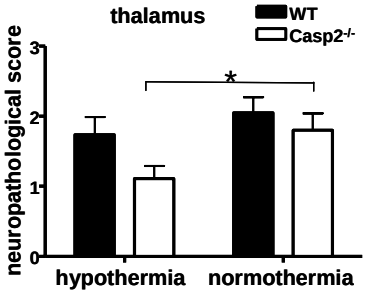


Fig.6B

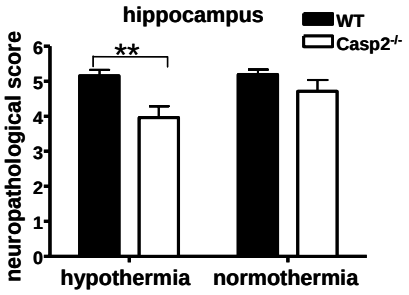


**Fig.6 Regional HI brain injury in WT and casp2$^{-/-}$ mice after delayed hypothermia**
Hypothermia treatment, delayed by 2 hours, reduced thalamic (A, p=0.03) injury and offered additive protection with casp2 gene deletion in the hippocampus (B, p=0.004). Data were analyzed with two-Way ANOVA and values are expressed as means + SEM. Results from Bonferroni post hoc test are given in the figure; **p<0.01; * p≤0.05.

Hypothermia reduced white matter injury as assessed by MBP (p=0.002) and NF (p=0.027) staining, but no additive effect with Casp2 gene deficiency was detected (not shown).

*In conclusion: Deletion of initiator casp2, either through genetic knock-out or through siRNA decreases neonatal brain injury, both in gray and white matter, in two different animal models: HI as well as excitotoxic injury. Hypothermic treatment after neonatal HI adds further protection to casp2 gene deletion. The protection seems, however, region dependent and together with hypothermia no additive protection of the casp2 gene deletion was seen in white matter.*

# MLK inhibition attenuates neonatal brain injury in a caspase-dependent manner (III)

## *CEP-1347 reduces neonatal HI brain injury*

CEP-1347 is a MLK inhibitor. When CEP-1347 was administrated after HI, 6 hours after HI and once daily until the rats were sacrificed; there was a 28% decrease in overall tissue volume loss (p=0.019) in CEP-1347-treated as compared to vehicle-treated animals (III, Fig.1). This injury reduction was further supported when assessing injury through neuropathological score, where the total neuropathological injury score was reduced by 27% in CEP-1347 treated rats (p = 0.046, III, Fig.1). The number of isolectin-positive cells with the morphology of activated microglia, i.e. retracted processes and rounded cell body phenotype, was counted and was shown to be substantially increased after HI. CEP-1347, reduced the number of isolectin-positive cells at 7 days after HI as compared to vehicle-treated animals (p=0.038, III, Fig.1).

## *CEP-1347 reduces apoptosis*

TUNEL staining was significantly (p=0.021) decreased in the hippocampus 24 hours after HI in CEP-1347-treated animals and a similar tendency was found in the cerebral cortex and striatum (p=0.066, III, Fig.2). This is consistent with a significant (p=0.047) reduction of cells expressing the cleaved activated form of caspase-3 in the striatum 24 hours after HI and a similar tendency is found in the cortex and hippocampus (III, Fig.2). There was no significant difference in the number of AIF-positive cells between CEP-1347-treated and vehicle-treated animals. Total p-JNK immunoreactivity was significantly decreased at 4 hours (p=0.049) in CEP-1347-treated rats, but no statistically significant difference was detected 8 hours after HI (p=0.404). There was no significant change in c-jun levels after HI in CEP-treated animals compared to controls.

## *CEP-1347 effect on inflammation*

There was no significant difference in the expression of microglia markers OX-42 (CR3) and OX-18 (MHC I) immunoreactivity in CEP-1347-treated versus vehicle-

treated animals at 24 hours after HI (III, Table 1). The expression of mRNA for the pro-inflammatory cytokines IL-1β, IL-6 and MCP-1 was not different in CEP-1347-treated versus vehicle-treated animals at 4 and 8 hours after HI (III, Table 2). Similarly, protein expression of IL-1β and IL-6 was not different in CEP-1347-treated versus vehicle-treated animals at 8 hours after HI (III, Table 3).

*In conclusion: MLK inhibition reduces HI neonatal brain injury in a caspase-dependent fashion, but had no detectable effect on inflammatory cytokines.*

# Age dependent differences in cell death pathways (IV)

## *Effects of CypD deficiency on HI brain injury*

Analysis of brain injury in PND9 WT, CypD deficient mice ($CypD^{-/-}$), and CypD het ($CypD^{+/-}$) mice revealed a more pronounced damage in $CypD^{-/-}$ mice (IV, Fig.1). This is in sharp contrast to PND60 mice, where absence of the $CypD^{-/-}$ gene resulted in a significant decrease in HI brain injury. No sex dependency could be discerned.

## *Mitochondria characteristics in $CypD^{-/-}$ mice*

Mitochondrial morphology was not different comparing WT and $CypD^{-/-}$ at PND9 or PND60 and CypD was as abundant in immature as in mature brain (IV, Fig.2A-E). HI did not alter the expression of CypD itself in mitochondria enriched cell fractions. Brain mitochondria from both neonatal and adult $CypD^{-/-}$ mice were desensitized to $Ca^{2+}$, requiring approximately twice the amount of $Ca^{2+}$ to open the PTP than mitochondria from WT animals (IV, Fig.3). Most importantly, brain mitochondria from neonatal WT mice required far more $Ca^{2+}$ to open the PTP than mitochondria from adult mice, regardless of the presence of CypD. CsA was a more efficient desensitizer in adult than in neonatal WT mitochondria, whereas it was ineffective in mitochondria from $CypD^{-/-}$ animals.

## *Effect of CypD deficiency on apoptotic mechanisms after HI*

Casp2, caspase-3, and -9 were markedly induced in the brain after HI at PND9, whereas the activation and alterations were negligible at PND60 (IV, Fig.4A-C). At

PND9, casp2, caspase-3, and -9 were all significantly more activated in CypD$^{-/-}$ than WT mice, whereas at PND60 the opposite was observed. A decrease of Cyt C was observed in the mitochondrial fraction, matched by an increase in the cytosolic fraction after HI at both ages. At PND9, the release of Cyt C at 24 hours after HI was much more pronounced in CypD$^{-/-}$ compared with WT mice, whereas in adult mice, the opposite was found (IV, Fig. 5A, D-E). AIF decreased at 6 and 24 hours after HI in the mitochondria-enriched cell fractions in WT and CypD$^{-/-}$ mice at PND9, but the loss was most pronounced at 24 hours after HI in CypD$^{-/-}$ mice (IV, Fig.5A). AIF changes after HI in the adult brain were limited and independent of genotype. In PND9 CypD$^{-/-}$ mice, post-HI changes were further confirmed by immunohistochemistry. AIF immunoreactivity translocated from mitochondria to nuclei after HI. Double immunostaining showed that Cyt C expression shifted from a weak expression in the cytosol of normal control brain cells to a much stronger staining with a diffuse cytosolic localization in cells that highly expressed the cleaved active form of caspase-3 after HI (IV, Fig.5F).

## *Effect of CypD deficiency on Bax expression*

In normal control brains from WT mice, a marked decrease of mitochondrial Bax expression independent of CypD during the development from PND3 to PND60 was found (IV, Fig.6A). After HI at PND9, translocation of Bax to mitochondria increased at 1.5 and 3 hours in both genotypes, but the increase was more pronounced in CypD$^{-/-}$ than in WT individuals at 3 hours (IV, Fig.6B). In brains of PND60 mice, Bax expression was low and no obvious changes after HI were detected.

## *The role of Bax inhibition in HI brain injury*

A single injection of Bax inhibiting peptide (BIP) was administered intracerebroventricularly (i.c.v) immediately before HI in the WT mice. BIP reduced brain injury score by 79% and caspase-3 activation by 65% in PND9 WT mice, whereas the corresponding effects were negligible in PND60 WT mice on both the brain injury score as well as the caspase-3 activation (IV, Fig.6C-D). In addition, Cyt C release was assessed *in vitro* by adding BH3-peptide to isolated brain mitochondria

in a cytosol-like medium. Under these conditions, BH3-peptide induced Cyt C release in both the absence and presence of exogenous full-length recombinant Bax at PND9, whereas at PND60, BH3-peptide induced Cyt C release required the presence of exogenous Bax, probably because the endogenous level of Bax in adult brain mitochondria was too low. Moreover, BH3-peptide induced a more pronounced Cyt C release in $CypD^{-/-}$ than in WT mice in PND9 brain mitochondria. In contrast, BH3-induced Cyt C release was attenuated in mitochondria from $CypD^{-/-}$ compared with WT mice at PND60. Bax alone did not elicit Cyt C release from isolated PND9 brain mitochondria, but exogenous Bax enhanced the effect of the BH3-peptide (IV, Fig.7).

*In conclusion: $CypD^{-/-}$ increases HI brain injury in neonatal mice as opposed to adult mice, where the deletion is protective. This difference is neither due to a change in the mitochondrial morphology nor in the components/regulators of the PTP itself. However neonatal mitochondria are less sensitive to $Ca^{2+}$, demanding more $Ca^{2+}$ to open the PTP than adult mice mitochondria. Apoptotic pathways are activated to a higher degree in neonatal mice brain than in adult mice brain and utterly most in $CypD^{-/-}$ neonatal mice. Bax expression decreases during development and is high after HI in neonatal mice, with a more pronounced increase in $CypD^{-/-}$ mice as opposed to WT mice. Inhibiting Bax leads to a decrease in injury as well as a decrease of apoptotic markers in neonatal mice, but not in adult mice and a more pronounced release of the apoptotic marker Cyt C was seen in neonatal $CypD^{-/-}$ as compared to WT neonatal mice after adding a BH3-peptide mimicking Bax action.*

# Discussion

Perinatal brain injury leads to life-long handicaps. A way to decrease/prevent poor outcome after NE and to increase life quality for those who are affected would be the ultimate goal. Hypothermia treatment proves the theory about a therapeutic window, a time-frame where it is possible to influence the outcome and change the course of the disease. Hypothermia improves outcome by 15%. Some children, for example pre-term neonates, are so far not eligble for this treatment [Barrett, et al., 2007] and the treatment has been shown not to be effective in severly affected children, why additive or even preferbly synergistic or alternative treatment would be valuable. The aim for this thesis was to elucidate the mechanisms behind different apoptotic pathways that occur during perinatal asphyxia, pathways where manipulation would be possible and hence be used as pharmacological targets for potential treatments.

## Role of Casp2 in neonatal brain injury

This thesis demonstrates a broad range of neuroprotective strategies involving casp2. Genetic deficiency of casp2 is neuroprotective in newborn mice exposed to both HI as well as excitotoxicity. In all experiments performed casp2 gene deletion reduced gray matter injury by as much as 59% (Fig.3A) and white matter injury by 50% (Fig.3B) in comparison to WT mice after HI brain injury. Using siRNA to delete casp2 after excitotoxic injury also reduced both cortical and white matter damage in the neonatal brain. This is in conjunction with other reports in vitro showing that decrease or deletion of casp2 expression using casp2$^{-/-}$ cells [Tiwari, et al., 2011] or casp2-specific siRNA [Troy, et al., 1997] decrease injury after oxidative stress. Altogether, combined with our finding that casp2 mediates HI and excitotoxic

neonatal brain damage and the importance of casp2 in apoptotic cell death, at least in the immature brain, one can suggest that casp2 might be a potential target for future neuroprotective strategies in neonates.

Casp2 is an upstream caspase, which can initiate the release of mitochondrial constituents [Troy and Shelanski, 2003] and is known to be able to regulate diverse cell signalling pathways, such as DNA damage [Robertson, et al., 2004a; Kumar, 2009]. In WT animals, the brain expression of casp2 is high at birth and progressively decreases during postnatal development. Casp2 activity has been shown to increase after HI in immature mice as opposed to adult mice [Wang, et al., 2009]. Quite contrary to the protective effect in our neonatal setting; in a model for adult focal cerebral ischemia casp2$^{-/-}$ mice have the same amount of brain injury as WT mice [Bergeron, et al., 1998]. A non-selective caspase inhibition reduces injury and saves neurons from apoptosis in the neonatal brain [Cheng, et al., 1998; West, et al., 2006; Clark, et al., 2007]. A more selective inhibition would however be desirable, since particularly some caspases seem to play a very important role in normal brain development [Lu, et al., 2002; Gulyaeva, 2003]. Caspase-3$^{-/-}$ and caspase-9$^{-/-}$ mice, as opposed to casp2$^{-/-}$ mice, have enlarged brains with an overabundance of neurons resulting from lack of apoptosis [Kuida, et al., 1996; Kuida, et al., 1998]. Caspase-3 is involved in the regulation of GluR1, a subunit part of the AMPA receptor and as a consequence influences neuronal plasticity and is able to prevent excitotoxic provoked necrosis, through cleavage of GluR1 in conjunction with cell damage, hence preventing excess $Ca2^{+}$ influx into the cell. The modulation of targets must therefore be done carefully; the results may not always be the expected ones [Lu, et al., 2002]. The degree of protection provided by casp2 gene deficiency varies in the different studies that we have performed. We observed better protection of casp2$^{-/-}$ mice in the study involving a novel caspase-9 inhibitor, which may be due to that the HI brain injury was evaluated at an earlier time point in this study, 3 days as opposed to 7 days post HI. However, already at 24 hours after HI there is a marked decrease of glucose utilization paralleled by a loss of MAP-2 immunoreactivity suggesting that most of the injury has become irreversible at that point [Gilland, et al., 1998]. Thus, it is unlikely that there is a marked further

progression of injury between 3 and 7 days post HI. This is also supported by a neonatal hypothermia study, where no difference in injury is seen 1 week and 6 weeks post HI in neonatal rats [Bona, et al., 1998]. However, delayed injury occurring weeks after HI is reported [Geddes, et al., 2001]. Cerebral edema peaks at 72 hours in those animals with the largest damage [Yager and Ashwal, 2009], which could also have affected our evaluation. The variable degree of protection may also partly relate to the Rice-Vannucci HI model, which is variable with regard to the extent of injury within and between litters [Grafe, 1994]. Individual operation-times and hence a difference in isoflurane exposure could possible also contribute to the variation [Chen, et al., 2011]. Finally, it is reported that casp2$^{-/-}$ mouse neonates (PND 1), develop a compensatory cell death pathway in the brain involving Smac/Diablo. This leads to decreased inhibition of caspase-9, followed by a 3-fold up-regulation of caspase-9, finally leading to caspase-9 dependent cell death [Troy, et al., 2001]. Such a compensatory mechanism could of course be at play to a variable degree also in 9-day-old mice, explaining why the protection offered by casp2 gene deficiency varied from substantial to mild/moderate [Carlsson, et al., 2011] in the various studies that we have completed.

In order to explore the role of casp2 in neonatal HI, there is a need for development of a selective casp2 inhibitor. TRP601, which is a casp2, caspase-3 and -7 inhibitor, shows promising results in the Rice-Vannucci HI model, mouse ibotenate-model and neonatal stroke model decreasing injury without noticeable side-effects on the animals [Chauvier, et al., 2011]. A selective casp2 inhibitor is however currently not available. Meanwhile using transgenic mice carrying conditional (floxed) alleles (Cre-Lox), with an inducible deletion of casp2 specifically in neurons, would be a way to proceed, involving for example the CamKIIα promoter which is turned on at PND3-5 [Nijboer, et al., 2011] with subsequent deletion of the casp2 gene. One problem with non-conditional transgenic mice is that the deleted gene or inserted gene could cause secondary alterations in other proteins over time during brain development. This could be avoided using the Cre-Lox technology.

Studies performed in caspase-3$^{-/-}$ and caspase-9$^{-/-}$ mice show a compensatory up-regulation of other caspases [Zheng, et al., 2000]. Although casp2$^{-/-}$ mice develop

normally [Bergeron, et al., 1998], as mentioned above, a study published on casp2 gene deletion in sympathetic neurons indicates that casp2$^{-/-}$ mice develop a compensatory pathway leading to apoptotic and necrotic cell death [Troy, et al., 2001]. This gives a hint of the delicate balance as regards to the multiple apoptotic pathways in a cell as well as implying that traditional knockout animals might not be the best way to study those pathways, but instead to aim for an inducible gene deletion. Despite up-regulated caspases, casp2$^{-/-}$ mice are still very well protected after HI.

## *Casp2 and hypothermia*

Hypothermia in combination with casp2$^{-/-}$ gene deletion reduces neonatal brain injury further. Total brain injury score was reduced by 45% in hypothermic casp2$^{-/-}$ mice compared with normothermic WT mice, while hypothermia alone reduced injury by 20% in WT mice and by 36% in casp2$^{-/-}$ mice. Brain injury measured as infarction volume was reduced by 58% in hypothermic casp2$^{-/-}$ mice compared with normothermic WT mice. Pharmacological treatment with xenon, erythropoietin and topiramate all show additive effect in combination with hypothermia, which supports the hypothesis that neuroprotection offered by hypothermia can be further improved [Liu, et al., 2004; Hobbs, et al., 2008; Cilio and Ferriero, 2010]. The present work is, however, the first example showing that hypothermic protection can be enhanced also if combined with gene deficiency in a neonatal mouse model of HI. The present results strengthen the concept that casp2 contributes to perinatal brain injury.

The hypothesis was that the combination of hypothermia with casp2 deletion would be superior to any intervention alone because complementary mechanisms are targeted. This was confirmed by immediate hypothermia, but when the hypothermia was delayed, an additive effect was only shown in the hippocampus. The reason for this is unclear, but it is interesting to observe that some regions were better protected by one mode of treatment, probably related to different mechanistic profiles.

The casp2 gene deletion protected white matter from HI injury [Carlsson, et al., 2011] (Fig.2A-B), however when hypothermia and casp2 gene deletion was combined the casp2 gene deletion did not seem to have any effect in the white matter

(II). Somehow the protective mechanisms involving casp2 gene deletion and hypothermia might interact in white matter in particular. Caspase-6 seems to be important in mediating apoptosis in white matter and this might just reflect a difference in the apoptotic cascade due to different mechanistic profiles in lack of casp2 versus hypothermia induction [Park, et al., 2010]. In order to exclude the possibility that the difference observed is due to an already present difference in $casp2^{-/-}$ and WT mice; the contralateral hemisphere was used as an internal control and also analyzed [Grafe, 1994; Towfighi, et al., 1994]. Qualitatively the staining looks similar and when comparing the contralateral gray and white matter staining in the mixed breeding litters ($casp2^{-/-}$, $casp2^{+/-}$ and WT) there was no difference as regards to gray matter injury analyzed with MAP-2 or white matter injury using NF and MBP staining. In addition there was no difference concerning olig-2 staining in the contralateral hemisphere. This indicates that, since all groups were treated alike, it is only if the contralateral hemisphere staining is different in the different groups that a bias problem would be introduced, which is obviously not the case in the present studies.

To our knowledge, this is the first study on post HI hypothermia in a neonatal PND9 mouse model of HI. We chose to cool only 3ºC from 36ºC to 33ºC, which is slightly less than what is used clinically (3.5ºC) and less than what is done in some previous rat studies: 5ºC for 3-5 hours. Zhu et al. [Zhu, et al., 2006a] uses mild (34ºC) intraischemic hypothermia in PND7 mice resulting in a complete protection in the cerebral cortex, indicating that mice do not demand a higher degree of cooling than rats and since mice, due to a 3-times smaller body volume, could be expected to be more vulnerable than rats; we chose a milder treatment approach - 3ºC and 4 to 5 hours of cooling. The degree of duration of hypothermia is nevertheless something we could consider to change in future studies. After 5 hours of hypothermia we got a significant protection (20%) in the WT group (normothermic WT versus hypothermic WT) by this cooling strategy, however in the delayed setting this significant difference was lost. Normally in rats a mild insult and 5ºC for 5 hours would give at least 50% protection [Thoresen, et al., 1996b]. Furthermore, the mice seemed to endure the present treatment protocol well. On the other hand in the

delayed setting the temperature effect was still stronger than the genotype effect with a significant overall effect and we managed to demonstrate that under at least some experimental conditions hypothermia and casp2 gene deficiency are additive. In addition, in a clinical situation the cooling may not always be optimal, and maybe we should instead increase the duration of cooling in the delayed hypothermia group. Gunn et al. [Gunn and Thoresen, 2006] show that the duration of cooling is important in the delayed setting.

Due to the fact that sentinel animals might experience increased stress because of the rectal probe; these animals were only used as temperature sentinels and excluded from the rest of the study. Even if the probe diameter was small and precautions were taken to reduce the trauma, the stress involved affects outcome [Thoresen, et al., 1996a]. Indeed, analyzing the sentinels exposed to HI and 5 hours of hypothermia separately, as regards to MAP-2, NF and MBP, revealed that the damage is less pronounced in the sentinels, which is fully in accordance with previous work.

During carotid artery ligation operations are made sequentially, which means that some pups have a longer delay between ligation and hypoxia than others. If the delay between ligation and hypoxia is prolonged, some studies show that injury decreases and that the variability in the model increases. This is due to circulation being partly restored again [Lee, et al., 2004; Taniguchi and Andreasson, 2008]. We took this into consideration and the time from first ligation to hypoxia was in no case longer than 130 minutes, often shorter. In order to decrease this confounding effect further, the ligation was always performed mixing casp2$^{-/-}$ and WT mice.

As mentioned previously we discovered that mouse pups easily lose body temperature when they are away from the dam, but could also quickly re-warm again. This is probably due to their small body volume, but gave us a problem in the experimental situation. Every bit of handling meant a decrease in body temperature and this might implicate that the difference in heating by the dam after HI could contribute to the damage variability that we observe in this model.

# Role of JNK after HI in perinatal brain injury

CEP-1347 has passed clinical trials in adult humans as a safe and well tolerated drug [Parkinson Study Group, 2004]. It has been tested in a clinical trial in patients diagnosed with Parkinson at an early stage, but failed to show a decrease in disease progress [2007]. However in a neonatal animal model CEP-1347 reduced HI brain injury in a caspase-dependent fashion. Indeed, in another recently published article by Nijboer et al., they use a JNK inhibitor in PND7 rats in the Rice-Vannucci model of neonatal HI [Nijboer, et al., 2010], and show 30% reduction in injury, which is comparable to our results.

In this study, they also demonstrate that JNK and c-jun increase 3 to 6 hours after HI and return to baseline 12 hours after HI. TNF-α, IL-1β, IL-6 as well as several other inflammatory cytokines are not affected by the JNK inhibitor [Nijboer, et al., 2010]. Interestingly they show a decrease in α-fodrin, a marker for necrotic cell death, and imply that JNK inhibition decreases necrosis instead of apoptosis. However JNK has been proven to be involved in apoptosis; where JNK inhibition reduces Cyt C release as well as caspase-9 and -2 activation and subsequent DNA fragmentation, probably via Bim, a Bcl-2 family protein [Bogoyevitch, et al., 2004; Dirsch, et al., 2004; Gao, et al., 2005; Pirianov, et al., 2007]. CEP-1347 has been shown to decrease apoptotic cell death by us and others [Mathiasen, et al., 2004], which means that the mild effect might possibly be a reflection of cell death passing through another pathway, when signalling via JNK is blocked or simply that the JNK inhibition is not sufficiently complete or specific. CEP-1347 inhibits pJNK activation completely 30 min to 4 hours after insult, however resulting in only 70% reduction in cell death speaking in favour of the theory that an alternative pathway of cell death is being activated [Mathiasen, et al., 2004]. JNK seems to be activated mostly after oxidative stress and the activation in stress-induced cell death seems to be highly dependent on cell type and signalling context [Dirsch, et al., 2004]. As mentioned previously, JNK can also cleave Bid, resulting in jBid releasing Smac/Diablo from the mitochondria [Deng, et al., 2003] and the expression of smac/Diablo after HI in animals treated with the JNK inhibitor by Nijboer is decreased [Nijboer, et al., 2010]. It would be interesting to evaluate the expression of Smac/Diablo after CEP-1347 as well.

In addition the decrease in p-JNK expression in our study was marginal and no significant drop in c-jun expression was detected. The lack of effect of CEP-1347 on c-jun in our study could be due to the fact that whole-brain tissue analysis was performed, disguising regional differences. Further JNK activation is quite transient and therefore difficult to detect [Pirianov, et al., 2007]. One study shows a peak of c-jun at 2-4 hours after HI in neonatal rats, which could explain the trend in our study at 4 hours and the lack of effect at 8 hours [Jiang, et al., 2004], but other studies show that JNK and p-c-jun are visible at 4 and 8 hours [Dirsch, et al., 2004; Jiang, et al., 2004; Nijboer, et al., 2010]. A way to approach this is to investigate the expression of AP-1 as Nijboer et al. do [Nijboer, et al., 2010]. Something we should bear in mind is that it is the phosphorylation of c-jun that is critical and not the level of the total protein itself [Saporito, et al., 2002].

We did not observe any effect of CEP-1347 on inflammatory cytokines. We have used the same dose as in all other studies showing significant effect involving CEP-1347; 1mg/kg [Saporito, et al., 2002], hence the lack of effect is unlikely due to wrong dosage. It could also be due to a lack in our methods in detecting the cytokines as well as too few animals in each group. The results however, is fully in concurrence with the recently published study by Nijboer as well as a study investigating the effect of CEP-1347 in human and murine microglial cultures after LPS treatment [Lund, et al., 2005; Nijboer, et al., 2010]. The reduction in brain injury after CEP-1347 was also accompanied by a marked reduction of microglia at 7 days offering additional indirect support of the cerebroprotective efficacy of CEP-1347 in this model. Decreased microgliosis has been shown to be associated with less damage; however a decrease in microgliosis might not always be beneficial [Colton, 2009; Vexler and Yenari, 2009].

CEP-1347, an MLK inhibitor, attenuates injury and decreases caspase-dependent cell death, but does not seem to have a major effect on the inflammatory response in a neonatal rat model of HI. This is confirmed by a recent published study, which importantly includes long-term outcome speaking in favour for JNK inhibition in a neonatal setting after HI. The drug may have potential as a clinical neuroprotective treatment in neonates as it is well tolerated in humans [Parkinson Study Group, 2004;

Parkinson Study Group, 2007], but its mechanisms of action remains to be explored further.

## Age different response after HI - the importance of mitochondria

Mitochondria are the power source of the cell. Several studies show that the mitochondria are critically involved in necrosis and execution of apoptosis and thereby in determination of cell fate [Hagberg, et al., 2009]. Mitochondrial permeabilization appears to be of utmost importance in the cell death pathway in response to HI in both the immature and the adult brain. However, in what way the permeability transition works after HI and what the pores consist of seem to be elusive. Studies show that CypD does not seem to be part of the pore, but rather regulates it and experiments in transgenic animals rule out that VDAC and ANT are molecular components of the pore [Kokoszka, et al., 2004; Rasola, et al., 2010]. Instead of being part of the pore, CypD seems to mask an inhibitory site, $P_i$, which is the actual permeability transition desensitizing agent. The hypothesis is thus that CypD favours pore opening by making the $P_i$ site on the pore not available [Rasola, et al., 2010]. The present study showed that permeability transition pore opening depended on brain maturation and adds to previous studies that brain damage mechanisms depend on the age of the brain. A treatment that could protect the adult brain, in this case the deletion of CypD, increased instead damage in the immature brain. This difference was neither due to a change in mitochondrial morphology nor in the assembly of the pore itself or age-dependent differences in CypD levels [Eliseev, et al., 2007]. Adding to the age dependent differences; neonatal mitochondria were less sensitive to $Ca^{2+}$, demanding more $Ca^{2+}$ to open the permeability transition pore than adult mouse mitochondria and $CypD^{-/-}$ mouse mitochondria demanded about twice as much $Ca^{2+}$ as WT mice.

CypD prevented rather than enhanced mitochondrial permeabilization in the immature brain. Our hypothesis is that CypD indirectly interacts with and inhibits Bax dependent permeabilization of the outer mitochondrial membrane. Our data suggest that CypD is not essential for physiological apoptosis in the early stages of

development. Bax on the other hand seems crucial to development, since Bax/Bak double-knock-out mice display defective apoptosis [Roset, et al., 2007]. Bax expression decreases during development [Northington, et al., 2001] and is known to translocates to mitochondria after HI in the neonatal mouse brain, with a more pronounced increase in $CypD^{-/-}$ mice as opposed to WT mice. Inhibiting Bax led to a decrease in injury as well as a decrease in apoptotic markers after HI in neonatal mice, but not in adult mice and a more pronounced release of the apoptotic marker Cyt C was seen in neonatal $CypD^{-/-}$ as compared to WT neonatal mice after adding a BH3-peptide mimicking Bax action. BIP administered immediately before HI reduced brain injury and caspase-3 activation. Indeed, a follow-up study subsequently demonstrates that BIP offers a reduction in white matter injury, reducing Cyt C and also AIF release and provides an improvement in functional recovery 7 weeks post HI [Wang, et al., 2010]. This confirms the results from a study using BIP in an in vitro model for neonatal excitotoxic injury, which shows a reduction in glutamate-induced neuronal cell death, as well as a decreased number of TUNEL-positive cells and suppression of the increase in caspase-3 and –9. In addition BIP prevents Bax translocation to the mitochondria [Iriyama, et al., 2009]. We suggest that inhibition of Bax translocation to mitochondria blocks mitochondrial permeabilization and could thereby be an effective strategy to protect the immature brain from damage after asphyxia.

# Clinical implications and future perspectives

In conclusion this thesis has shown that apoptosis seems far more important in the immature brain as opposed to the adult brain in conjunction with HI brain damage. It seems like we lose some of this remarkable ability of apoptosis with increasing age. This could imply that pharmacological drugs aimed for treatment of HI in the adult brain might not be beneficial in the immature brain and vice versa. It seems hopeful however that we have possibilities to affect apoptosis after HI. Data from studies in adults show that excitotoxic drugs have a short therapeutic time frame, while caspase inhibitors have a longer ability to intervene [Dirnagl, et al., 1999]. Hence, a substance inhibiting casp2 might be a useful therapy after perinatal asphyxia.

How casp2 is activated in the neonatal brain remains to be established in order to find out a way to best inhibit its activation and to be able to prevent unwanted side-effects in the developing brain. Casp2 induces mitochondrial outer membrane permeabilization and cell death [Guo, et al., 2002]. Genotoxic cell stress, as induced by doxorubicin and etoposide, triggers activation of casp2 in a complex involving PIDD, whose expression is induced by p53, and RAIDD [Tinel and Tschopp, 2004]. Genetic down-regulation of PIDD is reported to reduce hippocampal brain damage after transient global ischemia [Niizuma, et al., 2008], and inhibiting p53 after neonatal HI reduces brain injury together with mitochondrial permeabilization [Nijboer, et al., 2011]. Several other studies also points in the direction that PIDD is involved in the activation of casp2 [Zhivotovsky and Orrenius, 2005; Baptiste-Okoh, et al., 2008; Vakifahmetoglu-Norberg and Zhivotovsky, 2010]. This speaks in favour for casp2 being important after ischemic injury and could point towards perinatal HI brain injury triggering PIDDosome activation and consequently casp2 activation, which in turn cleaves Bid and triggers mitochondrial permeabilization and the intrinsic pathway of apoptosis. The next step would be to investigate brain damage in transgenic mice with an inducible p53 deletion or to inhibit PIDD/RAIDD in the neonatal HI model to further explore the mechanisms. PIDD has however been

shown not to be essential for casp2 activation [Bouchier-Hayes, et al., 2009], which could mean that a dual inhibition would be preferable.

Although having compensatory up-regulation of other pathways involving apoptosis, the casp2$^{-/-}$ mice are well protected after HI. A way to further explore this is to investigate if they do have less expression of smac/DIABLO, Cyt C, Bid, Bax as well as less protein expression of caspase-3 as one would hypothesize [Guo, et al., 2002; Kumar and Vaux, 2002; Robertson, et al., 2004a; Chauvier, et al., 2005]. Casp2 can also induce the release of AIF [Guo, et al., 2002]. Is the protection based on the fact that both caspase-dependent- as well as independent pathways are affected? Does hypothermia have any influence on those caspase-independent pathways in the casp2$^{-/-}$ mice? Where does the additive effect lie? In order to truly explore what casp2 really does, one way would be to investigate both casp2 as an inducible gene deletion to avoid any compensatory pathways evolved during development as well as using a selective casp2 inhibitor. TRP601, a caspase-2/-3 and-7 inhibitor shows promising results [Chauvier, et al., 2011].

The study by Nijboer et al. [Nijboer, et al., 2010] indicates that JNK inhibition would decrease necrosis. This opens up, at least in theory, an interesting possibility of combining JNK inhibitor affecting necrosis with casp2 inhibition mainly decreasing apoptosis in order to enhance the protective effect . Something that speaks in favour of this is also that an interaction between casp2 and JNK has been observed in some cells and that JNK inhibition has been shown to partially inhibit casp2 processing [Zhivotovsky and Orrenius, 2005], suggesting that JNK contributes to casp2 dependent apoptotic signalling upstream of mitochondria [Dirsch, et al., 2004] and a dual treatment would strengthen the inhibition.

This thesis emphasizes the important difference between the adult and the immature brain and puts the attention to mitochondrial permeabilization and Bax as important targets in order to decrease neonatal brain damage. Bax has the ability to heterodimerize with multiple Bcl-2 family members [Knudson, et al., 1995] and Bax/Bak double-knock-out mice display defective apoptosis in multiple tissues [Lindsten, et al., 2000] suggesting that Bax may have a central role in regulating

apoptosis in early development. However one concern is thus that Bax inhibition might block apoptosis important for normal brain development as well. Further studies are needed to rule out this concern, preferably using Bax cre-lox mice.

Hypothermia has become the standard of care after NE, saving one infant in nine from death or neurologic disability. Many questions remain however, such as *can* we provide added protection? Therapeutics targeting apoptosis may prevent delayed cell death, but would not affect earlier necrotic or excitotoxic injury. A combination therapy seems the choice of the future, but there is a need for better understanding *when* to treat and who to treat. Preventing apoptosis also includes the risk of saving neurons without function, or of imparing physiological mechanisms that suppress inflammation, which might not be benefical as discussed before. Gender is something else we need to take into consideration as well as the age of the child – preterm or term. The recent progress in the use of MRI will surely help us decide who to treat. Also several other treatments such as Xenon, EPO as well systemic injection of cord blood cells seem like promising strategies in the treatment of NE. Finally, given the number of different pathways involved in HIE, it is likely that the best outcome will be achieved by a multi-modal therapeutic approach such as a combination of an inhibitor of mitochondrial permeabilization with anti-apoptotic treatment and hypothermia.

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