Perinatal Hypoxic-Ischemic Brain Injury: Detection & Intervention

An experimental study in newborn piglets

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I **Garberg HT, Huun MU, Escobar J, Martinez-Orgado J, Løberg EM, Solberg R, Saugstad OD**

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II **Garberg HT**, **Solberg R, Barlinn J, Martinez-Orgado J, Løberg EM , Saugstad OD**

High-dose cannabidiol induced hypotension after global hypoxia-ischemia in piglets. Accepted manuscript in Neonatology, april 2017.

III **Garberg HT, Huun MU, Lars O Baumbusch, Atneosen-åsegg M, Solberg R, Saugstad OD**

Temporal profile of circulating microRNAs after global hypoxia-ischemia in newborn piglets. Neonatology 2017;111(2):133-139.

What is this thesis about?

Hypoxic-ischemic insults in the perinatal period rank globally among the three leading causes of newborn mortality [1]. Many of the newborns that survive the initial insult are developing disordered brain function known as hypoxic-ischemic encephalopathy (HIE). Those with moderate to severe HIE have a high risk of lifelong neurodisability with severe psychosocial and socioeconomic consequences for the child, the families involved and for society in general [2,3].

Currently, the only available treatment to minimize the consequences of HIE is therapeutic hypothermia. However, despite cooling the risk of death or severe neurodisability is still high [4] and additional neuroprotective strategies are greatly needed [5].

Further, determining the exact etiology and the timing of brain injury is often challenging and might preclude optimal treatment. Identifying novel biomarkers that could provide reliable information about the timing and nature of brain injury could potentially improve treatment and outcome for these newborns [2,6].

In this thesis, we have used a well-established piglet model of perinatal hypoxia-ischemia to explore the effects of a promising neuroprotectant, cannabidiol, as well as the potential of circulating microRNAs to be markers of hypoxic-ischemic injury.

Introduction

Hypoxic-ischemic encephalopathy (HIE)

The clinical syndrome of disordered brain function occurring in the first days of life in some term and near term-born neonates is referred to as neonatal encephalopathy (NE). Newborns with NE typically present with an abnormal level of consciousness, difficulty initiating and maintaining respiration, depressed muscle tone and reflexes, difficulty eating, often accompanied by seizures. This set of symptoms is an important predictor of perinatal death and a major contributor to long-term adverse neurological outcomes, including cerebral palsy (CP) [7].

A considerable proportion of NE is caused by acute peripartum hypoxic-ischemic events with estimates ranging from 30% in high-income countries to 60% in low-income countries [7]. Reliably identifying whether acute hypoxia-ischemia is the cause of NE is often challenging. Hence some recommend that the term NE is used to describe all encephalopathic newborns [8]. Others argue that when there is sufficient evidence of an acute hypoxic-ischemic event, as measured by clinical, chemical and neurophysiological variables accompanied by a characteristic topography of acute lesions demonstrable by MRI [9] the term hypoxic-ischemic encephalopathy (HIE) is a more accurate term for this sub-group .

In the studies included in this thesis we apply a controlled hypoxic-ischemic insult to term-born piglets with aim of studying brain damage of hypoxic-ischemic origin in term infants. Hence, from this point on the term HIE is used.

Etiology

HIE can result from either ante partum or peripartum hypoxic-ischemic insults. Among the antepartum risk factors are gestational age of more than 41+5 weeks, perinatal infection

and maternal disease. Among the intrapartum risk factors are prolonged rupture of membranes, abnormal cardiotocography, thick meconium stained amniotic fluid, sentinel event such as cord-prolapse or disruption of the placenta, shoulder dystocia, tight nuchal cord and failed vacuum [10]. HIE can also result from postnatal insults caused by, among others neuromuscular disease, cardiac malformations and lung/airway malformations that could prevent the normal adaptation to extra-uterine life.

Incidence

The reported incidence of HIE range from 1.3 in high-income countries to 26 per 1000 live births in low-income countries. HIE has a large impact on global child health with an estimated 700 000 deaths in the world annually [1].

Prevention of perinatal hypoxic-ischemic brain damage

Auscultation of the fetal heart rate gives information about fetal well-being. In high-income countries this is achieved electronically by cardiotocography and/or fetal ECG and is standard care. The purpose is prevention of intra-partum hypoxia-ischemia (HI). Recent meta-analyses have shown that, although reducing the burden of neonatal seizures, such monitoring have no clear benefits in regards to the other outcome variables, such as cerebral palsy or infant mortality [11,12]. Thus, it is likely that intra-partum hypoxia-ischemia will remain a major concern in the foreseeable future and that its consequences will need to be addressed by neonatologists after birth.

Early detection and grading the severity of brain injury

Early and reliable identification of the cause of neonatal encephalopathy is important for optimal decision-making regarding neuroprotective interventions and for tailoring supportive treatment [2,6,13]. The existing tools for the early prediction of brain injury have limited accuracy, especially in the acute phase after delivery [13,14], and a universal marker of hypoxic-ischemic brain injury has yet to be discovered. There is an ongoing search for more

accurate and reliable biomarkers, but currently the best approach is a multi-modal assessment applying both clinical, biochemical and neurophysiological tools[2].

Acid-base status – biochemical markers

Intra-partum acidosis (pH, lactate, base excess) measured in fetal scalp, umbilical or fetal arterial blood gives valuable information on the presence, severity and timing of HI. Hypoxanthine have also been thoroughly demonstrated to be a sensitive marker of hypoxia[15] and maybe should be considered for routine clinical use. However, these markers in general correlate poorly with the severity of brain injury and outcome [16].

Clinical evaluation and scoring

The condition of the newborn infant immediately after delivery is assessed by heart rate, breathing rate, muscle tone, response to stimuli and skin color - components of the APGAR-score. Further, the need for resuscitation and the response to resuscitation (e.g. time before spontaneous breathing) give an indication of the condition of the newborn. The Apgar score has a high inter-observer variability and a relatively poor specificity and sensitivity. However, it is still a very useful tool and at the extremes the specificity and sensitivity is better to detect future neurodisability[17]. E.g. an Apgar score of 0 at 10 min gives an 80% chance of death before or moderate/severe disability at school age [18].

After successful resuscitation a neurological assessment including level of consciousness, posture, muscle tone, tendon/complex reflexes and autonomic function is performed. Different scoring systems have been developed to grade severity with the most well known scores being versions of the Sarnat and Sarnat score [19,20] and the Thompson Encephalopathy score [21,22]. These scores are highly dependent on the examiner and reliably assessing the different clinical signs can be difficult. Further, the degree of HIE assessed by this score may fluctuate over time and be precluded by medications and treatment with therapeutic hypothermia. Despite this, clinical grading remains essential in the evaluation of the encephalopathic newborn and has shown a strong association with adverse clinical neurological outcome. E.g. a Thompson-score of >16 had a high specificity in identifying

infants who died or had a severely abnormal aEEG at 48 hours, which again is a strong predictor of abnormal outcome[23].

aEEG

The use of amplitude integrated electroencephalography (aEEG) has greatly improved diagnostic accuracy. aEEG provides a continuous reflection of the electrical activity in the brain and can be classified as normal or pathological based on voltage criteria and background pattern. Before the introduction of therapeutic hypothermia aEEG was shown in several studies to have excellent ability to predict neurological damage at early time points (3-6 hours after birth) [24]. After introduction of cooling the predictive values of aEEG has been shown to be lower in the early phase after HI (< 6 hours), but moderately/severely abnormal aEEG at 48 hours post HI strongly predicts poor outcome and aEEG remains a very good adjunct to clinical evaluation [25].

Biomarkers

Various biomarkers measured in blood and other body fluids, such as S100B, have also demonstrated promise in predicting brain injury and outcome after perinatal HI [14,26,27]. MicroRNAs are a novel class of biomarkers that might prove to be useful in this setting. Further studies are needed to establish the validity of these biomarkers[28].

Outcome

Infants, who are in need of resuscitation at birth, but recover quickly with only mild or no signs of encephalopathy and fully recover within the first week, will most likely have a normal outcome [29]. There are, however, data indicating impairment also in this group [30]. In contrast, those infants with evidence of moderate or severe HIE have a significant risk of death or severe disability. Before therapeutic hypothermia became standard care 62% of infants with severe HIE died or survived with moderate/severe disability compared to 25% of those with moderate HIE [31].

Disability includes motor deficits such as cerebral palsy [32], sensory deficits such as vision and hearing loss, cognitive deficits, epilepsy and neurodevelopmental problems (reviewed by Ahearne CE *et al.* [33]).

Therapeutic hypothermia has significantly improved outcome in high-income countries with improved survival without neurological abnormalities [34] while in low-income countries, however, efficacy is yet to be demonstrated[35].

Pathophysiology of HIE

HIE results from a complex set of pathophysiological mechanisms evolving in time from the acute hypoxic-ischemic insult. The following chapter is mainly based on the extensive reviews by Wassink *et al.* [36], Hassel KJ *et al.* [37] and Rainaldi Ma *et al.* [38].

Acute hypoxia-ischemia (HI)

The features of acute intra-partum HI are insufficient delivery of oxygen (hypoxemia) and blood (ischemia) to the fetus/neonate as well as inadequate clearance of carbon dioxide (hypercapnia). If prolonged this leads to a lack of substrates (glucose & oxygen) for cellular energy production and severe metabolic acidosis. In the brain the depletion of high-energy metabolites such as ATP results in failure of the ATP dependent Na+/K+ pump, massive sodium influx and cellular depolarization. The depolarization leads to flooding of the synaptic cleft with glutamate, a prominent excitatory neurotransmitter. In addition the energy dependent re-uptake of glutamate by astrocytes is reduced and this produces an accumulation of excitatory amino acids in the synaptic cleft, over-activation of glutamate receptors and massive calcium influx into cells. Together sodium and calcium overload results in hyperosmolarity which produce cytotoxic edema and ultimately cell lysis. Calcium is also released from damaged mitochondria and endoplasmic reticulum and the massive calcium overload also triggers several neurotoxic cascades. Further toxic reactive oxygen species, generated through activation of nitric oxide synthetase and the hypoxanthine-xanthine oxidase system[15,39], damage lipoproteins, DNA/RNA and mitochondria.

Most of the effects of the primary energy failure lead to cellular necrosis through impaired cellular integrity, disruption of the cytoskeleton and cell membrane, but programmed cell-death pathways (apoptosis) are also involved.

Latent phase

After reoxygenation/reperfusion, when cerebral circulation and oxygenation are restored, oxidative metabolism rapidly recovers in surviving cells and cytotoxic edema resolves over approximately 30 to 60 minutes. The levels of excitatory amino acids rapidly fall in parallel with resolution of the acute cell swelling. The rapid restoration of tissue oxygenation can be associated with a rapid burst of reactive oxygen species producing oxidative stress and breakdown of the blood-brain barrier (BBB), allowing large proteins to leak out in the extracellular space which may maintain brain swelling. The neurotoxic cascade is in general believed to be inhibited in these first hours after resuscitation and this period is also called the “therapeutic window”.

Secondary energy failure

Despite adequate perfusion and oxygenation deterioration in the cerebral oxidative metabolism has been demonstrated 6-24 hours after the initial hypoxic-ischemic insult. The exact mechanism of the injury in secondary energy failure remains incompletely understood, but the key event is believed to be HI-induced permeabilization of the mitochondrial membranes, leading to progressive failure of mitochondrial oxidative phosphorylation and ultimately delayed programmed cell death both via necrotic and apoptotic pathways [40]. Potent inflammation and oxidative stress by generation of reactive oxygen species and free radicals are also involved.

Tertiary phase

There is evidence suggesting that the brain injury continues to evolve even months and years after the initial insult. Mechanisms of this tertiary brain injury involve neural scarring (gliosis), epigenetic changes and persistent inflammation. Further, brain pH has been shown to be closely related to the degree of injury and outcome. This is due to its effects on

mitochondria and energy metabolism, NMDA receptors and excitability of neurons and the activity of proteases and lipases. Brain alkalosis persists for several weeks in babies with severely abnormal outcome and is associated with brain atrophy on MRI [41,42].

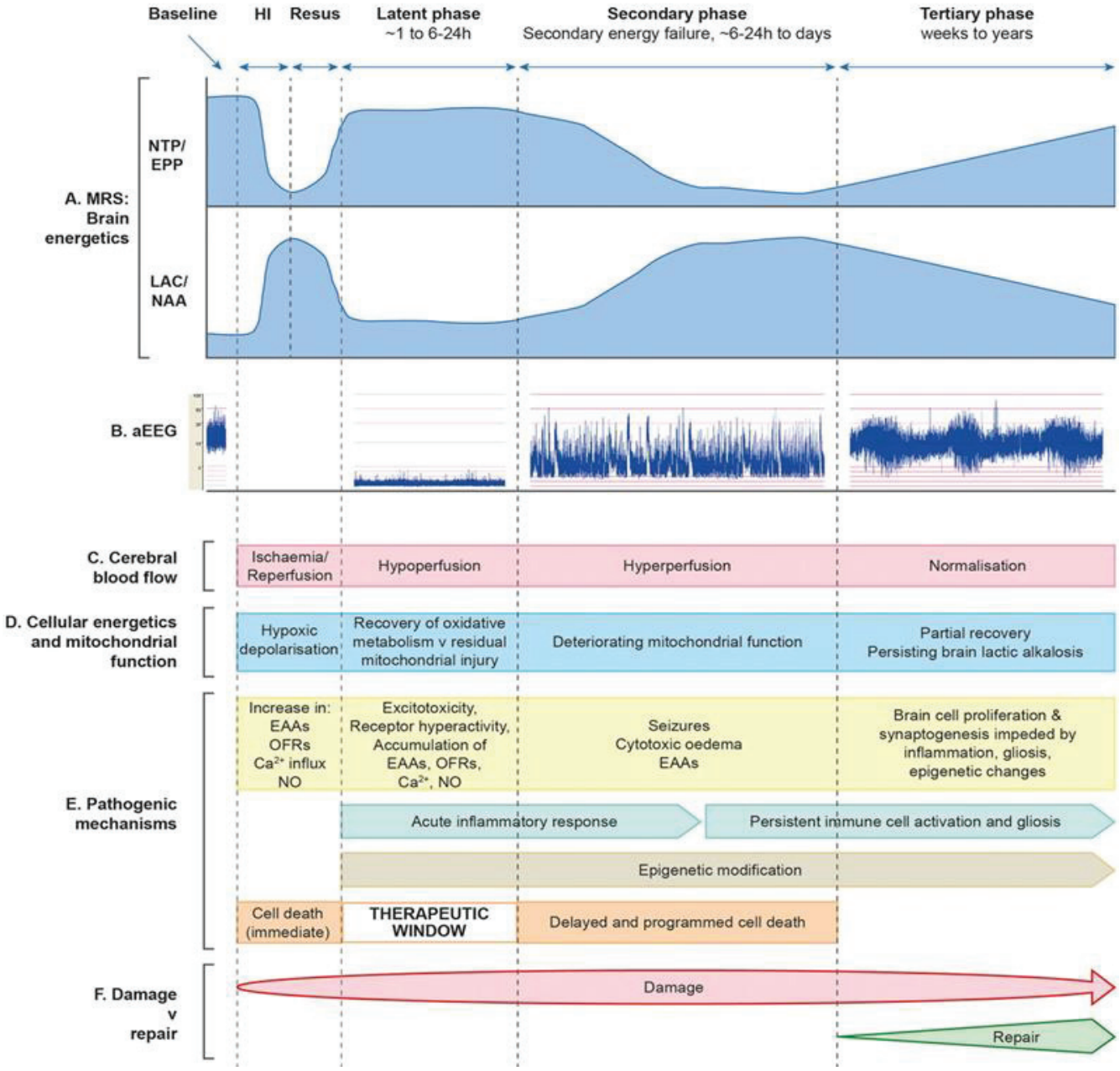

Figure 1. Phases of injury after perinatal HI.
Adapted from K Jane Hassell *et al.* Arch Dis Child Fetal Neonatal Ed 2015;100:F541-F552. Reproduced with permission.

Distribution of brain damage

The brain is not uniform, but contains a variety of cell types like neurons, astrocytes, microglia and endothelial cells. Different cell populations and regions of the brain display different vulnerability to hypoxic-ischemic insults. The distribution of damage after HI further depends on the level of maturation (gestational age) and nature of the insult, namely acute, sub-acute or chronic. The knowledge of the distribution of brain damage after perinatal HI is mainly derived from post-mortem autopsy and magnetic resonance imaging (MRI) studies [43,44] as well as animal studies.

In term infants exposed to acute HI, injury involving the basal ganglia, thalamus, and cortex is most typically seen, but also the midbrain, brain stem, and hippocampus can be involved. After more prolonged, chronic sub-acute HI, injury to white matter in the watershed areas and, if severe, also the overlying cortex, is often seen [32,45-47]. In the current animal model, we aimed to reflect an acute global hypoxic-ischemic insult in the term infant and have thus mainly evaluated the cortex, basal ganglia and hippocampus.

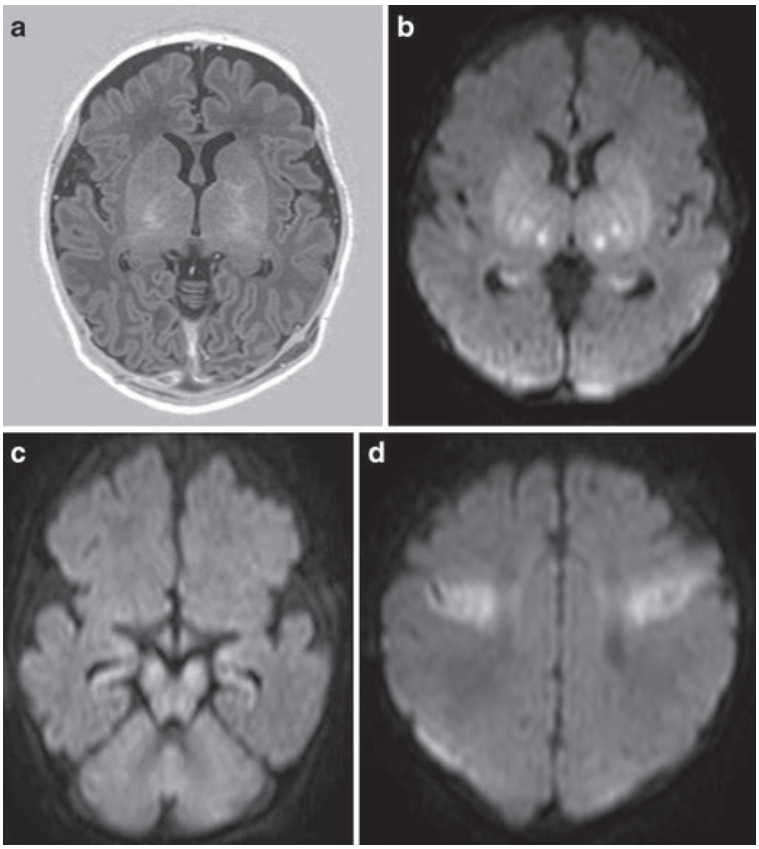

Figure 2. MRI images of a full-term infant after an acute, severe hypoxic-ischemic event. The MRI pattern is suggestive of acute near total asphyxia: **a** Inversion recovery sequence (TR 5038/TE 30/TI 600) does not show a normal signal within the posterior limb of the internal capsule, but areas of increased signal intensity within thalami and basal ganglia. DWI (**b**–**d**) shows restricted diffusion in the ventrolateral thalami, lentiform nuclei, cerebral peduncles, and in the perirolandic cortex. Also note involvement of the hippocampi.
Adapted figure from: Patterns of neonatal hypoxic–ischemic brain injury, De Vries et al., Neuroradiology. 2010 Jun; 52(6): 555–566.
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Neuroprotective interventions

MRS studies in asphyxiated newborns[48,49] and animals[50] more than 20 years ago gave rise to the concept of "secondary energy failure". These studies opened up to the idea therapeutic intervention as the latent phase before secondary energy failure represented a "therapeutic window", where cells not irreversibly damaged in the initial insult potentially could be saved. The only clinically established intervention to date is therapeutic hypothermia. Yet many neonates do not benefit from cooling and there is a continuous search for new and adjuvant neuroprotective strategies [5,37,51]. A brief summary of some of these strategies is presented below.

Delivery room management and the avoidance of hyperoxemia

Correct handling in the delivery room along with optimal supportive care is essential in minimizing brain injury after HI [52]. It has been demonstrated that hyperoxemia, as opposed to normoxemia, worsens brain damage [53] and is associated with a higher incidence of HIE[54].

Therapeutic hypothermia

In animal models mild cerebral hypothermia started within 6 h of birth, before the onset of secondary energy failure, and continued until resolution of secondary events such as seizures, has been shown to reduce injury and improve recovery after HI [55]. Therapeutic hypothermia is believed to exert neuroprotection by acting on several mechanisms simultaneously. Firstly cooling results in a general reduction of metabolism, reduced energy demands and thus conservation of energy reserves. Further, hypothermia is believed to mitigate exitotoxicity, inflammation and programmed cell death, among other mechanisms [56]. The pre-clinical evidence has been confirmed in randomized clinical trials in full-term infants with moderate-to-severe hypoxic ischemic encephalopathy, demonstrating improved survival and reduced disability. However, despite treatment 50 % of infants still have adverse outcomes [4]. In low and middle-income countries, where the burden of HIE is greatest, therapeutic hypothermia has not yet been shown to reduce mortality or morbidity [35,57,58]. This might be due to higher rates of intercurrent infection as it has been

demonstrated that the effects of hypothermia are reduced or even lost in the presence of infection and/or inflammation [59,60].

Erythropoietin (Epo)

Epo is a glycoprotein originally identified for its role in erythropoiesis, but is also produced endogenously in the brain. Numerous studies have demonstrated Epo`s neuroprotective effects. In the setting of acute HI Epo-receptor expression is rapidly up-regulated and if HI is prolonged Epo production increases. If there are sufficient levels of Epo to bind Epo receptors, cell survival is promoted, while in the absence of Epo apoptotic pathways pre-dominate. Epo is also believed to reduce inflammation and in the later phases and to beneficially modulate remodeling in the brain [5].

Melatonin

Melatonin is a naturally occurring neurohormone secreted by the pineal gland. Melatonin act on specific cell membrane and nuclear receptors, and exert neuroprotection via anti-oxidant, anti-apoptotic and anti-inflammatory effects [61]. Melatonin`s safety profile and ability to cross both placenta and blood-brain barrier also open up to the possibility of antenatal administration to prevent brain damage in the fetus. Melatonin has been shown to augment the protective effects of therapeutic hypothermia in a piglet model of perinatal HI[62]. Yet, more evidence is needed before melatonin can be implemented to a clinical setting [63].

Stem cells

Based on animal data stem cell therapies hold great potential in treatment of newborns with NE and HIE [64]. In addition to replacing damaged cells, stem cell therapies are likely to exert additional neuroprotective effects that promote neuronal survival and repair[65], may be by paracrine effects through the secretion of extra-cellular vesicles[66]. Our understanding regarding optimal stem cell type, route of delivery, safety profile and outcome is still limited

and ongoing clinical trials will provide valuable knowledge about the full potential of this therapy [5].

Remote ischemic post-conditioning

There is an increased awareness about the potential of stimulating endogenous protective mechanisms after HI. This is based on the knowledge that a small, sub-lethal dose of a harmful agent can protect an organism against a lethal dose of the same agent[67]. In remote ischemic post-conditioning blood flow in a peripheral limb is repeatedly reduced producing sub-lethal "doses" of ischemia. This is thought to produce neuroprotection by release of endogenous autocoids from skeletal muscle that activates both systemic and humoral pathways that lead to conservation of mitochondrial integrity, reduced energy demands, increased cell survival and promotion of repair mechanism [37].

Noble gases

Xenon easily crosses the placenta and the blood-brain barrier and mitigates apoptosis by binding to and inhibiting glutamate receptors. Preclinical studies have demonstrated that xenon augments the neuroprotective effects of therapeutic hypothermia [68], but it has yet to prove efficacy in a clinical setting [69]. Argon, another noble-gas that, in pre-clinical models, also has shown neuroprotective effects after perinatal HI [70]. Further molecular hydrogen has demonstrated efficacy in pre-clinical studies[71] and is currently being evaluated by our group. Argon and hydrogen have the advantage over Xenon in being at least 200 times less costly.

N-acetylcysteine (NAC) and N-acetylcysteine amide (NACA)

NAC is a free radical scavenger and major contributor to maintenance of glutathione levels in cells. Thus NAC can potentially ameliorate the massive oxidative stress after perinatal HI and it has shown neuroprotective effects in pre-clinical models [72,73]. Recently, it has been revealed that NAC amide (NACA), a NAC derivate, has higher bioavailability and enhanced antioxidant properties. Benterud *et al.*[74] recently demonstrated possible neuroprotective effects of NACA in the current animal model. As for many of the novel neuroprotectants clinical data of NAC/NACA`s efficacy is still lacking.

Many of the above neuroprotective strategies have overlapping effects, but might also complement, or add to the effects of therapeutic hypothermia and each other. It is likely that in the future, the treatment of HIE will involve a cocktail of different neuroprotectants.

Cannabidiol – a promising novel neuroprotectant

Cannabinoids and the endocannabinoid system

The Cannabis sativa plant has been used for medicinal purposes for millennia, treating a wide range of conditions including neurological disorders such as convulsions and pain. It was first introduced in the western world in the mid 19th century and was widely used up to late 20th century, when it was banned mainly due to its psychoactivity and abuse as a recreational drug [75]. However, despite the prohibition against medical use, there has been increasing interest and research into the therapeutic potential of cannabinoids. Since the 1964s, when delta-9 tetrahydrocannabinol (THC) was first isolated, a vast number of cannabinoids have been found [76] .

In 1990 an endogenous binding site for cannabinoids was discovered in the human brain [77] and shortly thereafter the cannabinoid receptor CB_1 was cloned [78]. This led to the discovery and characterization of an endogenous receptor and ligand system named the endocannabinoid system (ECS). The ECS consists of the cannabinoid receptors, the endocannabinoids as the endogenous lipid ligands and the machinery for their synthesis and degradation. It s widely distributed throughout the body, especially in the brain and spinal cord, and plays a role in many regulatory physiological processes including inflammation, metabolism, thermogenesis, neural development, immune function, cardiovascular function, synaptic plasticity, nociception, psychomotor behavior, sleep/wake cycles, regulation of stress and emotion [79]. Endocannabinoids are also involved in the endogenous neuroprotective response to hypoxic-ischemic injury.

The discovery of the involvement of the ECS in different disease states opened up for the idea of exogenous modulation. Despite the discovery of the therapeutic potential of phytocannabinoids, there are only a few licensed cannabinoid drugs to date. Synthetically produced THC and its analogues are used clinically as Dronabinol and Nabilone, for cancer chemotherapy-induced nausea and vomiting and in HIV/AIDS patients to stimulate appetite. The potential of THC is, however, limited by its unwanted psychotropic effects. An example

is Rimonabant, an anti-obesity agent that was withdrawn from the market due to adverse psychiatric side effects [80]. However, Sativex that is currently used to treat pain and spasticity in patients with multiple sclerosis, is well tolerated [81].

Cannabidiol (CBD)

1940`s and its complete stereochemistry established in the late 1960`s [82]. Because of the belief that THC was the only "active" component of cannabis it was presumed that all cannabis drugs would have unwanted psychotropic effects and research on CBD was therefore for a long time non existing. However, CBD is devoid of psychotropic effects [83-85] and possesses multiple actions with potential therapeutic benefit [81,83,86,87]. In the last 10-15 years there has been a considerable interest in the therapeutic potential of CBD and searching in PubMed we find 1004 publications in the period from 2005 to 10.10.2016 compared to 182 in the period from 1995-2005. A CBD/THC combination (1:1 ratio, Sativex/Nabiximol, GW Pharmaceuticals UK) is currently licensed internationally in more than 20 countries for the treatment of spasticity in multiple sclerosis, and a product containing only CBD (Epidiolex, GW Pharmaceuticals, UK) has entered an expanded access program in children with intractable epilepsies.

Medicinal chemistry

Both CBD and THC are C_{21} terpenophenols with pentyl alkyl tails and are syntethised by the same enzymes in the cannabis plant [88]. However, CBD`s conformational structure has important differences compared to THC. Where THC exists in a planar conformation, CBD has a conformation where the two phenol rings are more or less at right angles to each other (figure 3). As a results CBD binds to different receptors and have different biological actions, e.g. the lack of psychoactivity [86].

Pharmacology after intravenous administration

After intravenous administration CBD is rapidly distributed into the brain, adipose tissue, and other organs, governed by its high lipophilicity ($K_{octanol\text{-}water}$ ~6–7), and estimated high volume of distribution (~32 L/kg) [89,90]. CBD is highly protein bound, with ~10% bound to

circulating red blood cells. CBD is metabolized extensively by the liver, where it is hydroxylated to 6 and 7-OH-CBD by P450 enzymes, predominantly the CYP3A and CYP2C families of isozymes. These metabolites then undergoes significant further metabolism in the liver, and the resulting metabolites are excreted in the feces and, to some extent, in the urine. The half-life of CBD is estimated at 18–32 hours with a clearance of 960–1560 ml/min [89-91].

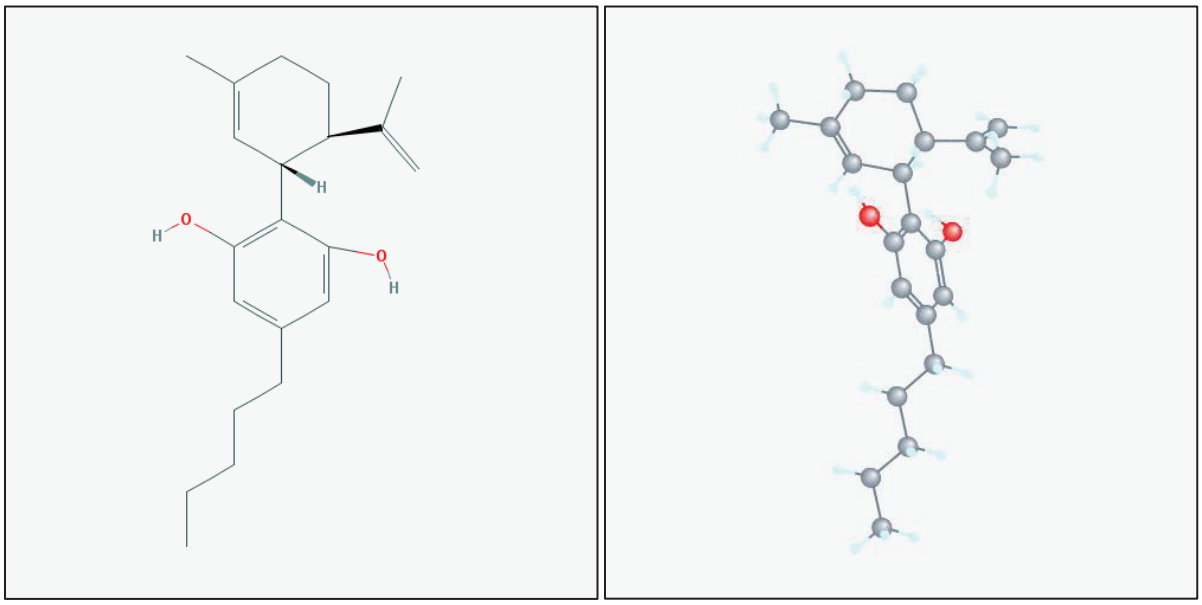

Figure 3**.** The chemical structure of CBD. Note CBDs bent conformation (right).

Molecular targets in neurological disorders

A review by Bih *et al.* [83] on the molecular targets of CBD in neurological disorders divide the action of CBD on various targets into the following groups: receptors (15%), ion channels (15%), transporters (20%) and enzymes (49%). Another systematic review by McPartland *et al*. [92] classify CBD`s targets into; direct and indirect effects on the classic endocannabinoid receptor CB_1, the "expanded endocannabinoid system" including G-protein coupled receptors (e.g. CB_2,GPR55) and transient receptor potential channels (e.g. TRPV1, TRPV2), and thirdly other molecular targets and effects such as $GABA_a$ receptors, dopamine receptors, inhibition of adenosine uptake and modulation of intracellular calcium levels, via T-type and L-type voltage-regulated Ca^{2+}channels and mitochondrial Na^{+}/Ca^{2+} exchange.

Bih *et al.* conclude that the most likely targets of CBD are involved in the regulation of, and responses to, intracellular calcium levels such as mitochondrial (VDAC1) and G-protein coupled receptor 55 (GPR55). Both reviews state that CBD is highly unlikely to exert direct effects through the classic endocannabinoid receptors CB_1 and CB_2. However, according to Mc Partland et al., CBD might indirectly affect these receptors through modulation of endocannabinoid levels. Mainly by inhibition of fatty acid amide hydrolase (FAAH) which is the primary enzyme responsible for endocannabinoid breakdown. As previously mentioned, endocannabinoids have a wide range of neuromodulatory effects, including neuroprotection.

Among the other plausible targets at physiological concentrations are; modulation of mitochondrial enzymes involved in the electron transport chain, inhibition of adenosine uptake through equilibrative nucleoside transporter 1 (ENT1), the serotonin receptor (5-HT_{1A}), glycine receptors (GlyR) and transient receptor vanilloid-type (TRVP). Further, the formation and interaction with CB_2/5-HT_{1A} heteromers have been postulated as a possible mechanism responsible for the effects of CBD [93].

Despite extensive preclinical evidence of the biological effects of CBD, establishing the exact molecular targets of CBD is still a work in progress. It is clear that CBD is a promiscuous compound with activity at multiple targets and with effects largely dependent on its molar concentration as well as the experimental and physiological setting. Results from *in-vitro* studies with CBD are not necessarily translatable to functional activity *in-vivo* something which highlights the need for *in vivo* testing before making conclusions on its functional activity at different targets [83,92].

CBD as a neuroprotectant after perinatal HI

CBD is believed to exert neuroprotection by modulating several of the key pathogenic processes leading to brain injury after perinatal HI, such as by reducing oxidative and nitrosative stress, mitigating exitotoxicity and hyperexitability, ameliorating neuroinflammation, preserving blood-brain barrier integrity and limiting mitochondrial failure and programmed cell death [83,94]. Many of these pathways are in part overlapping with the effects of therapeutic hypothermia, but CBD also involves independent pathways and could potentially be a useful complement to cooling.

The basis for our decision to evaluate CBD was based on previous in-vivo studies, rather than postulated effects on specific targets involved in neuroprotection. Mainly on the studies carried out in other pre-clinical models of perinatal HI [93,95-99]. However, we were also inspired by studies performed in other models of neuroprotection, such as stroke, neurotoxicity, neurodegenerative disease [94,100-107]. Below we present some of the hypothesized mechanisms of CBD`s neuroprotective effects in the setting of perinatal HI and we have tried to summarize them in figure 4:

Reducing oxidative and nitrosative stress

CBD can donate electrons under a variable voltage potential as well as prevent dihydrorhodamine in the Fenton reaction and has demonstrated potent anti-oxidative effects as a free-radical scavenger in a glutamate toxicity model [108], after H_2O_2 induced oxidative stress [106] and to reduce protein carbonylation after HI in piglets [93]. CBD might also exert anti-oxidative effects through inhibition of enzymes such as inducible nitric oxide synthetase (iNOS) [109,110] and myeloperoxidase (MPO) [111].

Mitigating exitotoxicity and hyperexitability and controlling calcium homeostasis

The massive increase in glutamate and other excitatory amino acids after perinatal HI lead to over-stimulation of ionotropic receptors, most importantly the NMDA receptors. As a result, there is a massive Ca^{2+} influx into cells forming an osmotic gradient that along with

Na^{2+} influx, produce cell swelling. Calcium influx also lead to direct injury of the mitochondria and formation of free radicals, and the inappropriate activation of proteases, lipases and endonucleases leading to the breakdown of cellular components. Together this is known as exitotoxicity and has been demonstrated to be associated with the severity of encephalopathy in newborns [112-114].

Cannabidiol have been shown to block NMDA and AMPA receptor-mediated neurotoxicity in rat cortical neurons exposed to glutamate[108]. Under certain conditions, modeling increased excitability in hippocampal neurons, CBD has been demonstrated to reduce intracellular Ca^{2+} levels maybe by targeting the mitochondrial Na^{+}/Ca^{2+}-exchanger (NCX) [115]. It has also been observed that CBD decreased glutamate levels after oxygen-glucose deprivation in mice brain slices [98] and mitigated the increase in Glutamate/NAA ratio in piglets after HI[93]. At the same time CBD has been shown to inhibit glutamate uptake so the explanation for these effects are unclear. Further, CBD is an agonist of the $5\text{-}HT_{1A}$ receptor and stimulation of this receptor exerts inhibitory effects on neurons and might reduce the detrimental effects of glutamate overload [93,116,117] .

Preventing seizures

Although glutamate and Ca^{2+} levels quickly return to normal after reperfusion, there is a subsequent rise following the latent phase[118]. There is also a persistent hyperexitability of NMDA receptors after HI and as a consequence seizures are common [36] and might *per se* be detrimental to the newborn brain [119]. CBDs anti-convulsant properties are well documented [91] and there are currently clinical trials evaluating its potential use in treatment-resistant childhood epilepsies [100]. However, through which molecular targets CBD exert these effects are still unclear. Interaction with $5\text{-}HT_{1A}$, VDAC1, GPR55 and modulation of adenosine homeostasis through ENT1 are some plausible targets.

Preserving the blood-brain barrier integrity

The blood–brain barrier (BBB) allows the body to control which substances and cells can gain access to the brain and thus maintain homeostasis. After perinatal HI this barrier is

often disrupted leading to worsening of brain edema [36,120]. In a model of ischemic stroke CBD reduced BBB hyper-permeability and thus maintained integrity through mechanisms involving activation of PPARγ and 5-HT_{1A} receptors [117].

Preventing mitochondrial failure, limiting programmed cell death

Mitochondrial collapse is considered the hallmark of secondary energy failure and might be the key event in the initiation of cell death pathways leading to irreversible brain injury after perinatal HI[56]. It has been suggested that CBD interacts with complexes in the electron transport chain to indirectly improve mitochondrial bioenergetics and function under pathological conditions [115,121]. CBD might ameliorate mitochondrial failure and apoptosis by mitigating several of these key initiating steps, such as calcium overload that cause uncoupling of the mitochondrial electron transfer and activation of enzymes that injure the mitochondrial membrane, both directly and indirectly through production of reactive oxygen and nitrogen species[122,123]. Further, the increase in pro vs. anti-apoptotic proteins (e.g. Bax vs. Bcl-2) lead to permeabilization of the outer mitochondrial membrane and release of cytochrome C that initiates the pro-apoptotic cascade [122,124], which is an important contributor to the overall neuronal loss after HI [40,125]. CBD has been shown to reduce levels of caspase-3, the "final executioner" of apoptosis, in both *in-vitro* and *in-vivo* models of neurotoxicity [102,126].

Ameliorating neuroinflammation

An excessive immune response exacerbates brain injury after perinatal HI [56,127] and CBD has demonstrated anti-inflammatory effects through different mechanisms [86,128]. CBD can attenuate microglial activation and migration [107,129], probably as a result of modulating adenosine transport through interaction with the equilibrative nucleoside transporter 1 (ENT1) and also by interaction with the adenosine receptors [130]. CBD has also been shown to reduce the levels of inflammatory cytokines, e.g. TNFα and IL-1β, after HI injury [93,97,98,130]. Furthermore, inhibition by CBD of the transcription of NF-kappa-B, an essential transcription factor in the inflammatory pathway, has been observed [109,131].

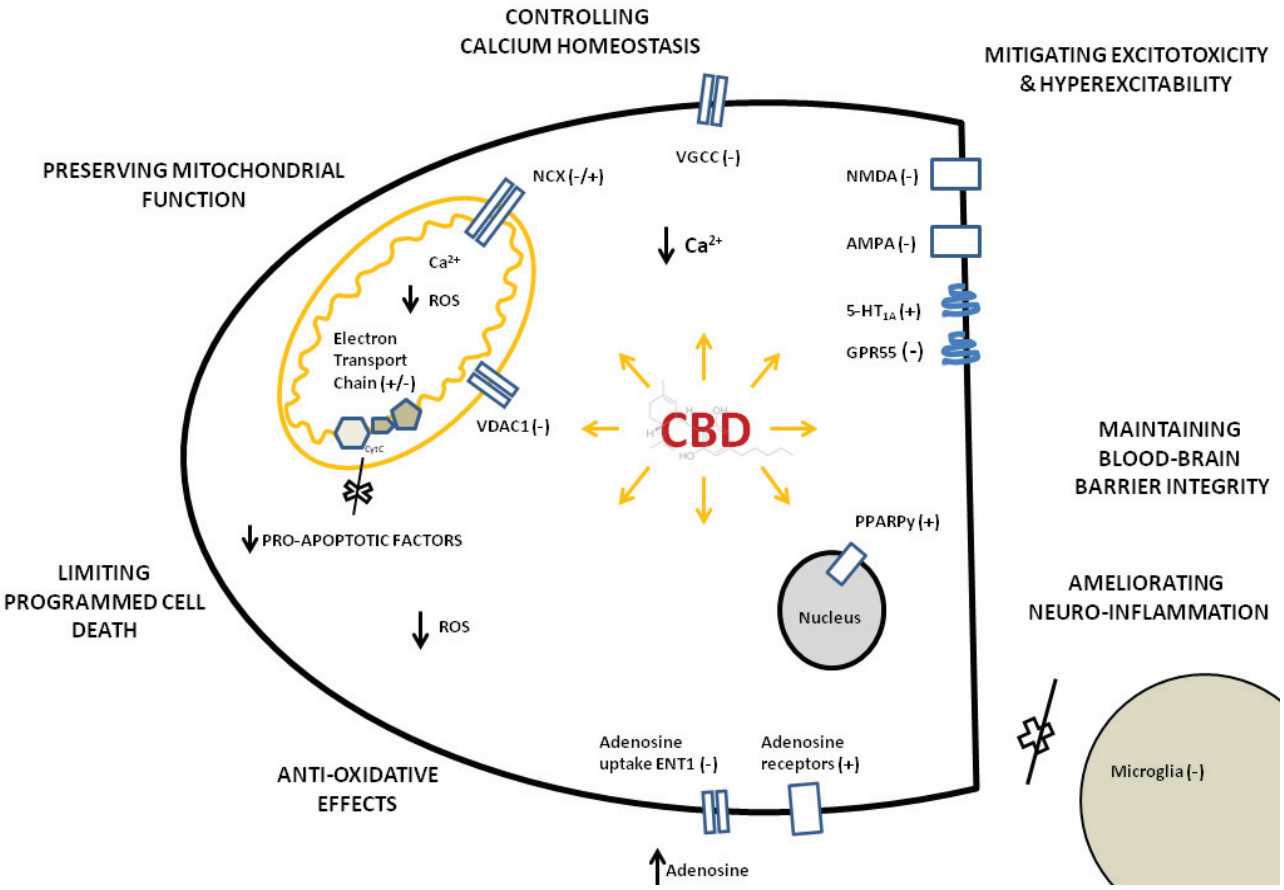

Figure 4. This is a schematic presentation of a neuronal cell illustrating some of the possible molecular targets and mechanisms that could be involved neuroprotection by CBD. [83]. Pluss (+) indicate stimulation/binding, minus (-) indicate inhibition/blockage. CBD has anti-oxidative effects mainly based on its chemical conformation and ability to act as a free radical scavenger. *ROS= reactive oxygen species, NCX= mitochondrial Na^{+}/Ca^{2+}-exchanger, CytC= cytochrome C, VDAC= voltage dependent anion channel, VGCC= voltage gate calcium channel, 5-HT_{1A}= serotonin receptor, AMPA= α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, NMDA = N-methyl-D-aspartate receptor, ENT1= Equilibrative nucleoside transporter 1, PPAR= peroxisome proliferator-activated receptor.*

Conflicting effects of CBD

We are still far from determining the exact molecular actions and effects of CBD. When searching the literature, we find conflicting effects of CBD on several mechanisms involved in neuroprotection. For instance; in the brain CBD has been shown both to ameliorate and exacerbate oxidative stress [108,132], to reduce and increase intracellular calcium levels [115,132], and to inhibit and to activate microglia and other immune cells [107,133]. In other pathophysiological settings, such as cancer, CBD has been shown to reduce proliferation by the exact opposite mechanisms of which by it prevents cell death in studies on neuroprotection. In a study of human glioma cells CBD increased ROS production and triggered apoptosis by caspase activation [134]. In fact, numerous studies have demonstrated CBD`s ability to induce apoptosis in cancer cells [135].

It becomes evident that the effects of CBD seem highly dependent on cell type (healthy vs. cancer cells), and the biophysical and pathophysiological setting (e.g. in vitro vs. in vivo) in which it is administered. A very fascinating observation in this regard is that cannabinoids can mediate distinct signalling mechanisms even in a single neuron, depending on the state of the neuron, meaning that the physiological condition of the neuron at the time of drug delivery may affect the outcome of treatment [136,137]. Further, the dose and concentration of CBD can produce differential effects, as demonstrated for other phytocannabinoids (reviewed in Sarne *et al.* [138]). This highlight the importance of testing cannabidiol in well established pre-clinical models, with sufficient biological resemblance to the condition one intend to treat.

MicroRNAs as biomarkers of perinatal hypoxic-ischemic brain damage

MicroRNAs (miRNA) are short (~22 nucleotides) non-coding RNAs that are essential regulators in the post-translational regulation of gene expression, tissue development and homeostasis. MiRNAs are integral to almost all known biological processes, including cell growth, proliferation and differentiation, as well as metabolism and development [139,140].

MiRNA - biogenesis and function

MiRNA biogenesis is a complex process, but in the classic understanding miRNAs are initially expressed as precursors (pre-miRNAs) in the form of double stranded RNA hairpins and are then cleaved in the nucleus by the ribonuclease *Drosha* to produce primary miRNAs (pri-miRNAs). Pri-miRNAs are transported by Exportin-5 from the nucleus to the cytoplasm, and the terminal loop is then cleaved by Dicer RNase III to form a double-stranded mature RNA. Mature miRNA may associate with argonuate proteins into the RNA silencing complex (RISC) where miRNA guide RISC to specific mRNA`s and inhibits the translation to protein Alternatively mature miRNA may either be degraded or released from the cell through various export mechanisms. Either in small vesicles (exosomes), bound to high-density lipoproteins or RNA binding proteins or in a microparticle free form. This release is believed to be either passive e.g. as a by-product of damaged cells or active through interaction with specific membrane channels and proteins and as such miRNA might be involved in cell-to-cell communication [141,142].

MiRNA as biomarkers

The expression of miRNAs has been found to be specific for tissues, developmental stages, and various pathological conditions [143,144]. This, along with the discovery of their presence and remarkable stability in the circulation, sparked the interest for miRNAs as biomarkers[145]. An increasing number of studies to date have demonstrated the ability of circulating miRNAs to reflect tissue pathology in various conditions, among others, stroke, myocardial infarction, and brain damage after cardiac arrest [146-148].

miRNAs as biomarkers after perinatal HI

Very few studies have yet investigated miRNAs as biomarkers in the setting of perinatal HI. Whitehead *et al.* showed that circulating miRNAs in maternal blood could predict fetal hypoxia in-utero [149]. Qui *et al.* found that miR-210 protected PC12 cells from dying after oxygen glucose deprivation by inhibition of apoptosis[150] and also reproduced these finding in neonatal rats[151]. Interestingly Ma *et al.* found different effects with inhibition of miR-210 leading to neuroprotection after hypoxia-ischemia in neonatal rats [152]. Looney *et al.* showed a significant step-wise downregulation of hsa-miR-374a expression in cord blood of infants with perinatal asphyxia and subsequent HIE [153]. And recently Ponnusamy *et al.* demonstrated the feasibility of analyzing circulating miRNAs in dried blood spots sampled from asphyxiated newborns [154]. Despite a limited number of studies miRNAs are considered to be promising candidates also in the setting of perinatal brain injury [155].

The selected miRNAs in our study

This was considered as a "pilot study" of microRNAs in our animal model. Due to financial constraints, we chose to focus on selected miRNAs in our study rather than performing a microarray study, well aware that the latter probably is a preferred approach. Despite focusing on only a few microRNAs, the number of analyses was considerable due to the four time-points evaluated. The following miRNA candidates were chosen based on a literature review.

MiR-124 is the most abundant miRNA in the brain and is considered to be brain specific. MiR-124 has a crucial role in neurogenesis, neuronal homeostasis and differentiation [156]. It`s dysregulation has therefore, not surprisingly, been implicated in neurological diseases and injury to the nervous system [157]. In animal models it has been shown that miR-124 is up-regulated in the ischemic penumbra and also in plasma after middle cerebral artery occlusion [158]. Further circulating miR-124 has recently been put forward as a biomarker of neurological outcome after cardiac arrest in humans [159,160].

MiR-125b is highly enriched in the brain, and in addition to expression in neurons it is also expressed in glia [161]. MiR-125b is believed to play a role in neuronal differentiation and synaptogenesis [162]. Circulating miR-125b has been studied in the setting of various cancers and neurodegenerative disease [163,164], but has also been suggested as a biomarker of stroke [165].

MiR-374a has mostly been studied in the setting of various cancers where it predicts survival [166,167]. Its exact biological function is unknown. It has however, by Looney *et al.* [153], been linked to perinatal HI and HIE. They showed a stepwise downregulation in cord-blood expression of miR-374a in infants with asphyxia and in those who developed HIE, respectively, and target analysis identified several plausible pathways linked to neurological injury [153].

MiR-210 is also known as "the master hypoxiamiR" and is believed to be an essential regulator in the cellular response to hypoxia [168,169]. In fact it has been demonstrated that circulating miR-210 act as a messenger to coordinate the hypoxic response among cells [170]. MiR-210 is ubiquitously expressed, but also highly expressed in the brain and has been linked to perinatal hypoxic-ischemic injury in several animal studies [152,171]. Circulating miR-210 has been found to predict fetal hypoxia when measured in maternal blood [149] as to predict survival in critically ill patients with acute kidney injury [172] among others.

Methodological considerations

The study of miRNAs as biomarkers is still a novel area, and despite their great promise they have yet to prove diagnostic specificity, reproducibility and thus clinical usefulness as biomarkers. This is probably partly due to methodological challenges with optimization and standardization of sampling, miRNA extraction, isolation and analysis still being a work in progress [173,174]. We applied an approach used previously in studies published in high-quality journals [159,160,175] and we aimed to standardize the handling of samples and methods of extraction and analysis as much as possible.

Aims of the studies

Previous animal studies have consistently demonstrated neuroprotection by CBD in the setting of perinatal HI. Before considering clinical trials, however, it is essential to reproduce the promising findings in other large animal models independently. Further, there is a need for new tools to improve the detection of perinatal hypoxic-ischemic brain injury.

In the studies included in this thesis we have, in a well-established piglet model of global perinatal hypoxia-ischemia, aimed to evaluate the following:

1. Possible neuroprotective effects of CBD (Paper I, II)

2. Possible synergic or additive effects of CBD on therapeutic hypothermia (Paper I)

3. Possible dose-related effects of CBD (Paper II)

4. Potential of circulating miRNAs to be biomarkers of hypoxic-ischemic injury (Paper III)

Methods

The animal model

Background

Animal models have contributed significantly to our understanding of the pathophysiology of HIE and possible new interventions [176]. Different preclinical models of HIE exists all with inherent strengths and weaknesses. The type of models range from *in-vitro* models using cell-cultures [177] and organotypic brain slices [178], small animal models using mice [179] and rats [180] and large animal models using lambs [127,181] and piglets [182-186]. Also other animals such as non-human primates, rabbits and dogs have been used.[187]

The advantage of the piglet model is its similarity to human neonates in several important aspects. Piglets are comparable in body size and weight which make them easy to work with in an experimental neonatal intensive care setting. Piglets are comparable to human neonates in brain anatomy, growth and myelinisation, and development [188,189]. Further, compared to humans, piglets have a similar response, pattern of organ injury, as well as timeline of secondary energy failure after HI [50,176,188,190].

Different piglet models of perinatal HI are in use. They vary in how HI is induced, the method of monitoring HI and how they "quantify" the hypoxic-ischemic insult. As a consequence, they also vary to some extent in the degree and distribution of injury. In some models, HI is achieved by carotid occlusion along with a reduction the FiO_2 [62,95,97,99,191]. Others rely solely on global hypoxia with a fixed or with variable FiO_2 [182,185,186,192]. Some models apply a fixed duration of HI [191] while others quantify HI by amplitude integrated EEG [193], in vivo magnetic resonance spectroscopy [62] or by biochemical and physiological parameters [186]. The various piglet models also vary in survival time after HI, from moderate/long (24-72 hours) [62,194] to shorter duration, as the current model and the majority of studies on the effects of CBD (6-9.5 hours) [185,191].

Animal welfare

The Norwegian Council for Animal Research approved the experimental protocols (approval number 5723 and 7359). The animals were cared for and handled in accordance with the European Guidelines for Use of Experimental Animals and everyone involved in the animal experiments in the current study were certified FELASA (Federation of European Laboratory Animals Science Associations) Category C researchers. These studies are classified as non-recovery studies that have a low severity grade in animal research. All procedures were performed under general anesthesia and the animals did not recover consciousness before being euthanized. Further the experiments were planned and conducted with the principles of reduction, refinement and replacement (3Rs) in mind [195].

Inclusion criteria

The piglets included in our studies fulfilled the following criteria: age of 12–36 h, hemoglobin levels >5 g/dl and good general condition. The general condition was assessed subjectively by looking at general appearance (skin color, effort of breathing, muscle tone) and level of activity.

Anesthesia and procedures

Adequate anesthesia is essential in animal experiments to minimize stress, pain and discomfort. Before the induction of anesthesia 2-3 piglets were kept together in a thermoneutral environment and exposure to noise, light and movements were kept to a minimum. Piglets were then gently removed from their container, held in a warm blanket while an ear vein was cannulated and anesthesia was induced by administration of Fentanyl 50 µg/kg, Midazolam 1 mg/kg, and Pentobarbital 15 mg/kg. Anesthesia was maintained by continuous infusion of Fentanyl 50 µg/kg/hour and midazolam 0.25 mg/kg/h in mixtures, giving 1 ml/kg/hour for each drug. This protocol is well established in the current animal

model [182,185,186] and the effects of the drugs used have been extensively evaluated in the newborn pig [196-199].

We aimed to closely monitor the depth of anesthesia by assessing changes in heart rate, active movements and the motor response to painful stimuli (retraction when pinching toes). When considered insufficient, an i.v bolus of midazolam 1 mg/kg and Fentanyl 50 µg/kg were given. If ineffective, pentobarbital 15 mg/kg was added. In rare cases where excessive shivering was observed despite adequate depth of anesthesia pancuronium 0.1 mg/kg, was added. In our experience shivering occur in anesthetized piglets despite adequate depth of anesthesia, probably as a response to a low body temperature, and thus was a challenge especially in animals treated with hypothermia.

We were well aware of the potential confounding effects of these drugs e.g. by their actions on the cardiovascular system and cerebral blood flow [196-198] and also by their possible neuroprotective effects [200,201]. The fact that we observed changes in the outcome variables also in the control/SHAM animals (paper I) can, in addition to mechanical ventilation and instrumentation, probably be attributed to anesthesia. Therefore a strict protocol was followed to ensure equal dosages among animals and groups. Regardless, the strict randomization to the different treatment groups should have limited the possible confounding effects of anesthesia.

Due to some concerns regarding the unwanted cardiovascular effects of midazolam [198,202] we modified this protocol slightly in study II. Here midazolam was given only for induction while maintenance anesthesia was achieved by fentanyl alone. Boluses of midazolam and pentobarbital were given “on-demand” as in the original protocol. We are aware that fentanyl cannot be considered a sedative, and that this approach could be considered problematic from an animal welfare perspective. However, we carefully assessed signs indicating insufficient depth of anesthesia and our initial experience is that the depth of anesthesia was sufficient with this approach.

Throughout the experiments, there was a continuous surveillance of blood pressure (measured through the indwelling carotid artery-catheter), saturation (Masimo Pulsoxymeter), pulse (BioPac ECG) and temperature (measured by electronic thermometer) Further, temperature-corrected arterial acid/base status, glucose and hemoglobin were regularly measured throughout the experiment on a Blood Gas Analyzer 860 (Ciba Corning Diagnostics, Midfield, Mass., USA).

The animals in the two experiments that have produced the data for this thesis were randomized by sealed envelopes. To reduce the number of animals needed, we designed the first study to allow for the control groups (controls/SHAM, VEH and VEH + hypothermia) to be shared with another experiment evaluating DHA, another novel neuroprotectant. This was achieved by prospective allocation to the different study groups including the groups assessing DHA by block randomization.

Cannabidiol preparation and administration

Pure CBD (GW Pharmaceuticals, Cambridge, UK) was dissolved in a vehicle consisting of ethanol:solutol:saline at a ratio of 2:1:17. In the first study a 5 mg/ml solution was prepared a dose of 1 mg/kg, equal to 0.4 ml in a 2 kg pig, was given as a bolus in the central i.v line. In the high-dose study a solution of CBD in vehicle 10 mg/ml was prepared and, according to desired dose, mixed with saline to give a total volume of 20 ml. CBD and vehicle were given 30 minutes after the end of hypoxia as a slow intravenous infusion over 15 minutes. All vehicle treated animals in the high-dose study received an equivalent amount of vehicle to the highest CBD dose regimen, namely 10 ml, mixed with saline to give the same total volume of 20 ml.

Methodological considerations

In the current piglet model we apply a global hypoxic-ischemic insult with a fixed FiO_2 (8%) and a variable duration, quantifying the insult based on biochemical (base excess) and physiological (mean arterial blood pressure (MABP)) variables. Ischemia is achieved by

induction of hypoxemia until the point of cardiovascular de-compensation, and a drop in MABP below the lower limit of cerebral auto-regulation for newborn piglets (~30-40 mmHg) [203-205]. The aim is to mimic an acute global hypoxic-ischemic insult in the term born infant.

Apart from being well established in our research group, the strength of our model is its ability to reflect a global hypoxic-ischemic insult with multi-organ involvement, which is common in newborns after perinatal HI [206,207]. Looking at the severity of metabolic acidosis and hypotension, the insult in this model can be classified as severe and mirrors global asphyxia rather than more localized ischemia. This permits the study of different aspects compared to animal models applying a more targeted and milder insult [70,191,208,209]. In fact, according to the current criteria for therapeutic hypothermia, the animals in these models would not be eligible for cooling based on degree of metabolic acidosis.

One of the challenges in this model is the considerable variation among piglets in the tolerance and response to HI which is reflected in the degree of injury. For this reason, some piglets are potentially too severely injured for neuroprotection to be found, regardless of treatment. On the other hand, there are also some piglets that do not exhibit a significant drop in blood-pressure (below the limit of cerebral auto-regulation) and as such cannot be said to have suffered significant ischemia. Further, the short survival time is a major limitation regarding the translation of our findings to a clinical setting. A survival time of 9.5 hours after HI only permits the study of effects an intervention has on pathophysiological mechanisms involved in the latent and initial phase of secondary energy failure. Another important consideration is that the study animals already have completed the fetal to neonatal transition and adapted to extra-uterine life, as it has been shown that the response to HI is different in-utero as compared to ex-utero [210]. Thus this model reflects post-partum HI better than intra-partum HI. In conclusion, one should be cautious when interpreting the findings regarding long-term outcome and careful when applying the knowledge gained to the clinical setting.

To improve efficacy and translatability of the current animal model there are some modifications that could be considered. Most importantly we could aim for a longer survival

time to better reflect effects on secondary energy failure. However, in its current form, this is challenging for several reasons. The piglet model, in general, is highly energy intensive and after being exposed to HI, piglets are in need of full intensive care and monitoring around the clock. Understandably this requires a lot of resources and due to the severity of HI and multi-organ injury in the current model, this is even more challenging. We could consider a milder insult measured by degree of hypotension and metabolic derangement, and the use of additional tools to monitor and more precisely grade the hypoxic-ischemic insult, to prevent excessive injury while at the same time ensuring significant damage [193]. The current research in this model on the metabolome after HI could potentially provide markers to indicate severity and help grade HI [211,212]. Being able to produce more homogenous injury would have reduced variability [192,193] and the number of animals needed to achieve adequate statistical power.

Analyses

CBD concentrations in brain and plasma

In the first paper the concentration of CBD in plasma was measured by gas chromatography-mass spectrometry at the Norwegian Institute of Public Health (Oslo, Norway). In the second paper CBD concentrations in brain and plasma were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at LGC (Fordham, UK).

Histology

Histopathological evidence of neuronal damage can be found early after HI [182,213]. Hematoxylin and eosin (H&E) staining is a well-established method for evaluating morphological changes in neuronal tissue. Analysis of H&E sections was performed by an experienced pathologist blinded to the randomization and clinical details. Areas assessed in the first study were cortex (3 levels), hippocampus and cerebellum and in the second study cortex, white matter and striatum. In cerebrum areas with vacuolated neuropil, shrunken

neurons with pyknotic nuclei and scattered eosinophilic neurons were defined as early neuronal damage. In the cerebellum eosinophilic Purkinje cells were the indicators of damage. A modified version of a validated scoring system was used [184,214]. Based on all regions assessed a global score was given to each piglet. Zero indicating no damage, 1: mild/moderate, 2: moderate/severe, 3: severe and 4: massive damage.

A semi-quantitative approach rather than cell-counting in specific brain areas (quantitative) was preferred when assessing the brain damage in this animal model. Firstly the degree and distribution of damage varies considerably between piglets and choosing one specific area for cell counting is therefore vulnerable to bias. Also we are not able to, with millimeter precision; reproduce the exact same plane of tissue section when preparing the tissue blocks. Thus evaluating the whole brain slide rather than one specific area is a more robust assessment in our opinion and importantly; this approach has previously been validated in piglets [214].

Biomarkers of glial (CSF derived), myocardial and renal injury

S100B

S100B is a calcium-binding protein mainly found in glial cells of the central nervous system (CNS). Damage to the CNS is associated with a continuous release of S100B which can be detected in different biological fluids including cerebrospinal fluid [215]. Numerous studies have documented a correlation between the extent of brain damage due to hypoxia/asphyxia and elevated concentrations of S100B [216-218] and thus an increase in S100B protein level is considered a promising early index of perinatal brain damage [219,220]. We measured S100B using electrochemiluminescent immunometric assay on the Cobas e601 immunoassay platform (Roche Diagnostics, Mannheim, Germany).

Troponin-T

Plasma troponin-T is a well-documented marker of myocardial ischemia in asphyxiated neonates [206,207]. Studies have also demonstrated that troponin may aid in the prediction of

the severity of neonatal HIE as well as mortality [221] as significantly higher troponin levels are found in neonates with severe asphyxia compared with those mildly affected [222,223]. We measured plasma troponin-T using electrochemiluminescent immunometric assay on the Cobas e601 immunoassay platform (Roche Diagnostics, Mannheim, Germany).

Neutrophil gelatinase associated lipocalin (NGAL)

NGAL is a lipocalin secreted by activated neutrophils and released from different cell types in response to injury (e.g. kidney, lungs and colon). Urinary NGAL levels are increased by 25–100-fold in response to renal epithelial injury and are considered an excellent marker of acute kidney injury [224]. In asphyxiated newborns urinary NGAL can differentiate between those who develop and do not develop acute kidney injury, and does also to some degree predict the severity of HIE [225]. Urinary NGAL was measured by a porcine specific ELISA (Bioporte, Hellerup, Denmark).

Ex-vivo magnetic resonance spectroscopy (H+-MRS)

Cerebral magnetic resonance spectroscopy biomarkers , and especially the Lac/NAA ratio, are considered accurate biomarkers in neonatal HIE [226]. The Lac/NAA ratio, as well as NAA concentration, have been put forward as potential bridging biomarkers between pre-clinical and clinical studies [227]. In-vivo MRS have been used in the current animal model previously (although with the use of bilateral carotid artery occlusion) showing significant alterations in several metabolites, including lactate and n-acetylaspartate (shown in figure the pictures below from the study by Munkeby *et al.* [182].)

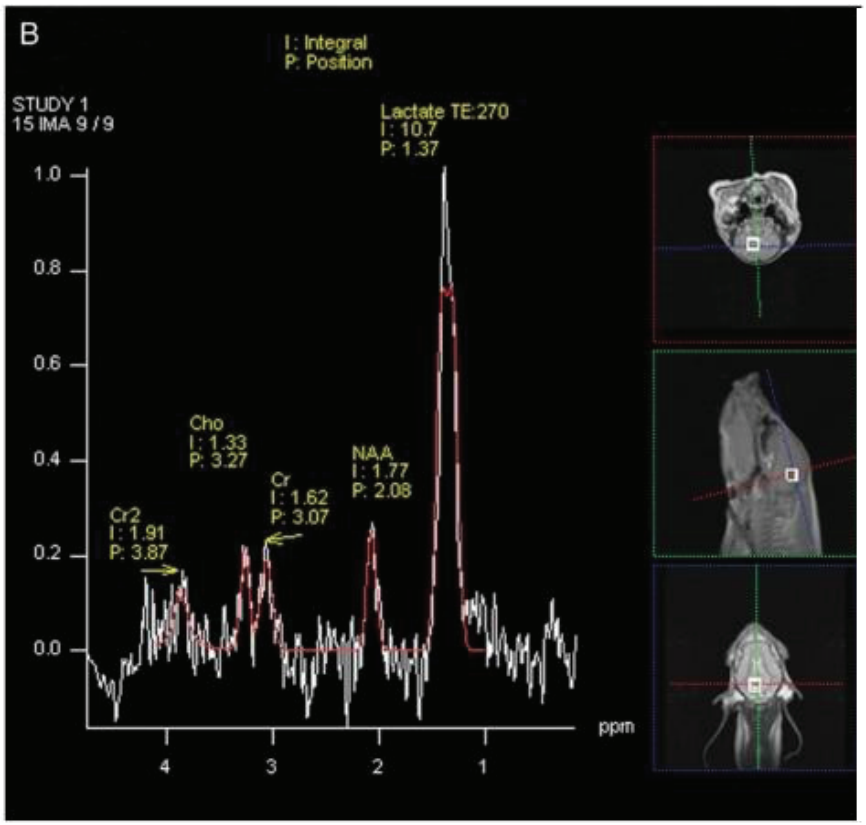

Figure 5. Proton MRS spectra (single voxel, PRESS) recorded in the basal ganglia of a piglet 7 hours after exposure to global HI. Note especially the lactate (lac) peak and the low N-acetylaspartate (NAA) peak. Choline (Cho), creatine (Cr). Adapted figure from a previous study by our group; Munkeby et al., Acta Radiol. 2008 Nov;49(9):1049-57. Used with permission.

We did not have access to *in-vivo* H+-MRS in the current studies, but instead performed ex-vivo H+-MRS which has been used in previous piglet studies to demonstrate neuroprotection by CBD [95,191]. The sensitivity and spectral resolution of ex-vivo H+-MRS on fresh frozen tissue have been shown to be comparable to *in-vivo* H+-MRS [228]. Our analyses were carried out at the same location as previous CBD studies in piglets (MRI Unit of the Instituto Pluridisciplinar (Universidad Complutense, Madrid, Spain)) and the details of the method can be found in the publications from Pazos *et al.* [191] and Lafuente *et al.* [95] as well as in paper I.

Gene, mRNA and miRNA expression

Quantitative real time polymerase chain reaction (qPCR) was used to analyze gene expression in brain tissue (Paper I) and miRNA expression in plasma (Paper III). This is a widely used method for mRNA as well as miRNA studies giving a high sensitivity and specificity. The real time PCR uses a polymerase to amplify a specific target sequence, causing a chain reaction that is performed repeatedly to exponentially amplify the target. A

fluorescence signal increases proportionally to the amount of PCR product during each cycle and is detected at a certain threshold, the threshold cycle (C_T) [229].

For relative quantification of changes in gene and miRNA expression the comparative C_T method was used[230]. The C_T values of the genes of interest were normalized to endogenous controls and the mean C_T values in the non-hypoxic controls using the $2^{-\Delta\Delta CT}$ formula. For the miRNA expression, however, an exogenous spike-in (cel-miR-39) was used as a control because there is no universally accepted endogenous control for miRNA studies in plasma [231].

Protein expression

For measurement of cytokines in brain tissue and urinary NGAL the enzyme linked-immunosorbent assay (ELISA) was used. ELISA uses a specific antibody to bind an antigen in a sample. This antibody is linked to an enzyme that converts an additionally added antibody, generating a signal that is detectable by photometry [232].

Statistical analysis

Statistical analysis was performed in SPSS version 19 and 21 and in GraphPad Prism. The considerable variability among piglets in response to the hypoxic-ischemic insult is reflected in our data that often exhibit a large variance. Increased variance reduces the statistical power and hence the risk of type II errors and this is something we have considered when interpreting our findings. When data have shown a non-normal distribution we have used log-transformation to achieve a normal distribution. If unsuccessful, non-parametric statistics have been applied.

Summary of the results

Paper I: Short-term effects of cannabidiol after global hypoxia-ischemia in newborn piglets.

We found a significant effect of the hypoxic-ischemic insult on neuropathology score, levels of S100B in cerebrospinal fluid, plasma troponin-T, hippocampal H^+MRS biomarkers and levels of urinary NGAL. Gene and protein expression, as well as lipid peroxidation products in urine, were not significantly altered by HI.

Compared to vehicle we found no effects of CBD alone on the different outcome variables. CBD did not augment or add to the observed neuroprotective effects of therapeutic hypothermia, but we observed significantly reduced levels of urinary NGAL in this group. Therapeutic hypothermia alone significantly lowered the hippocampal Glu/NAA ratio and reduced plasma levels of troponin-T.

Paper II: High-dose cannabidiol induced hypotension after global hypoxia-ischemia in piglets.

In this study, we observed significant hypotension after treatment with the highest doses of CBD (50 and 25 mg/kg) in some animals, with a drop in MABP below 70% of baseline during infusion. Three out of four piglets in the 50 mg/kg group developed significant hypotension, and in one of these piglets this progressed into fatal cardiac arrest. In the other two piglets, the blood pressure slowly recovered to the level of vehicle. One out of four piglets in the 25 mg/kg group developed severe hypotension, and 2 had a milder drop in blood pressure. CBD 10 mg/kg did not induce significant cardiovascular effects. The mean drop in MABP in the 50 mg/kg group was significantly lower than in vehicle, and we found a significant overall correlation between plasma concentration of CBD and degree of hypotension.

High-dose CBD had no significant effects on the outcome variables related to neuroprotection, such as neuropathology score, levels of S100B in cerebrospinal fluid, plasma troponin-T or hippocampal H^+MRS biomarkers.

Paper III: Temporal profile of circulating microRNAs after global hypoxia-ischemia in newborn piglets.

In this study we characterized, in plasma, the temporal changes of four selected miRNAs starting at baseline (before HI); at the end of HI, and 0.5 h, 3.5 h and 9.5 hours post-HI. Compared to baseline the expression of the brain-specific/enriched miRNA 124 and 125b was not significantly altered. However, miR-374a and miR-210 displayed significantly alterations during and immediately after HI.

The absolute expression of miR-374a at 0.5 h post HI was significantly higher in hypoxic-ischemic piglets compared to controls. This was also observed when stratifying according to the presence of neuropathological damage at end of study, and according to low vs. high lactate levels. Finally, we observed a negative correlation between miR-374a and arterial blood pH and base excess, and a positive correlation with levels of lactate.

Discussion

Cannabidiol as a neuroprotectant after perinatal HI (I & II)

We have during two experiments studied the effects of intravenous CBD in a well-established piglet model of neonatal HI. This model permitted the elucidation of new aspects of CBD`s effects and limitations after hypoxia-ischemia. Both the "established" dose of CBD (1mg/kg) and high-dose CBD (10-50 mg/kg) were assessed and hence a relatively wide range of molar concentrations where neuroprotection should have been evident based on the existing literature.

In this animal model, CBD did not demonstrate significant effects on any of the outcome variables used to assess neuroprotection. This was primarily evaluated by inter-group comparison (I & II), but also, for the purpose of this thesis, we performed a linear regression analysis of all data to assess possible dose related effects of CBD on the various outcome variables (table 1). A significant correlation was found only for the CBD-induced reduction in blood pressure.

	Pearson r	95% CI	P (two-tailed)
Neuropathology score	0,17	-0,18 to 0,47	0,3348
S100B in CSF	-0,19	-0,49 to 0,14	0,2556
Lac/NAA ratio	0,076	-0,26 to 0,39	0,6614
Glu/NAA ratio	0,16	-0,18 to 0,47	0,3434
Plasma TnT	-0,14	-0,45 to 0,20	0,4241
Delta MABP (drug infusion)	-0,81	-0,90 to -0,66	< 0,0001

Table 1 Correlations between the concentrations of CBD in plasma 9 hours post-dose vs. different outcome variables. (Piglets receiving CBD 1 mg/kg + hypothermia are also included)

There are different possible explanations for the lack of effects observed in our studies. One is that CBD is not neuroprotective, especially after more severe insults, and that effects of this single molecule are insufficient to mitigate the complex cascade of injurious events

causing brain injury after perinatal HI. Previous *in-vitro* studies have, in fact, raised concerns regarding the efficacy and safety of CBD in the brain. Harvey *et al.* showed an inability of CBD to protect against Aβ induced neurotoxicity. Schonhofen *et al.* [233] revealed no neuroprotective effects of CBD, as measured by protection against redox-active neurotoxins, in a mature human neuroblastoma SH-SY5Y cell line. Further, they demonstrated that exposure to CBD during differentiation in this cell line sensitized the cells to further challenges with redox-active toxins. Mato *et al.* [132] showed that brief exposure to cannabidiol (100 nM-10 µM) led to a concentration-dependent decrease in the viability of oligodendrocytes. This effect was partially blocked by inhibitors of caspase-3, -8 and -9, PARP-1 and calpains, suggesting that CBD triggers caspase-dependent and -independent death pathways. CBD was also found to increase reactive oxygen species in this study. However, as discussed previously, it is difficult to extrapolate *in-vitro* results to an *in-vivo* setting and the available data from animal models show only positive reports. In particular, the studies in models of neonatal HI who all show remarkable effects of CBD [93,95-99]. It is thus clear that alternative explanations for the lack of effects seen in our studies have to be evaluated.

In respect to the existing evidence from piglets, we have largely used similar variables and time points for the assessment of neuroprotection[95,97,99,191]. However, there is a considerable dissimilarity in the nature and severity of the hypoxic-ischemic insult. The degree of hypotension and metabolic acidosis is much more profound in our model. In fact, according to current criteria for treatment with therapeutic hypothermia the piglets in previous studies would not be eligible for treatment based on the degree of metabolic derangement. Further, it has recently been questioned whether bilateral artery occlusion, as applied in these studies, create the ischemia it is intended to do in piglets [234,235]. If not, the discrepancy in insult severity between these two models is even larger.

As a result of the discrepancy in insult severity, we could expect that the state of the neurons is dissimilar in these two models. As demonstrated by Bologov *et al.* [236], the effects of different cannabinoids depend on the physiological conditions under which they are administered. Even in the same cell type the observed, dual and opposite effects of CBD on

the viability in a neuroblastoma cell line. Further, it is obvious that the insult severity influences the probability of finding an effect of the treatment. We do acknowledge the possibility that some of our piglets suffer intractable brain damage and thus are resistant to intervention. The limited effects of therapeutic hypothermia in our study could be interpreted as evidence in support of this, as both clinical and experimental data indicate that cooling is less effective after the most severe insults [4,237]. However, a considerable proportion of perinatal hypoxic-ischemic insults are severe with considerable multi-organ damage [206,207] and we thus argue that it is relevant to evaluate the effects of novel neuroprotectants also in models reflecting this spectrum of injury.

	Piglet model in other CBD studies (Madrid, Spain)	**The current piglet model (Oslo, Norway)**
Strain of piglet	Landrace-large White	Noroc (LyxLD)
Method of inducing HI	Ventilation with ≤10% O_2 and bilateral carotid occlusion (BCAO)	Global HI by ventilation with 8% O_2 until the point of cardiovascular de-compensation
Duration of HI	Fixed – 20-30 min	Variable 40-80 min
Degree of metabolic acidosis	Mild/moderate (pH 7.2, BE not reported)	Severe (pH 6.9, BE ~-20 mmol/l)
Degree of hypotension	Mild/moderate (>70% of baseline)	Severe (<70% of baseline)
Anesthesia	Propofol, midazolam, fentanyl	Pentobarbital, midazolam, fentanyl
End-point	6 hours (3 studies) & 72 hours (1 study)	9.5 hours
Histological analysis	Quantitative Counting necrotic cells in pre-defined areas.	Semi-quantitative Evaluation of whole brain slices for neuronal damage.

Table 2 Summary of some of the important differences in the current animal model compared to the one used in previous studies.

CBD and therapeutic hypothermia

The group in Madrid, Spain recently demonstrated that CBD 1 mg/kg augmented the effects of therapeutic hypothermia in their model [95]. We did not observe any significant additive or synergic neuroprotective effects of combining CBD and therapeutic hypothermia (Paper I). The limited effects of cooling alone in our study, with reduced Glu/NAA ratio and plasma Troponin-T levels as the only significant findings, do make the interpretation of these results

more challenging. However, given the severe global HI, our findings of reduced exitotoxicity are in line with previous studies in piglets are. We found only one other piglet study demonstrating early effects of cooling after comparably severe HI, where the authors assessed the effects of hypothermia on excitotoxicity and nitric oxide production, both of which were ameliorated by hypothermia [238]. Another study demonstrated effects only when animals were stratified according to injury severity [184]. Indeed the majority of studies showing hypothermic neuroprotection in piglets have applied milder insults in terms of global damage [208,209,239]. Further, the effects in previous models have mainly been studied after longer survival times, which in general make comparison difficult. Although not methodologically correct, when we combined both the hypothermic groups in our study (n=24), the difference reach significance also for the lactate/NAA ratio (hypothermia 1.9 [1.6 – 2.6] vs. normothermia 2.4 [2.1-3.1], p=0.02) and the percentage of animals with histopathological damage (hypothermia 50% vs. normothermia 78%, p=0.04).

The only significant observation of synergic effect of CBD and hypothermia was reduced levels of urinary NGAL. We interpret this as a possible synergic nephroprotective effect, although CBD or cooling alone had no effects on these levels. Meta-analyses have found no differences in renal or hepatic function in neonatal cooling trials, but a trend towards reduced risk of renal impairment, and for CBD it has previously been shown that could exert nephroprotection [240].

Safety

The majority of existing data classifies CBD as a safe drug [241]. However, the period following global HI with multi-organ involvement is precarious [207]. A meta-analysis was published just after the acceptance of paper II, concluding that CBD has no hemodynamic side effects under normal physiological conditions, but lowers blood pressure under stressful conditions [242]. Further, they state that more data are needed determine the hemodynamic effects of CBD fully. We observed that cardiovascular effects that could be considered beneficial under normal physiological conditions [243], led to unwanted effects in the setting of global HI. Indeed, piglets exposed to mild HI do not seem to display significant hypotension after CBD 50 mg/kg i.v. (personal communication from Prof. Orgado, collaborative unit in Madrid,

Spain). However, the cardiovascular dysfunction in our animal model is more pronounced (Table 2). CBD did not produce cardiac arrhythmia, and the heart rate was not significantly altered in these animals. Instead, we speculate that the observed hypotension resulted from a combination of CBD`s sympatolytic, vasorelaxant and cardio-depressive actions [243-245] under stressful conditions. At the molecular level CBDs interaction with ENT1, the serotonergic receptor (5-HT_{1A}),, and with the G-protein-receptor55 are probably involved in these effects[83].

Optimal dose

We cannot conclude on an optimal dose for neuroprotection based on our findings, simply because we have not observed any neuroprotective effects. We can, however, state that one should be cautious if considering doses above 10 mg/kg i.v in future studies of perinatal HI. Alternative strategies of drug infusion, such as a more diluted solution at an even slower infusion rate, could be considered to allow for improved tolerance to higher doses. Regardless, the possibility of inducing hypotension is an important limitation of high-dose CBD in this setting.

Limitations

As already discussed in the method section, the main limitation in the current studies regarding translatability is the short survival time. As secondary energy failure just has commenced at 9.5 hours, any effects CBD has on these processes have little time to manifest. Changes in many of the studied variables, like histopathology and the H^+MRS biomarker Lac/NAA ratio, would probably have been more pronounced if assessed at later end-points. It is thus possible that effects of both CBD and cooling could have become evident if assessed later. Regardless, as the existing literature in support of CBD as a neuroprotectant after neonatal HI [95,99,191] mainly come from studies using similar end-points and outcome variables; we argue that our results are relevant.

Circulating microRNAs as markers of hypoxic-ischemic brain injury (III)

We hypothesized that miRNAs could be altered in plasma in the early phase after HI and be potential markers of hypoxic-ischemic injury. Our "pilot study" demonstrated significant and rapid temporal alterations in two of the studied miRNAs, although not in those considered brain-specific.

Temporal changes

Early increase in circulating miRNAs after tissue injury or stress [175,246] as well as relatively rapid clearance when conditions normalize [247], has been demonstrated previously. However, we have not found studies describing the same swiftness of changes as observed for miR-374a and miR-210 in our study. This rapidity might imply their presence in plasma due to causes not directly related to release from injured tissues as it might be more reasonable to expect more sustained elevations after tissue injury. We can speculate that these microRNAs are actively secreted and taken up by cells to orchestrate the response to HI. In fact, *in-vitro* studies might suggest this as miR-210 is secreted to regulate adaptation to hypoxia [170] and miR-374a to regulate lactate dehydrogenase [167]. The observation that levels of miRNA show rapid and considerable temporal alterations clearly demonstrate that one should consider the timing of sampling in future studies.

Biomarkers of brain injury

After the literature review conducted before our study, we considered miR-124 as the most promising brain-specific biomarker. It did, however, not display significant alterations in our study and the same accounts for the brain-enriched miR-125. Although miR-124 has been able to predict hypoxic-ischemic brain injury in adults, both in humans [160] and pigs [248], the miRNA expression in the newborn might be distinctive. Also, the timing of sampling and nature of the insult is not directly comparable. Further, the sample size in our study is insufficient to reveal smaller differences that might hold relevance on a population level. Future studies might still find miR-124 to be a promising biomarker candidate in neonatal HI.

Neither miR-374a nor miR-210 is considered to be brain-specific, yet both have previously been linked to perinatal HI. Looney *et al.* found several possible pathways linking miR-374a to hypoxic-ischemic brain injury, and there are currently ongoing trials investigating the role of this miRNA in neonatal HIE (The Investigation and Validation of Predictive Biomarkers in Hypoxic-ischemic Encephalopathy. (BiHiVE2) clinical trials: NCT02019147). As discussed previously these miRNAs might be important regulators of the cellular adaptation and response to perinatal HI, and thus be promising biomarkers despite not being brain specific.

General considerations

Due to various reasons, the reproducibility of studies on circulating miRNA to date has been low. The full understanding of the biological function of miRNA as well as developing a methodology for analysis is still a work in progress [173,174]. We aimed to apply a methodology that was recommended for the conditions in our study [231] and used with success in previous studies published in high-impact journals [159,160]. To minimize the influence of potential haemolysis we selected miRNAs that, based on existing literature [249], were minimally influenced by haemolysis. Further, the experimental setting allowed us to tightly control the collection and handling of samples as opposed to a clinical setting. That said; the limited sample size permits us only to speculate on the function and potential of these miRNAs. This study has demonstrated the feasibility of analysing circulating miRNA in our animal model and hence, that it can be well suited for pre-clinical miRNA studies. A microarray study would be the natural next step, where a large number of potential miRNAs could be identified and the most promising candidates confirmed by PCR. To increase knowledge about the functional effects of miRNA, such studies could potentially investigate downstream targets as well. This could supplement the already initiated clinical studies on miRNA in asphyxiated newborns that use human cord blood [153], as well as elucidate differences between cord blood and neonatal blood.

Future perspectives and closing thoughts

The optimal way to reduce the impact of acute perinatal HI on infant mortality would obviously be to prevent it from occurring. Despite high-quality obstetric care and the routine use of advanced methods for detecting fetal distress acute intrapartum events occur [11]. More sensitive and non-invasive methods for the detection fetal hypoxia-ischemia are needed. Further, we also need better tools for early detection of hypoxic-ischemic brain damage in the newly born as well as improved strategies for ameliorating the consecutive brain damage.

Circulating miRNAs have shown promise both as markers of fetal hypoxia in maternal blood[149] and for the early identification of acute encephalopathy in the newborn[153]. We have contributed by showing the rapid temporal alterations of circulating miRNAs in this setting (paper III). Circulating miRNAs is an exciting area with great potential, however, still in its infancy and future studies will determine whether miRNAs will live up to their promise.

Despite an increased understanding of the complex nature of hypoxic-ischemic brain injury a considerable number of novel treatments translate poorly into clinical practice [69,250]. One of the reasons for this is probably inadequate pre-clinical evidence [227] and, in our opinion, further animal studies are also needed to determine the neuroprotective potential of CBD. Before our studies, the evidence of CBD`s neuroprotective effects in this setting came from a single large animal model and, except one study[97], had only been evaluated in the first 6 hours after HI. Our studies are relevant as they challenge existing evidence in a different pre-clinical model [227], but also have the limitation of short observation time. As a result, the current evidence of CBDs effects on secondary energy failure is still difficult to interpret. Future studies should aim for sufficient survival time, like in the models that contributed to the successful translation of therapeutic hypothermia into clinical use [55] and are used in many of the recent studies of novel neuroprotectants [62,70]. However, to further enhance the translational value of these models, one might consider applying more severe and global insults as this certainly would give a more close reflection of the sub-group of infants with most severe hypoxic-ischemic damage.

Along with the search for new technology and treatments for asphyxiated newborns, it is also vital to remember the global aspect. The low-income countries carry the greatest burden of perinatal HI and the vast majority them do not have access to even basic obstetric and neonatal care[251]. In fact, therapeutic hypothermia has yet to demonstrate efficacy and safety in low-resource settings[35]. Regardless, most of the deaths due to intrapartum events could have been prevented without the use of advanced technology, simply by ensuring early access to health services and education of health personnel in obstetric care and basic neonatal resuscitation[251,252]. As such, to achieve the global goals of reduced neonatal mortality, this is an area of great importance.

Conclusions

Based on the studies in this well-established piglet model of severe global neonatal HI, we make the following conclusions:

1. CBD 1 mg/kg or high-dose CBD (10-50 mg/kg) does not offer neuroprotection in the early phase after global HI (I & II)

2. CBD 1 mg/kg does not augment therapeutic hypothermia or produce additive neuroprotective effects in the early phase after global HI (I)

3. High-dose CBD can induce a dose-dependent drop in mean arterial blood pressure after global HI (II)

4. Circulating miRNAs can display rapid temporal alterations during and immediately after global HI and could be useful markers of hypoxic-ischemic injury (III)

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