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Cannabidiol for Neuroprotection in Newborn Hypoxic-Ischemic Encephalopathy: pharmacological aspects and mechanisms of action

Memoria para optar al Grado de Doctor presentada por
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CERTIFICAN: Que Nagat Mohammed El Demerdash, licenciada en Medicina, ha realizado bajo su dirección el trabajo titulado "Cannabidiol for Neuroprotection in Newborn Hypoxic-Ischemic Encephalopathy: pharmacological aspects and mechanisms of action". Consideramos que dicho trabajo reúne todos los requisitos necesarios para ser presentado como Tesis Doctoral en el Departamento de Pediatría, de la Facultad de Medicina de la Universidad Autónoma de Madrid.

Y para que así conste, firman el presente certificado en Madrid, a 12 de Mayo de dos mil catorce.

VºBº

Dr. José Martínez-Orgado

VºBº

Dra. Mª Ruth Pazos

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ABBREVIATIONS

AA……Arachidonic Acid

abnCBD……abnormal-Cannabidiol

AEA……*N*-arachidonoylethanolamine or Anandamide

2-AG……..2-arachidonoylglycerol

BBB……Blood brain barrier

BDNF……..Brain derived Neurotrophic Factor

CBD……..Cannabidiol

CB_1……Cannabinoid Receptor type 1

CB_2……Cannabinoid Receptor type 2

CBF……Cerebral blood flow

CBN……..Cannabinol

CBR……Cannabinoid Receptor

CNS……Central Nervous System

DAGL……Diacylglycerol Lipase

Δ^9-THC……Delta-9-tetrahydrocannabinol

eCB……Endocannabinoids

ECS……Endocannabinoid System

EPO……Erythropoietin

FAAH……Fatty Acid Amide Hydrolase

GABA……Gamma-aminobutyric Acid

GDNF……Glial cell derived Neurotrophic Factor

GPCR……G-protein coupled receptor

iNOS……...Inducible Nitric Oxide Synthase

MBP……Mean Blood Pressure

MGL……Monoacylglycerol lipase

NADA……..*N*-arachidonoyldopamine

NAT……..*N*-acyl-transferase

NBT……..Neuro-behavioral Test

NFκB……Nuclear factor kappa-light-chain-enhancer of activated B cells

NGF……..Nerve Growth Factor

NHIE……..Newborn hypoxic-ischemic encephalopathy

NMDA……..*N*-methyl-D-Aspartic Acid

nNOS……Neuronal Nitric Oxide Synthase

MRI……..Magnetic Resonance Imaging

OGD……..Oxygen and glucose deprivation

PEA……*N*-palmitoylethanolamine

PPAR……Peroxisome proliferator-activated receptor

SVZ……Subventricular zone

TH……Therapeutic hypothermia

TNFα……..Tumor Necrosis Factor-alpha

TRPV1……Transient Receptor Potential cation channel type 1

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INTRODUCTION

INTRODUCTION

1. NEWBORN HYPOXIC-ISCHEMIC ENCEPHALOPATHY

1.1. THE PROBLEM

Perinatal asphyxia resulting in newborn hypoxic-ischemic encephalopathy (NHIE) occurs in 2 to 9 in 1000 live births at term (Volpe, 2001; González & Ferriero, 2008; Fatemi *et al.*, 2009). In addition to inflicting direct brain damage, leading to acute brain dysfunction, such an insult may interfere with brain development, determining long-term morbidity. Thus, worldwide near 2 million babies die or remain with long-lasting disability because of NHIE each year (Volpe, 2001). In addition to the invaluable cost of losing human lives, developmental disabilities derived from NHIE have important socioeconomic costs for patients and their caregivers (usually, their family). The CDC estimated in 2004 a lifetime cost (in 2003 US$) of US$11.5 billion for persons with cerebral palsy, representing a lifetime cost per person of near US$ 1 million; near 20% of cost derives from direct medic and non-medic costs, but the largest percentage accounted for indirect costs, in terms of losses of works and social opportunities (CDC. Economic costs associated with mental retardation, cerebral palsy, hearing loss and vision impairment – United States 2003. MMWR 2004; 53:57-59).

Despite the continuous progress in Neonatology and Perinatology in the late years, the aforementioned numbers have not substantially changed, and perinatal asphyxia remains an outstanding health problem even in developed countries. With not small frustration, neonatologists accept that, nowadays, there is no therapy successfully preventing o reducing the consequences of perinatal asphyxia.

1.2. PATHOPHYSIOLOGY OF NHIE

The brain requires a continuous supply of oxygen and glucose to maintain normal function and viability. When this supply is interrupted, a cascade of events takes place leading to cellular injury (Fig. 1) (Volpe, 2001; Johnston, 2001; Allan & Rothwell, 2001; du Plessis-Volpe, 2002; Ferriero, 2004; Takuma *et al.*, 2004; Chisari *et al.*, 2004; Martinez-Orgado *et al.*, 2006; Fatemi *et al.*, 2009; Rees *et al.*, 2011). The determining event is the energetic failure, which completes two periods: the early energetic failure, just after the start of hypoxia-ischemia, and the late energetic failure, during reperfusion and after a period of apparent recovery; this late energetic failure is proportional to the early one, and is of a high prognostic value as it is related to the starting of apoptotic processes (Volpe, 2001; Ferriero, 2004). Energetic failure leads to the dysfunction of ATP-dependent ionic bombs for Na^+, K^+, H^+ and Ca^{+2}, which alters the membrane polarity and determines the intracellular accumulation of cations as Na^+ and Ca^{+2}, resulting in cytotoxic oedema (du Plessis-Volpe, 2002; Martinez-Orgado *et al.*, 2006).

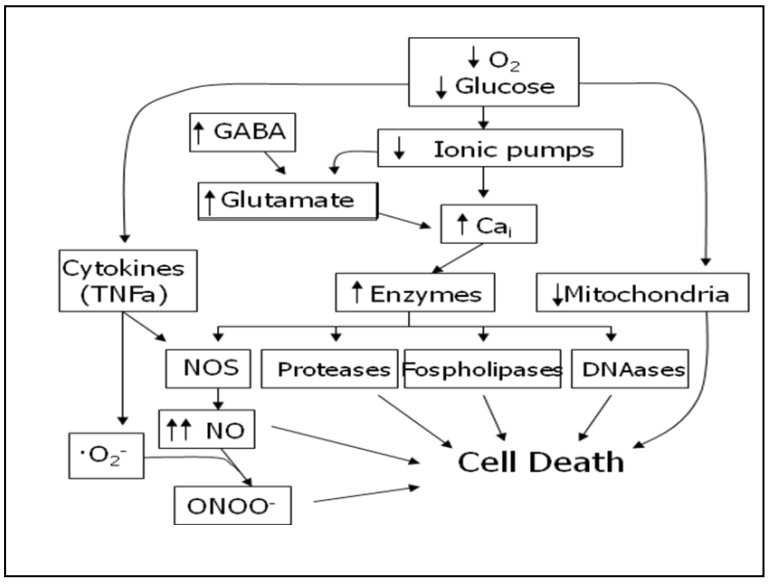

Figure 1. Mechanisms of cell damage after oxygen and glucose deprivation that lead to cell death (Martínez-Orgado *et al.*, 2007).

In addition, there is an accumulation of purines, products from anaerobic metabolism of ATP, which constitute the substrate for free radicals generation during reoxygenation (Saugstad, 1996). Production of oxygen free radicals is enhanced during asphyxia, an effect attributed to the dysfunction of some components of the respiratory chain, particularly the cytochrome oxidase complex; in addition, there is an inhibition of superoxide dismutase activity, preventing the neutralization of superoxide anion (Warner *et al.*, 2004). These changes, together with specific changes in enzymatic activity, mitochondrial function, membrane transporters and, particularly in the immature brain, the scarcity of antioxidant defences, make brain very vulnerable to reoxygenation (Warner *et al.*, 2004).

Changes in membrane polarity led to the release and accumulation of excitotoxic aminoacids, in particular glutamate. Glutamate plays an important role in brain damage, increasing dramatically intracellular levels of cations through the opening of channels coupled to NMDA or AMPA receptors (Johnston, 2001); in *in vitro* models of NHIE glutamate release show a direct relationship with the severity of cellular necrosis (Fernández-López *et al.*, 2005). Glutamate enhances TNFα synthesis and induces the expression of inducible NO synthase (iNOS), both relevant factors in NHIE (Fernández-López *et al.*, 2005; Martinez-Orgado *et al.*, 2006). The latter effect is of particular relevance as glutamate receptors and NOS are co-expressed in the same areas of immature brain (Johnston *et al.*, 2001).

The increase in intracellular Ca^{+2} activates some enzymes as phospholipases, endonucleases, proteases and caspases; these enzymes induce structural damage, worsen the energetic failure, increase oxidative stress and are involved in apoptosis (du Plessis-Volpe, 2002; Ferriero, 2004). Another enzyme activated is the neuronal NOS (nNOS), which together with iNOS leads to a massive production of NO (du Plessis-Volpe, 2002; Ferriero, 2004; Fernández-López *et al.*, 2005; Martinez-Orgado *et al.*, 2006); other factors as cytokines

or glutamate further enhance NO production (Martínez-Orgado *et al.*, 2006). NO massively produced further impairs the energetic status by irreversible blocking mitochondrial respiration, and induces DNA damage (du Plessis-Volpe, 2002; Ferriero, 2004; Martinez-Orgado *et al.*, 2006).

During reperfusion, two major events take place increasing and aggravating brain damage: inflammation and oxidative stress (Volpe, 2001; Johnston, 2001; Allan & Rothwell, 2001; du Plessis-Volpe, 2002; Ferriero, 2004; Takuma *et al.*, 2004; Chisari *et al.*, 2004; Martinez-Orgado *et al.*, 2006; Rees *et al.*, 2011). Cytokines, mostly TNFα and interleukin-1: i) alter blood-brain barrier integrity, enhancing the infiltration and proliferation of immune cells; ii) increase excitotoxicity by inhibiting astroglial glutamate reuptake; iii) increase oxidative stress by inducing the expression of iNOS; iv) are involved in processes leading to apoptosis by activating FAS receptors; v) impair mechanisms of repair by reducing astroglial production of neurotrophic factors as BDNF and NGF; and vi) lead to vacuolar degeneration of myelin because of oligodendrocyte injury. Inflammation, together with the dysfunction of some components of the respiratory chain, particularly the cytochrome oxidase complex and the inhibition of antioxidant defences as superoxide dismutase, increases oxidative stress.

Lately, the importance paid to glial cells, particularly astrocytes, in NHIE pathophysiology is growing (Chen & Swanson, 2003). Astrocytes guarantee the energetic supply to neurons by accumulating glycogen, synthesize and release neurotrophic factors, release antioxidant molecules, and remove the excess of NO or glutamate from the interneuronal space (Yong, 1998; Chen & Swanson, 2003; Villapol *et al.*, 2008; Belanger *et al.*, 2011). In addition, it has been recently shown that astrocytes play an important role in activation flow coupling and local blood flow preservation during ischemic insults (Belanger *et al.*, 2011). After a HI insult, astrocytes die by early necrosis or by apoptosis; thus,

preservation of astrocyte survival has become an endpoint of similar importance to neuronal survival (Takuma *et al.*, 2004). The role of microglia is still more controversial since two different phenotypes have been described (M1, cytotoxic, and M2, cytoprotective) and switches between both phenotypes are possible depending on inflammation, oxidative stress and apoptosis processes in brain tissue (Faustino *et al.*, 2011). Thus, depletion of microglia in post-HI brain leads to the increase of damage; therefore, it becomes apparent that microglia might somehow pertain to the natural protective mechanisms of brain (Faustino *et al.*, 2011).

1.3. PARTICULARITIES OF IMMATURE BRAIN

Although all the aforementioned components are acting similarly to induce cellular death in mature and immature brain, the latter shows some particularities determining its highly selective vulnerability to hypoxic-ischemic damage. Immature brain is particularly resistant to hypoxia: prenatal brain development takes place in a physiologically "hypoxic" environment, determining that the immature brain metabolism was adapted to low oxygen concentrations. By contrast, the rapid growth of brain during the perinatal period determines a high metabolic rate, which together with the immaturity of glucose uptake mechanisms makes hypoglycaemia particularly harmful for the perinatal brain (Volpe, 2001; Ferriero, 2004). Thus, if a failure in glucose supply is over imposed to hypoxia, immature brain damage occurs. In fact, removing glucose and oxygen for 30 min from the medium of incubation of forebrain slices from newborn rats leads to brain damage, closely similar both histopathologically and biochemically to that observed after *in vivo* hypoxia-ischemia (Fernández-López *et al.*, 2005).

Of high importance is the fact that immature brain is particularly sensitive to excitotoxicity (Johnston, 2001; Mishra *et al.*, 2001; Vexler & Ferriero, 2001; Fatemi *et al.*, 2009; Johnston et al., 2011): since NMDA receptors are involved in processes of brain maturation and plasticity, they are over expressed in immature brain (Johnston, 2001). Besides, in immature brain, NMDA receptor subunits are more sensitive to glutamate, in particular after hypoxia (Johnston *et al.*, 2001; Mishra *et al.*, 2001), showing a longer open time, which results in larger Ca^{+2} influx into the cell (Johnston, 2001). Finally, GABA release, which can modulate glutamate effects, becomes exhausted earlier (Johnston, 2001). The very significant role played by excitotoxicity in NHIE is supported by the direct relationship between extracellular glutamate levels and the severity of cellular death, as observed in an in vitro model of NHIE, the oxygen and glucose deprivation (OGD) of forebrain slices from newborn rats (Fernández-López *et al.*, 2005). As the regional distribution of glutamate receptors in the brain of immature rats corresponds to the pattern of selective vulnerability during hypoxia-ischemia (Vexler & Ferriero, 2001; Ferriero, 2004), it can be concluded that the age-dependent regional vulnerability to hypoxic–ischemic insults seen in the immature brain can be related, at least in part, to regional vulnerability to excitotoxicity.

Immature brain is extremely sensitive to inflammatory damage as well (Fatemi *et al.*, 2009; Johnston *et al.*, 2011; Rees *et al.*, 2011). It is well known that cerebral HI selectively stimulates IL-1ß and TNFα gene expression in brain regions susceptible to irreversible injury in perinatal rats (Szaflarski *et al.*, 1995). Although most of the pathways related to inflammation-induced exacerbation of post-HI brain damage are similar in mature and immature brain, the latter is particularly sensitive due to the immaturity of the immune system, the apoptosis pathways enhancement, the disbalance between pro- and anti-oxidant enzymes, differences in leukocyte-endothelial cell communication and distinct

intracellular signalling within inflammatory pathways (NF-κB and MAPK) (Vexler & Yaris, 2011). In newborn rats, previous administration of lipopolysaccharide dramatically increases HI-induced brain damage (Coumans *et al.*, 2003). Thus, intrauterine infection/inflammation is a major factor underlying perinatal brain damage (Volpe, 2009; Yoon *et al.*, 2000), with an increased risk of cerebral palsy in term infants exposed to chorioamnionitis (Wu, 2002).

Other relevant aspect is that immature brain is much more vulnerable to oxidative stress than mature brain, as antioxidant defences are only partially developed at birth (Fatemi *et al.*, 2009; Gitto *et al.*, 2009; Johnston *et al.*, 2011). This situation is particularly harmful for the immature oligodendroglial cells, because these cells accumulate excessive amount of iron as the active acquisition of iron is required for oligodendroglial proliferation to occur, thus determining the production of high quantities of hydroxyl radicals by Fenton's reaction (Volpe, 2001; du Plessis & Volpe, 2002; Rees *et al.*, 2011); as a result, white matter damage is usually wider and more severe in immature brain after hypoxia-ischemia than in mature brain (Volpe, 2001).Therefore, excitotoxicity, inflammation and oxidative stress constitute the triad of major factors leading to HI damage in immature brain (Johnston *et al.*, 2011).

It is well known that apoptosis plays a significant role in normal brain development; in particular for brain plasticity; thus, proapoptotic molecules as the Bcl-2 family member Bax or proapoptotic enzymes as caspases are highly expressed in immature brain (Roth & D'Sa, 2001). This determines that after a hypoxic-ischemic insult, apoptotic cell death plays a more relevant role in immature than in mature brain (du Plessis & Volpe, 2002). Nevertheless, in immature brain, necrosis occurs swiftly during the HI insult with apoptosis starting early after. The pattern of necrosis-apoptosis has geographical variations, being the former predominant in subcortical and the latter in cortical regions as demonstrated in term fetal

lambs in which HI brain damage was induced by global ischemia after reducing umbilical blood flow (Goñi de Cerio *et al.*, 2007). Necrosis and apoptosis are considered now a continuum, with cells showing either feature at the same time or even mixed morphologies (Fatemi *et al.*, 2009).

Cerebral blood flow (CBF) shows also some particularities in immature brain. Autoregulatory range of CBF is narrower in newborns than in older children and adults, particularly in preterm babies (Volpe, 2001; Martinez-Orgado *et al.*, 2006). There are dramatic regional variations in the amount of blood flow as well as in metabolism-microcirculation coupling, influencing the regional differences in the vulnerability to hypoxia-ischemia (Volpe, 2001; Martínez-Orgado *et al.*, 2006). These responses are particularly dependent on endothelial function in newborn brain arteries (Martínez-Orgado *et al.*, 1998); thus, CBF autoregulation is very vulnerable to hypoxic-ischemia-induced endothelial dysfunction in newborns (Volpe, 2001).

Finally, immature brain has demonstrated a particularly strong capacity to recover from hypoxic-ischemic injury by producing new neurons in the subventricular zone that migrate to injured areas in neocortex (Ong *et al.*, 2005; Yang *et al.*, 2007; Fernández-López *et al.*, 2010). Neocortical neuron migration coincides with proliferation and migration of glial cells (Zaidi *et al.*, 2004); this process is of great importance to start remyelinization of injured areas, but also to guarantee the survival of the new neurons (Ong *et al.*, 2005). Thus, an important spontaneous recovery of myelinization can be demonstrated in the external capsule of newborn rats 7 days after an HI insult (Fernández-López *et al.*, 2010).

2. NEUROPROTECTION IN NHIE

Several factors account for the feasibility or unfeasibility of preventing or reducing the consequences of NHIE (Volpe, 2001; Ferriero, 2004; Martínez-Orgado *et al.*, 2007; Fatemi *et al.*, 2009; Johnston *et al.*, 2011):

***Favourable aspects**:*

- There is the so-called "therapeutic window", defined as the lapse between the start of the HI insult and the activation of the different processes leading to late neuronal death.
- Perinatal asphyxia often is the result of a well-recognized episode occurring in the presence of health caregivers, prompting an immediate obstetric response. Thus, neonatological management of asphyxiated newborns often can be started between the limits of the therapeutic window.

Unfavourable aspects:

- Occurrence of perinatal asphyxia is unpredictable. Therefore, it often occurs outside well-equipped health institutions. Those neuroprotective strategies needing strong investments either in technology or in human resources – as is the case of the most promissory neuroprotective strategy so far, hypothermia- won't be universal.
- Neuroprotective substances must reach brain parenchyma –thus crossing the blood-brain barrier- being effective without significant side effects. In the case of newborns, in addition, they must avoid interferences with developmental processes: in other words, safety must be demonstrated in the short as well as in the long term.
- Many neuroprotective strategies successfully tested in animals have failed to show significant benefit in humans (for instance, allopurinol, antioxidants) or have showed

unexpected side effects (for instance Ca^{+2} channel blockers, Mg sulphate, NMDA antagonists, etc). Multicenter studies on hypothermia consistently show trends to better results than placebo, but significant differences –excepting for death and/or major disabilities in mild encephalopathy- have not been reported.

- The complex pathophysiology of NHIE demands the use of treatments acting at different levels. Very likely the optimal results will be obtained with combined therapies.

2.1. GENERAL MANAGEMENT

General management of an asphyxiated infant is aimed to prevent the occurrence of homeostatic disbalances that could further increase brain damage (Perlman, 2006; Martinez-Orgado *et al.*, 2007).

- Blood pressure must be maintained in the normal range, because after HI brain arteries autoregulation is lost, so CBF become passive.
- Hyperthermia must be avoided, because it increases brain excitability and metabolic consumption.
- Ionic (in particular, sodium and calcium) disbalance as well as hypoglycaemia must be avoided.
- It is mandatory to maintain normoxemia, because hypoxemia extends HI damage whereas hyperoxemia increases oxidative stress. In addition, hypocapnia must be avoided because it reduces CBF.
- Seizures must be vigorously treated, because epileptic activity increases brain metabolic consumption, increases excitotoxicity and impairs cardio-respiratory homeostasis.

- Brain oedema must be treated if so severe as to induce a “non-reflux” phenomenon that is the reduction of CBF because of brain arteries compression.
- There is no indication for prophylactic antibiotic treatment, but infection must be diligently treated, in particular if produced by LPS-producing bacteria.

2.2. SPECIFIC NEUROPROTECTIVE STRATEGIES

Magnesium sulphate: This compound blocks NMDA receptors, blunting their activation by glutamate, and is anti-oxidant (Gonzalez & Ferriero, 2008). In animal models, however, magnesium sulphate has been effective just when administered before the HI insult (Levene, 2010). In agreement, clinical trials in asphyxiated newborns showed no benefit but instead an increase of the risk of hypotension (Whitelaw & Thoresen, 2002). However, prenatal administration in very preterm deliveries seems to have some neuroprotective effects, reducing the risk of cerebral palsy (Nguyen *et al.*, 2013).

Therapeutic hypothermia (TH): TH is the only treatment having demonstrated clinical efficacy so far (Laptook, 2009; Edwards *et al.*, 2010). TH reduces cell metabolism (5% per ºC), reduces ionic influx and glutamate release, reduces inflammation and oxidative stress and stops apoptotic processes (Fatemi *et al.*, 2009; Johnston *et al.*, 2011). Two modalities have been proved: selective hypothermia, in which head temperature is reduced by some kind of cap filled with cooled water, together with mild body temperature decrease (Gluckman *et al.*, 2005); and whole body hypothermia, in which body temperature is decreased to 33-35 ºC by a cool water-filled blanket (Azzopardi, 2009). TH has to be started in the first 6 h after birth and discontinued after 72 h (Laptook, 2009). A recent systematic review demonstrates that this procedure reduces death and/or severe neurological impairment by the age of 18 month, but just in mild NHIE cases (Edwards et al, 2010). Thus, it is well accepted now the

need for synergistic neuroprotective strategies such as pharmacological agents being delivered either during or after the hypothermic treatment (Cilio & Ferriero, 2010; Levene, 2010).

Xenon: The neuroprotective effect of this anaesthetic gas, demonstrated in HI models in newborn animals (Dingley *et al.*, 2006), relies on its effect as antagonist of NMDA receptors (Cilio & Ferriero, 2010; Levene, 2010). Xenon inhibits apoptotic processes and show cardioprotective effects (Roberston *et al.*, 2012). Interestingly, xenon easily crosses the brain-blood barrier (BBB) and there is substantial experience on its use in human newborns (Levene, 2010; Roberston *et al.*, 2012). The main caveat against xenon is its very high cost, although there are ongoing experiments with re-circling devices that might make xenon less expensive (Chakkaparani *et al.*, 2009). Xenon has demonstrated to enhance TH neuroprotection in newborn animals (Hobbs *et al.*, 2008; Faulkner *et al.*, 2011). There are some ongoing clinical trials testing xenon plus TH in asphyxiated infants.

Erythropoietin (EPO): EPO is a very pleiotropic substance. Studies in animal models of NHIE demonstrated that EPO, acting on JK/Stat5 pathways and inhibiting NF-□B, blunts different pro-apoptotic pathways and modulates toxic NO synthesis; in addition, EPO shows anti-inflammatory, antioxidant and anti-excitotoxic properties and improves post-HI perfusion (Sola *et al.*, 2005a; Sola *et al.*, 2005b; McPherson & Juul, 2010). Interestingly, EPO is not only reducing post-HI brain damage but is enhancing neuro-repair by enhancing angiogenesis and neurogenesis (McPherson & Juul, 2010; Gonzalez *et al.*, 2013). There is a long experience of using EPO in newborn infants (Sola *et al.*, 2005b). There is some concern regarding the possible increase of the risk for retinopathy after EPO administration in newborns, but this effect has not been demonstrated yet in term infants receiving EPO at the usual dose (McPherson & Juul, 2010). Unfortunately, recent studies in newborn rats

failed to demonstrate any additive effect of EPO in HI animals treated with TH (Fan *et al.*, 2013; Fang *et al.*, 2013). There are promising data coming from clinical trials on EPO for newborn with NHIE (Zhu *et al.*, 2009).

Melatonin: The interest on melatonin as a neuroprotectant for HI infants has risen lately. Melatonin is a mighty antioxidant substance that exerts immunomodulatory effects too (Gitto *et al.*, 2009; Hardeland *et al.*, 2011; Robertson *et al.*, 2012; Robertson *et al.*, 2013). Melatonin administration in experimental models demonstrated that this substance reduces apoptosis, at least in part by inhibiting NOS activity and stabilizing mitochondria (Hardeland *et al.*, 2011; Robertson *et al.*, 2012). Melatonin exerts some anticonvulsant effects by increasing GABA levels (Hardeland *et al.*, 2011). In addition, melatonin may enhance neuro-repair and myelogenesis because it increases the release of neurotrophins like GDNF or NGF (Villapol *et al.*, 2010; Hardeland *et al.*, 2011). There is a long experience on its safe use in humans, it may be administered by IV as well as by oral route, and it easily crosses the BBB too (Hardeland *et al.*, 2011; Robertson *et al.*, 2013). The main caveat is the possible effect on the hormonal production of the neuro-axe but this is likely to be insignificant in short-term treatments (Hardeland *et al.*, 2011; Robertson *et al.*, 2012). Melatonin has demonstrated to enhance TH neuroprotection in HI models in newborn pigs (Robertson *et al.*, 2013). There are some ongoing clinical trials testing melatonin plus TH in asphyxiated infants.

Anticonvulsants: Although early reports suggesting that administration of high doses of Phenobarbital might have neuroprotective effects in NHIE were very promissory, further clinical trials did not support that effect (Whitelaw&Thoresen, 2002; Evans *et al.*, 2007). Thus, Phenobarbital is currently accepted in NHIE patients just for the treatment of seizures and at conventional dose (Evans *et al.*, 2007).

Topiramate is a well-known anticonvulsant already used in newborns that has resulted

neuroprotective in animal models of NHIE (Schubert *et al.*, 2005; Levene, 2010). Interestingly, some synergistic effect with hypothermia has been reported (Johnston *et al.*, 2011); therefore, there are some ongoing clinical trials on topiramate plus TH.

3. ENDOCANNABINOID SYSTEM

3.1. GENERAL CONCEPTS

The Asiatic plant *Cannabis sativa* (Fig. 2) has been used for more than 8000 years due to its medical and psycotropic effects (Cannabinoids and the brain, Ed. Kofalvi A, 2007). The first written note about the medical use of cannabis was discovered in China which dates back to 2727 B.C., and lengthwise the History it was used as a medicine in several countries. However in the 20th century the plant was declared harmless and its use was prohibited.

Figure 2.*Cannabis sativa*. The plant cannabis belongs to the family *Cannabaceae* and its leaves and flowering tops are used to produce marijuana and hashish. The medical use of cannabis it is extensively known and even Queen Victoria was prescribed cannabis by her doctor in 1890. However in 1901 it was declared harmful and illegal.

In 1940 the first phytocannabinoids were discovered (Adams *et al.*, 1940): Cannabinol (CBN) and Cannabidiol (CBD), although these compounds have not psycotropic effects. A few years later Gaoni and Mechoulam (Gaoni & Mechoulam, 1964) isolated and described the most important active constituent of cannabis, a benzopyran derivative isolated from the

yellow resin of the leaves and inflorescences, named Δ^9-tetrahydrocannabinol (THC); this substance made the way for the discovery of a group of molecules with similar effects, the cannabinoids (Howlett et *al.*, 2002; Mechoulam & Lichtman, 2003; Fowler, 2003; Stella, 2004; Pazos *et al.*, 2005; Martinez-Orgado *et al.*, 2007).

The existence of an endocannabinoid system (ECS) was confirmed with the discovery of two different cannabinoid receptors (CBRs) at late 80s (Devane *et al.*, 1988; Matsuda *et al.*, 1990) and the two main endocannabinoids: *N*-arachidonoylethanolamine (AEA) (Devane *et al.*, 1992) and 2-arachidonoyl glycerol (2-AG) (Mechoulam *et al.*, 1995).

In the last forty years, a lot of knowledge has emerged about the *Cannabis sativa* and its beneficial effects. Currently, many scientific evidences suggest the huge importance of the ECS in physiological and pathological conditions, so the pharmacological manipulation of different components of this system could be very useful in several pathologies.

The endocannabinoid system (ECS) is comprised of several elements:

- At least two different G-protein coupled membrane receptors (termed CB_1 and CB_2)
- Endogenous ligands or endocannabinoids (eCB)
- Mechanisms for the synthesis and degradation of eCB.

3.2. CANNABINOID RECEPTORS

Two cannabinoid receptors were cloned so far: CB_1 and CB_2. Both receptors are G-protein coupled receptors and share an overall identity of 44% (Fig. 3) but they exhibit dramatical differences in their tissue distribution.

Besides, studies carried out on knock-out mice have demonstrated that some effects of cannabinoids are not mediated by CB_1-CB_2 receptors, suggesting that more unknown

cannabinoid receptors could exist. In addition, cannabinoids can activate other receptors such as vanilloid receptors (TRPV1), orphan GPCRs (GPR55, GPR119), and also nuclear receptors as peroxisome proliferator-activated receptors (PPARs) (Pertwee *et al.*, 2010).

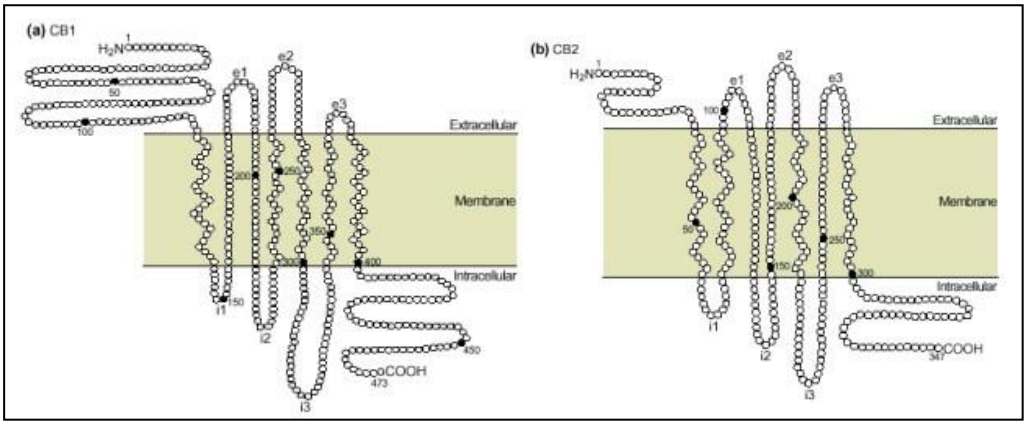

Figure 3. Cannabinoid receptors structure. Both receptors are G-protein coupled receptors and share an overall identity of 44%.

Finally, some effects could be receptor-independent, that is the case of the antioxidant properties of ECB, in which the chemical structure of these compounds is involved.

CB_1R: CB_1 receptor belongs to the GPCR superfamily, it contains seven-transmembrane domains and it is coupled to Gi protein. This receptor is located in several peripheral tissues such as heart, liver, spleen, and reproductive organs, urinary and gastrointestinal tracts (Galiegue *et al.*, 1995; Gerard *et al.*, 1991; Mackie, 2005).However, CB_1 is mainly distributed in nervous tissues, in fact, in brain is known to be the most profuse and ubiquitous of Gi-protein coupled receptors, with a distribution comparable to that of glutamate or GABA receptors.

In adult rodents, CB_1 receptor was detected preferentially in hippocampus, cerebral cortex, cerebellum and basal ganglia (substantia nigra and globus pallidus); it is less distributed in limbic areas such as acumbens nucleus and tonsil (Fig. 4; Herkenham *et al.*,

1991). This specific distribution probably is the reason why CB_1R is involved in the control of neuronal circuits related to coordination and modulation of movement; superior cognitive functions as memory and reward mechanisms; response to stress and pain; regulation of sleep, body temperature, appetite, nausea and vomiting (López de Jesús *et al.*, 2006).

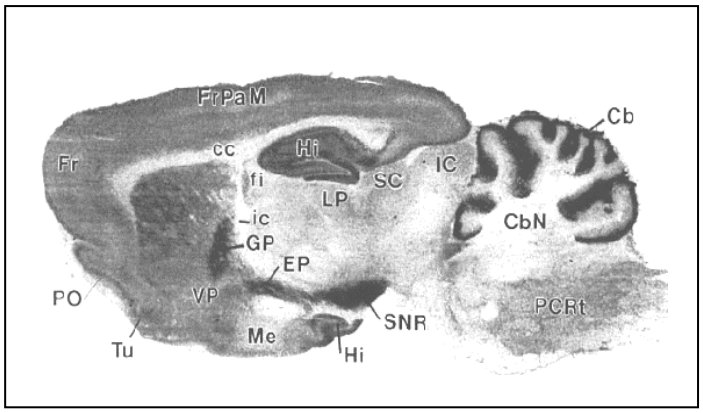

Figure 4. CB_1 receptor distribution in adult rat brain (Herkenham *et al.*, 1991). Cb: cerebellum; CbN: cerebelar nucleus; cc: corpus callosum; EP: entopeduncularnucleus; Fr: frontal cortex; GP: globus pallidus; Hi: hippocampus; Sn: substancia nigra.

Regarding to cell populations, in nervous tissues CB_1R is expressed primarily by neurons where it modulates the release of several neurotransmitters (GABA, glutamate, dopamine, adrenaline, etc). CB_1R can also be found in glial cells, both astrocytes and oligondendrocytes (Howlett *et al.*, 2002; Mechoulam & Lichtman, 2003; Stella, 2004; Pazos *et al.*, 2005; Pertwee *et al.*, 2010).

CB_2R: CB_2 receptor seems to be restricted to cell types related with the immune system (Munro *et al.*, 1993) (spleen and tonsil) and in immune cells such as monocytes, B and T lymphocytes (Schazt *et al.*, 1997). It is thought that CB_2R could be involved in the immunomodulatory effect of cannabinoids (Howlett *et al.*, 2002; Mechoulam & Lichtman, 2003; Fowler, 2003; Stella, 2004; Pazos *et al.*, 2005; Klein, 2005; Pertwee *et al.*, 2010).

Recent reports have also described the presence of CB_2 receptor in brain cells, including neurons of brain stem that could be responsible for emesis control (Van Sickle *et al.,* 2005). Although it is accepted that CB_2 receptor is not expressed in forebrain neurons,

controversial studies were published due to appropriate biochemical tools are not available, and this receptor was detected in hippocampus, cerebellum and some cortical areas involved in memory and cognitive processes (Gong *et al.*, 2006; Morgan *et al.*, 2009).

Under some pathological conditions such as ischemia (Zhang *et al.*, 2007), Alzheimer's disease (Benito *et al.*, 2003), Huntington's disease (Sagredo *et al.*, 2009) and multiple sclerosis (Benito *et al.*, 2007), CB_2 receptor has been described in activated glia.

Both CB_1 and CB_2 receptors are also expressed in blood vessels. Vascular effects of cannabinoids are mediated primarily by TRPV1 receptors, although recently it has been described that functional CB_1 and CB_2 receptors are constitutively expressed in the endothelial cells of human brain microvessels (Golech *et al.*, 2004).

Non CB_1-CB_2 receptors: Other non-CB_1-non-CB_2 receptors have been proposed to exist in brain for cannabinoids. The activation of endothelial receptor for Anandamide (AEA), the *abnormal*-cannabidiol receptor (*abn*-CBD), enhances vasorelaxation and potenciates microglial migration (Járai *et al.*, 1999).

The orphan GPCR receptor, GPR55 has been described as a novel metabotropic cannabinoid receptor. It is widely distributed in peripheral tissues (spleen, liver, intestine) and also in nervous tissues (hippocampus, cerebellum, striatum). An endogenous acetilethanolamide structurally related to AEA, *N*-palmitoylethanolamine (PEA), could be the endogenous ligand for GRP55 (Godlewski *et al.*, 2009; Pertwee, 2007).

Recently, it has been published that some endogenous cannabinoid ligands can activate some nuclear receptors such as PPARs (De Petrocellis *et al.*, 2009a). These results open a new role for cannabinoids; they could directly modulate gene expression.

Finally, cannabinoids are known to non-specifically bind different receptors present in brain, as NMDA receptors and others (Howlett *et al.*, 2002).

3.3. ENDOGENOUS LIGANDS

After the description of the CB receptors,two types of arachidonic acid-derivated molecules were identified as endogenous ligands or endocannabinoids (eCB) for the cannabinoid receptors: *N*-arachidonoylethanolamine (AEA) and 2-arachidonoyl glycerol (2-AG).AEA was isolated from pig brain and it has functional properties similar to THC; this substance, received the evocative name of anandamide (from “ananda”: “inner bliss”, in Sanskrit) (Devane *et al.*, 1992).The second endocannabinoid, 2-AG was isolated from rat brain (Sugiura *et al.*, 1995) and from canine gut (Mechoulam *et al.*, 1995).

These two lipophylic compounds exhibit important differences in their quantitative distribution as well as in their properties as endogenous agonists for cannabinoid receptors; 2-AG is more abundant (200-fold higher in some brain regions) than AEA in brain tissue and behaves as a full agonist for CB_1 and CB_2receptors, while AEA acts as a partial agonist for CB1 receptors and as a weak partial agonist-antagonist.

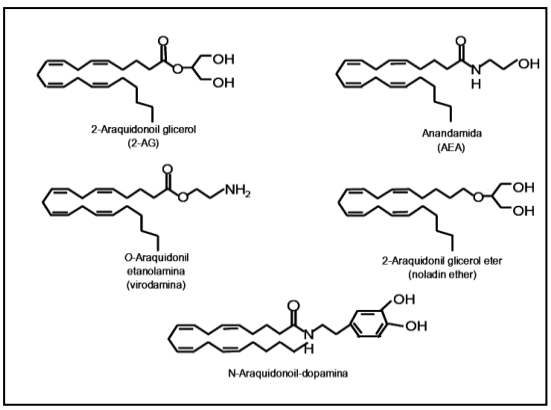

Figure 5. Chemical structures of endogenous ligands of ECS

From this moment, a number of molecules with cannabimimetic activity (Fig. 5) have been described (Noladin-eter, Virodhamine; Porter *et al.*, 2002), sharing a common basic structure consisting in a lipidic radical, usually derived from arachidonic acid, and a polar group.

3.4. BIOSYNTHETIC AND HIDROLYZING ENZYMES

Different proteins are involved both in the synthesis and inactivation of the eCB signal. Characteristically, synthesis of eCB is an on-demand process, so that eCB are synthesized when needed from lipid precursors located in the plasma membrane, but they are not stored.In relation to eCB synthesis, the most knowledge is about AEA and 2-AG formation, the increase of intracellular Ca^{+2} induced by the nervous impulse activates some enzymes involved in eCB synthesis.

Biosynthesis of AEA. Two main pathways were described for the AEA synthesis *in vivo*:

- Condensation of arachidonic acid (AA) and ethanolamine through Fatty Acid Amide Hydrolase (FAAH) action (Ueda *et al.*, 1995).
- Activation of *N*-acyl-transferase (NAT) that facilitates the transference of arachidonic acid to phosphatidylethanolamine (PE) to obtain *N*-arachidonoyl-PE (NArPE).NArPE is hydrolyzed by phospholipase D and AEA is released (Di Marzo *et al.*, 1994).

Biosynthesis of 2-AG. 2-AG is formed from arachidonic acid-containing membrane phospholipids through the combined actions of phospholipase C and diacylglycerol lipase (DAGL).

Once eCB are synthesized, they are released to synaptic cleft in a calcium-dependent way, quickly activate CB receptors and modulate synaptic activity (Wilson & Nicoll, 2002;

Stella, 2004).Finally, eCB are re-uptaked and subsequently degradated inside the cells (Pertwee *et al.*, 2010). AEA is hydrolyzed to AA and ethanolamine by FAAH action (Cravatt *et al.*, 1996). 2-AG can be metabolized also by FAAH activation but monoacylglycerol lipase (MAGL) is the main enzyme involved in 2-AG degradation (De Petrocellis *et al.*, 2004).

Both AEA and 2-AG can be also metabolized by enzymes involved in eicosanoids general metabolism, such as ciclooxygenases, lipooxygenases and P450 oxidases. These reactions produce a huge variety of bioactive molecules (Guindon & Hohmann, 2008).

3.5. SIGNAL TRANSDUCTION PATHWAYS

The activation, in particular of CB_1 receptor, inhibits the adenylyl cyclase through $G_{i/0}$ activation; hyperpolarizes the presynaptic cell due to the closure of voltage-dependent Ca^{+2} channels and the opening of K^+ channels; induces the expression of kinases as extracellular signal regulated kinase, c-Jun-N-terminal kinase, mitogen p38-activated protein kinase or protein kinase B; inhibits the nuclear factor κ-B (NF-κB); and generates ceramides. The closure of Ca^{+2} channels finishes the synaptic transmission and the release of the neurotransmitter is interrupted (Wilson & Nicoll, 2002; Howlett *et al.*, 2002; Mechoulam & Lichtman, 2003; Fowler, 2003; Stella, 2004; Martinez-Orgado *et al.*, 2007).

By activating cannabinoid receptors, eCB can modulate various signal transduction pathways involved in controlling cell proliferation, differentiation and survival. Thus, by coupling to $G_{i/0}$ proteins, the CBR inhibit adenylyl cyclase and the cAMP pathway in several cell types, stimulating then the mitogen-activated protein kinase (MAPK) cascades, specifically the extracellular-signal-regulated kinase (ERK) cascade and the p38 MAPK cascade.

On the other hand, CB_2 receptor also inhibits the adenylyl cyclase but through G_0 activation; activates MAPK and PI3K/PKB pathways (Diaz-Laviada & Ruiz-Llorente, 2005; Sánchez *et al.*, 2003). CB_2 activation does not modify the ionic conductance but, in the same way that CB_1, increases the Ca^{+2}concentrations inside the cell (Demuth & Molleman, 2006).

In addition to their effect on the synaptic transmission, eCB also bind to receptors located in glial cells (Howlett *et al.*, 2002; Fowler, 2003; Stella, 2004; Pertwee *et al.*, 2010). Thus, eCB enhance the rate of glucose oxidation and ketogenesis of astrocytes, and modulate the production of nitric oxide (NO) and cytokines by astrocytes or microglial cells.

Pharmacological stimulation of CBR also activates the pro-survival phosphatidylinositol 3-kinase and Akt (PI3K–Akt) pathway in rat oligodendroglial cells (Fernández-Ruiz *et al.*, 2004). Despite the deep knowledge on ECS physiology, several issues remain unresolved, as the characterization of eCB transporters or the existence of additional CBR (Pertwee *et al.*, 2010). Besides, new discoveries offer new perspectives for the understanding of the role and particularities of the ECS in the central nervous system; some examples are the oligomerization of CBR, or the presence of CBR in mitochondrial membranes.

4. PHARMACOLOGY OF ECS

After that huge amount of knowledge, a lot of synthetic or exogenous substances that interact with most of the main elements of the endogenous cannabinoid system have been described (Howlett *et al.*, 2002; Mechoulam & Lichtman, 2003; Fowler, 2003; Stella, 2004; Pazos *et al.*, 2005; Martinez-Orgado *et al.*, 2007).

Currently we have available drugs that bind to the CB_1 / CB_2 receptors as agonists or antagonists, drugs that block the endocannabinoid transport and drugs that inhibit the

synthesis or degradation of eCB. Cannabinoid receptor agonists may be designed to mimic the signaling processes mediated by AEA and 2-AG, mainly in pathological situations where a boost in cannabinoid receptor stimulation might be needed. Cannabinoid receptor antagonism might be the approach selected in conditions with enhanced endocannabinoid signaling. Transport inhibition and inhibition of degradation are more sophisticated approaches, both oriented towards magnifying the tonic actions of endocannabinoids (Rodríguez de Fonseca *et al.*, 2005). As a summary of cannabinoid pharmacology, Table 1 shows the reference compound for each molecular target.

Table 1. Pharmacological characteristics of ECS. Adapted from Rodríguez de Fonseca *et al.*, 2005.

Name	Target	Action	Ki/IC50 (nM)	Reference
ACEA	CB_1	Agonist	1.4	Hillard *et al*., 1999
SR141716A	CB_1	Antagonist	5.6	Rinaldi-Carmona *et al*., 1994
HU-308	CB_2	Agonist	22.7	Hanus *et al*., 1999
SR 144528	CB_2	Antagonist	0.60	Rinaldi-Carmona *et al*., 1998
UCM 707	AT	Blocker	800	Lopez-Rodriguez *et al*., 2001
OL-135	FAAH	Inhibitor (reversible)	2.1	Lichtman *et al*., 2004
URB 597	FAAH	Inhibitor (irreversible)	4.6	Kathuria *et al*., 2003

5. PARTICULAR CHARACTERISTICS OF CANNABINOIDS IN IMMATURE BRAIN

5.1. ROLE IN NEURAL PROLIFERATION AND MYELOGENESIS

Several data suggest that the ECS might play a significant role in brain development. Thus, CBR are found very early in brain structures: in human brain CB_1R is expressed in brain

neurons at 9 weeks of gestational age, whereas in rodents it is detected at G12 (Fernández-Ruiz *et al.*, 2004). Neural progenitors express FAAH too and are able to synthesize eCB.

In addition, CB_1R is found in developing brain in "atypical" areas related to proliferation and migration of neural cells: the subventricular zone, the cortical plaque or commissural fibers from white matter (Fernández-Ruiz *et al.*, 2004). As CB_1R expression is not found in these areas in mature brain, such distribution is considered as an evidence for the ECS involvement on brain development. Further findings suggest that the eCB signaling: 1) might regulate the proliferation of progenitor cells and promote their differentiation into glial cells, in an attempt to maintain the neuron/glia balance during brain development; 2) plays a role in the generation of neurons from neural progenitors; and 3) are involved in the control of neuritic elongation, the establishment of synaptic communication, and the acquisition of a specific neurotransmitter phenotype (Gómez *et al.*, 2008).

5.2. LACK OF PSYCHOACTIVE EFFECTS

Psychoactive effects of cannabinoids are determined by the characteristic localization of CB_1 receptors in adults in areas as prefrontal cortex; as in immature brain CB_1 receptors are not expressed in these areas, cannabinoids are expected to not induce psychoactive effects in immature brain. In agreement, clinical trials of THC in children with cancer showed an almost complete absence of psychoactive effect (Martinez-Orgado *et al.*, 2005).

5.3. HYPERSENSITIVITY TO CB_1-MEDIATED APOPTOSIS

There are some data suggesting that some functional differences in ECS function between immature and mature brain could exist. Bernard *et al.* (2005) reported that either blockade or over activation of CB_1 receptor has a strong impact on network activity in newborn rat brain slices; by contrast, in the adult CB_1 receptor antagonists and agonists have little effects on network activity in physiological conditions. This difference could be due, at least in part, to a constitutive activity of CB_1 receptors in immature brain.

Downer *et al.* (2007) reported that, *in vitro*, Δ^9-THC induces the activation of JNK and caspase-3 in the cerebral cortex isolated from the neonatal rat, via activation of the CB_1 receptor. In contrast, cortical slices obtained from the adult rat brain are less susceptible to the Δ^9-THC-induced activation of JNK and caspase-3. A similar profile is observed *in vivo*, with newborn rat brain being more vulnerable than adult rat to the activation of JNK, caspase-3, cathepsin-D, and DNA fragmentation following an acute peripheral administration of Δ^9-THC. .

In addition, rimonabant reduces brain damage in newborn rats after intracerebral injection of NMDA (Martínez-Orgado *et al.*, 2007). These data suggest that CB_1R activation in immature brain might activate signaling pathways in a different manner than in mature one.

5.4. ROLE OF CB_2 RECEPTORS

There are some evidences suggesting a more important role for CB_2 receptor in immature than in mature brain. CB_2 expression has been reported in embryonic chick brain; our group has demonstrated by Western-blot the presence of CB_2 receptors in forebrain

slices and whole brain homogenates from 7-day-old rats (Martinez-Orgado *et al.*, 2005; Fernández-López *et al.*, 2006).

It has been reported that the CB_1-CB_2R agonist WIN55212 reduces glutamate release in an *in vitro* model of hypoxic-ischemic brain damage on newborn rat forebrain slices and that either the CB_1R antagonist rimonabant or the CB_2R antagonist SR144558 blunt such a reduction (Fernández-López *et al.*, 2007). However, in adult rodent brain the cannabinoid-induced reduction of glutamate release is abolished by CB_1R but not by CB_2R antagonists (Pertwee *et al.*, 2010). In addition, it has been reported that the CB_2R antagonist AM630 reverses the neuroprotective effects of CBD observed in the *in vitro* model of hypoxic-ischemic brain damage on newborn mice forebrain slices (Castillo *et al.*, 2010). In mature brain, however, it is accepted that CBD does not bind CB_2R and that CB_2R antagonists did not modify CBD effects (Pertwee, 2004; Mechoulam *et al.*, 2007). All those results might be explained by a different activity of CB_2R in immature and mature brain.

6. CANNABINOIDS AND NEUROPROTECTION

Some of the aforementioned characteristics of cannabinoids account for a theoretical neuroprotective effect (Fig. 6) (Mechoulam & Lichtman, 2003; Fowler, 2003; Stella, 2004; Klein, 2005; Martínez-Orgado *et al.*, 2007; Martinez-Orgado *et al.*, 2009; Pertwee *et al.*, 2010):

- Prevention of intracellular Ca^{+2} increases: activation of cannabinoid receptors induces the closure of Ca^{+2} channels.
- Reduction of glutamate release: the closure of Ca^{+2} channels determines the reduction of glutamate release. In addition, cannabinoids reduce direct NMDA toxicity by downstream

inhibition of protein Kinase A signaling and NO generation. Drugs reducing glutamate release are of particular value in neuroprotection in NHIE, as glutamate receptor blockers are neurotoxic in immature brains.

- Reduction of toxic NO production: cannabinoids inhibit iNOS expression in glial cells after different stimuli, by inhibiting of transcriptional activity of NF-κB (the mechanism by which glutamate induces the expression of iNOS) and enhancing the release of the IL-1 receptor antagonist (IL-1ra). In addition, cannabinoid-induced reduction of Ca^{+2}influx reduces the activation of nNOS.
- Reduction of inflammatory insult: cannabinoids are potent immunomodulators, inhibiting TNFα production in cultured astrocytes or microglial cells after immunological stimuli by modulating different transcriptional factors and enhancing the release of the endogenous IL-1ra.
- Reduction of oxidative stress: endocannabinoids are potent antioxidants, an effect related to their molecular structure. Several studies have reported neuroprotective effects of cannabinoids related to their antioxidant effect.
- Repair: as it has been discussed before, cannabinoids promote cell proliferation and neurosphere generation and induce remyelinization in animal models of demyelinizating diseases. In addition, cannabinoids promote the differentiation of glial precursors into astroglial cells.
- Glioprotection: cannabinoids enhance energy metabolism of astrocytes, and are protective of these glial cells against cytotoxic and proapoptotic stimuli.

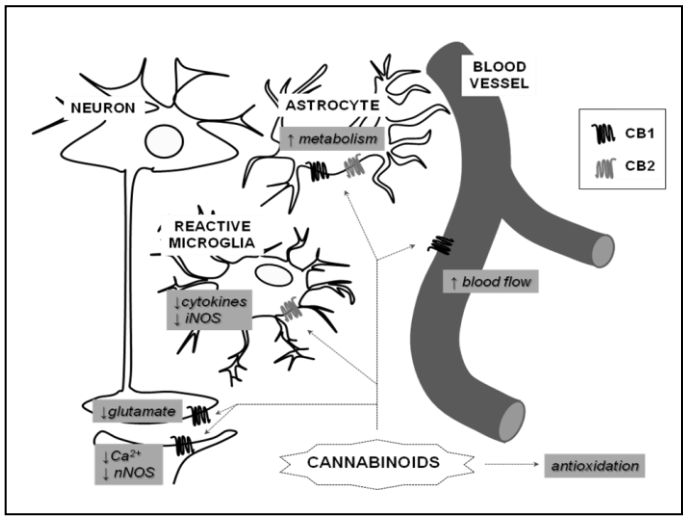

Figure 6. Neuroprotective effects of cannabinoids and the mechanisms involved.

- Other effects:

  - Hypothermia: it is known that cannabinoids reduce body temperature. Studies in adult rats have demonstrated that hypothermia is substantial part of the neuroprotective effect of different cannabinoids, as warming reduces or even abolishes that beneficial effect.

  - Vasodilatation.

  - Blood-brain barrier stabilization.

6.1. REPORTS ON CANNABINOID NEUROPROTECTION

Ten years ago, *in vitro* studies by the group of David Greenberg demonstrated that cannabinoids prevent cellular death in incubated neurons exposed to OGD (Nagayama *et al.*, 1999; Sinor *et al.*, 2000). Lately, a number of *in vivo* experiments supported the neuroprotective effect of cannabinoids in models of stroke, global ischemia or closed brain trauma (Mechoulam&Lichtman, 2003; Martinez-Orgado *et al.*, 2007; Martinez-Orgado *et al.*, 2010). As the number of these studies grows, it has become evident that this

neuroprotection varies according to the species, age, and the type and/or severity of brain insult.

Enhanced levels of endocannabinoids have been observed in the brain of the newborn rat after acute injury, both traumatic or excitotoxic, and in adult rat brain after focal ischemic injury; besides, increased CB_1 and CB_2 receptor expression soon after an acute ischemic insult has been observed in the rat brain cortex (Mechoulam & Lichtman, 2003; Fowler, 2003; Martinez-Orgado *et al.*, 2007). These data prompted several investigators to propose that the endogenous cannabinoid system constitute a natural system of neuroprotection (Mechoulam&Lichtman, 2003).

Cannabinoids have been also tested for treating neurodegenerative diseases in humans. However, the results should be considered with caution since most studies tested cannabinoids administered per os, a route with unpredictable results (Martinez-Orgado *et al.*, 2007; Pertwee, 2008):

- Alzheimer's disease: THC and dronabinol decrease nocturnal agitation by significantly improving several clinical parameters (such as nocturnal motor activity), without undesired side effects.
- Multiple sclerosis: Some recent studies have demonstrated that cannabinoid-induced amelioration in spasticity and other symptoms of MS reach significance after 10 weeks of treatment and remains over 1 year, with reported side effects usually mild and declining as the treatment continues
- Brain ischemia: The cannabinoid HU-211 administered after brain trauma in adults offered short term beneficial effects, but failed to demonstrate any benefit on long term neurologic outcome or survival at 6 months.

6.2. STUDIES ON CANNABINOIDS AS NEUROPROTECTANTS IN NHIE

Studies on neuroprotection by cannabinoids in immature brain are scarce, and limited to direct excitotoxic damage. Thus, it has been described that administration of THC or AEA reduces brain damage in newborn rats after intracerebral injection of ouabain or the AMPA agonist S-bromowillardiine (Van der Stelt *et al.*, 2001; Shouman 2006). After those early studies, some research has been carried out regarding cannabinoids and neuroprotection in models of NHIE (Martinez-Orgado *et al.*, 2003; Fernández-López *et al.*, 2006; Fernández-López *et al.*, 2007; Alonso-Alconada *et al.*, 2010; Fernández-López *et al.*, 2010; Alonso-Alconada *et al.*, 2012):

Rodents:

- *In vitro* models: in newborn rat forebrain slices exposed to oxygen-glucose deprivation (OGD), the CB_1- CB_2 agonist WIN55212-2 (50 µM) reduces cell death in OGD slices, as quantified in terms of LDH efflux and histopathological studies. Neuroprotection by WIN55212-2 is related with the decrease of glutamate and cytokines release as well as of iNOS expression. All these effects are abolished by either a CB_1 or CB_2 receptor antagonist. Moreover, the effect of WIN55212-2, a combined CB_1 and CB_2 agonist, is superior to the effect of a pure CB_1 agonist (ACEA) or a pure CB_2 agonist (JWH133). These data suggest that the simultaneous activation of both CB_1 and CB_2 receptors offers more benefits than CB_1 or CB_2 activation alone.
- *In vivo* models: in newborn rats exposed to severe anoxia, post-anoxic administration of WIN55212-2 0.1 mg/kg i.p. dramatically reduces both early and delayed neuronal death in cortex and hippocampus. In newborn rats exposed to acute hypoxia-ischemia (Rice-Vannucci model: left carotid artery ligation plus exposure to hypoxia -10% O2- for 90-120

min) (Rice *et al.*, 1981), post-insult administration of WIN55212-2 led to a strong neuroprotective effect, in which CB_1 and CB_2 receptors are involved since co-administration of WIN55212-2 with either a CB_1 or a CB_2 receptor antagonist abolishes the beneficial effect of WIN55212-2. Finally,WIN55212-2 increases cell proliferation and protein expression of the neuroblast marker doublecortin (Dcx) in the subventricular zone (SVZ) 7 days after neonatal HI, as well as the number of newly-generated neuroblasts (BrdU+/Dcx+ cells) in the ipsilateral striatum 14 days after HI. WIN55212-2 also promoted the remyelination of the injured external capsule, increasing the number of early oligodendrocyte progenitors (NG2+ cells) in this area.

Big mammals: in term fetal lambs exposed to HI by umbilical cord occlusion, post-insult administration of WIN55212-2 (0.01 mg/kg) improves cerebral blood flow and reduces apoptotic neural death, as demonstrated by the reduction of TUNEL+ neurons. Interestingly, WIN also demonstrated glioprotective effect, reducing astrocytic death. Those effects rely on the maintenance of mitochondrial integrity and functionality.

7. CANNABIDIOL AS A NEUROPROTECTANT IN NHIE

7.1. PARTICULARITIES OF THE NON-PSYCHOACTIVE PHYTOCANNABINOID CANNABIDIOL

Cannabidiol is the major non-psychoactive constituent of *Cannabis sativa*; the lack of psychoactive effects derives from the lack of significant binding to CB_1 receptors (Pertwee, 2004; Mechoulam *et al.*, 2007; Pertwee, 2008). However, it remains unclear whether some effects of CBD are mediated by CB_2 receptors, since CB_2 antagonists may reverse some of the

effects of CBD *in vitro* and *in vivo* (Sacerdote *et al.*, 2005; Ignatowska-Jankowska *et al.*, 2010), including its neuroprotective effect (Castillo *et al.*, 2010). CBD is also thought to be an agonist of serotonin 5HT1A receptors (Russo *et al.*, 2005), which have previously been implicated in the neuroprotective effects of CBD in adult rat models of stroke (Hayakawa *et al.*, 2010). Adenosine receptors are also involved in CBD-mediated neuroprotection in immature mouse brains exposed to OGD, in particular A2A receptors (Castillo *et al.*, 2010); CBD increases brain adenosine levels by reducing adenosine reuptake (Carrier *et al.*, 2006). In addition, CBD binds other receptors as TRPV1, TRPV2, TRPA1 and GPR5 (Pertwee, 2004; Mechoulam *et al.*, 2007; Pertwee, 2008).

CBD has shown neuroprotective effects in both *in vitro* and *in vivo* studies in adult animals (Fernández-Ruiz *et al.*, 2013):

- Hampson *et al.* (1998) showed that CBD protects against glutamate-induced neurotoxicity in primary cultures of rat cerebrocortical neurons, an effect attributed to its antioxidant properties.
- Braida *et al.* (2003) reported that administration of CBD 5 mg/kg to gerbils after bilateral carotid occlusion reduces brain damage as show by EEG, neurobehavioral and histological studies.
- Lately, the group of Fujiwara (Hayakawa *et al.*, 2007) has reported in a model of stroke in mice that post-insult administration of CBD 1-3 mg/kg results in long lasting neuroprotection -attributed to antioxidant, anti-inflammatory, and cerebral blood flow stabilizing effects-, without the development of tolerance after repeated doses.

The mechanisms by which CBD exerts these effects are not completely understood yet, but some of its properties may account for CBD-induced neuroprotection (Hampson *et al.*, 1998; Pertwee, 2004; Esposito *et al.*, 2006; Mechoulam *et al.*, 2007):

- CBD is a potent anti-inflammatory substance, modulating cytokine production, COX activity and cell infiltration in different paradigms of inflammatory damage.
- CBD modulates toxic NO production by inhibiting iNOS induction, at least in part by inhibiting NF-κB activation.
- CBD is a potent anti-oxidant substance, directly because of their molecular characteristics, and by reducing inflammatory responses and NO production.
- CBD increases brain adenosine levels by reducing adenosine reuptake. Adenosine is thought to play a neuroprotective role after HI and adenosine reuptake inhibitors have been shown to induce neuroprotective effects.
- Other effects with neuroprotective potential are the inhibition of calcium transport across membranes, the inhibition of anandamide uptake and enzymatic hydrolysis and the release of CGRP by TRPA1 activation.
- In addition, CBD shows a significant anti-convulsant activity.
- CBD is virtually free from side effects (Pertwee, 2004; Mechoulam *et al.*, 2007). It has been reported that CBD may induce mild sedation at doses of 40 to 100 mg/kg in rats or 600 mg in humans, which represents 10 times higher than the dose of THC needed to achieve a similar effect. In rats, CBD 5 mg/kg may decrease locomotor activity, a dose 5 times higher than of THC (Pertwee, 2004). In newborn pigs, CBD 0.1 mg/kg iv shows no significant side effects on cardiovascular and respiratory parameters; as the piglets were

sedated, however, no effects on locomotion or consciousness has been tested (Alvarez *et al.*, 2008).

- Early studies in the late 80's described that plasma level of CBD declines rapidly after i.v. administration, showing a terminal half life of 18-33 h, and being eliminated mostly by feces.

7.2. STUDIES CARRIED OUT ON CBD AS NEUROPROTECTANT IN NHIE

The aforementioned data suggesting a deleterious effect for CB_1 agonists in immature brain prompted the studies on non-CB_1 cannabinoids for the treatment of HI infants. CBD became quickly a candidate because of their lack of CB_1 activity and the emerging evidences for CBD neuroprotective effects. In this regard, several studies have demonstrated the neuroprotective effect of CBD both in *in vitro* and *in vivo* models of NHIE:

Mice (Castillo *et al.*, 2009): in newborn mice forebrain slices exposed to OGD, CBD 100 µM reduces cell death (reduction of LDH efflux). CBD also blunted apoptotic pathways, reducing the production of caspase-9. The neuroprotective effect is related with the decrease of glutamate and cytokines release as well as of iNOS expression. All these effects are independent from CB_1receptors, but are mediated by CB_2 receptors as well as by adenosine receptors, mainly A2A receptors.

Rats (Pazos *et al.*, 2012): in newborn rats undergoing a HI insult (Rice-Vannucci model), then receiving CBD 1 mg/kg or vehicle, and followed for 30 days (when rats become adults), CBD reduces the volume of brain infarct (as assessed by magnetic resonance imaging) by 17%, as well as the extent of the histological damage (mean neuropathological score) being 1 point lower in CBD than in vehicle-treated animals. Those neuroprotective effects are

associated with a neurofunctional restore: CBD treated animals score similarly to control animals in different neurobehavioral tests assessing motor (Rotarod, assessing motor coordination, and Cylinder Rear Test, assessing unilateral motor deficits) and cognitive (Novel Object Recognition, assessing work memory) whereas vehicle-treated animals show permanent deficits as scored in all tests. In those experiments, proton magnetic resonance spectroscopy (H^+-MRS) studies not only supported the neuroprotective effect but indicated that the n-acetylaspartate/choline ratio (reflecting neuronal density) was increased in CBD-treated animals, suggesting some neuroproliferative effect for CBD.

Piglets (Alvarez *et al.*, 2008; Lafuente *et al.*, 2011): in newborn piglets the administration of CBD 0.1 mg/kg i.v. after an HI insult (hypoxia and carotid occlusion) had neuroprotective effects, as shown by:

- Amplitude-integrated EEG studies: reporting a significant recovery of cerebral activity, the reduction of post-insult brain oedema (as reflected by the reduction of impedance increase in EEG) and the reduction of electrical seizure incidence.
- Near-infrared spectroscopy studies: reporting a significant improvement of brain metabolic activity, as reflected by the reduction of FTOE extraction fall, as well as the reduction of cerebral hemodynamic impairment, as reflected by nTHI .
- Histological studies: reporting the reduction of HI-induced brain damage as reflected by the increase of the number viable cell loss and the decrease of that of degenerating neurons (fluorojade B stained). In addition, CBD administration reduced astrocyte damage, blunting HI-induced reduction in number and size as observed by GFAP immunohistochemistry 72 h post-HI.

- Biochemical studies: reporting that CBD blunts of HI-induced increase of Neuronal Specific Enolase (reflecting neuronal damage) and S-100β protein (reflecting astrocyte damage) levels in cerebrospinal fluid 3 h after HI, thus supporting the neuroprotective and glioprotective effect of CBD.
- Neurobehavioral studies: CBD administration dramatically improves piglet neurobehavioral performance as early as 24 h after the HI insult. This improvement progresses in the following days so that at 72 h the neurobehavioral score in HI+CBD was similar to in SHAM. In HI+VEH there was a modest recovery in neurobehavioral performance in the first day after HI, but this recovery did not continue in subsequent days.

7.3. NON-ANSWERED QUESTIONS REGARDING CBD AS NEUROPROTECTANT IN NHIE

All the aforementioned studies strongly suggest that CBD is a valuable candidate for synergistic therapy with TH in HI infants. However, before testing CBD in human infants some issues have to be clarified:

- The mechanisms of action of CBD in vivo, including the involvement of cannabinoid and non-cannabinoid receptors.
- The optimal CBD dose.
- The therapeutic window (i.e., the longer time that CBD administration could be delayed without losing CBD neuroprotection)
- The effects of CBD on neuro-repair (proliferation and myelogenesis).

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Cannabidiol, administered at 1 mg/kg by i.v. route will achieve such levels in brain tissue as to protect neurones and glial cells by modulating inflammation, excitotoxicity and oxidative stress. This neuroprotective effect is mediated by CB_2 and $5HT_{1A}$ receptors and will be still apparent with CBD being administered up to 6 h after the hypoxic-ischemic insult.

OBJECTIVES

1. To develop a short follow-up (6 h) model of hypoxic-ischemic brain damage in piglets, to demonstrate cannabidiol neuroprotection by different ways:

    1.1. Demonstrating that cannabidiol recovers brain activity as assessed by electroencephalographic studies.

    1.2. Demonstrating that cannabidiol reduces brain damage as assessed by histological studies:

        1.2.1. Protecting neurones.

        1.2.2. Protecting glial cells.

    1.3. Demonstrating that cannabidiol reduces brain damage as assessed by magnetic resonance spectroscopy biomarkers.

2. To develop a medium follow-up (72 h) model of hypoxic-ischemic brain damage in piglets, to demonstrate:

    2.1. That cannabidiol-induced recovery of brain activity and prevention of seizures as assessed by electroencephalographic studies is sustained for 3 days.

2.2. That cannabidiol-induced reduction of histological brain damage is sustained for 3 days and includes:

2.2.1. Protecting neurones.

2.2.2. Protecting glial cells.

2.3. That cannabidiol-induced reduction of brain damage as assessed by magnetic resonance spectroscopy biomarkers is sustained for 3 days.

2.4. That cannabidiol recovers neurological function:

2.4.1. As assessed by motor tests.

2.4.2. As assessed by behaviour tests.

2.4.3. A assessed by anxiety tests.

3. To determine the mechanisms of cannabidiol neuroprotection:

3.1. Analyzing the effect ofcannabidiol on excitotoxicity.

3.2. Analyzing the effect of cannabidiol on oxidative stress.

3.3. Analyzing the effect of cannabidiol on neuroinflammation.

3.4. Analyzing the receptors involved in cannabidiol neuroprotection, in particular:

3.1.2. CB_2 receptors.

3.1.3. $5HT_{1A}$ receptors.

3.5. Analyzing the involvement of changes of endocannabinoid brain concentration

3.6. Analyzing the receptors involved in cannabidiol neurobehavioral effects, in particular $5HT_{1A}$ receptors.

4. To develop a model of hypoxic-ischemic brain damage in newborn mice:

4.1. To demonstrate the protective effect of CBD by different ways:

4.1.1. Demonstrating that cannabidiol reduces the volume of brain damage as assessed by magnetic resonance imaging.

4.1.2. Demonstrating that cannabidiol reduces the histological brain damage:

4.1.2.1. Preventing neuronal necrosis.

4.1.2.2. Preventing apoptosis.

4.1.2.3. Protecting glial cells.

4.2. To determine the temporary therapeutic window of cannabidiol.

5. To determine the pharmacological properties of cannabidiol in a intravenous formulation, assessing:

5.1. The optimal dose and interval.

5.2. The pharmacokinetic profile.

METHODS

METHODS

1. ANIMAL MODELS

All procedures met European and Spanish regulations for protection of experimental animals (86/609/EEC and RD 1201/2005) and were approved by the Ethical Committee for Animal Welfare of the Hospital Universitario Puerta de Hierro Majadahonda. The number of animals used was determined to be the minimum number necessary to achieve statistical significance.

The mice model was realized in Oslo (Norway) and the animal experiments were approved by the National Animal Research Authority (NAAR) in Norway.

1.1. PIGLET MODEL

Animals: Newborn piglets were provided by an authorized farm (aged 1 to 2 days old and body weight 1600-2000 g).Two different approaches were used in this model: in the first one, piglets were maintained for 6 hours under anaesthesia to get insights on the mechanisms of CBD neuroprotection; in the second one, piglets were observed for 72 hours to study short term effects of CBD.

1.1.1. 6 H-LONG FOLLOW-UP (6HFU)

***Animal preparation and instrumentation*:** At arrival piglets were weighted and then anesthetized with sevoflurane (5% induction, 1% maintenance), firstly by mask and then through an endotracheal tube (Portex 2.5, single lumen). One ear vein was cannulated (Introcan 24G, Braun, Melsungen, Germany) to infuse 4 mg/Kg/min of dextrose

intravenously. In each animal, each carotid artery was dissected, exposed and surrounded by an elastic band (Vessel Loops. Bard Nordic.Helsingborg. Sweden).

- Haemodynamic monitoring: Then, right jugular veins were dissected and an indwelling catheter (NutrilineTwinflo 2F, Vygon, Valencia, Spain) placed to continuously infusing drugs and measuring Cardiac Output (CO). To do this, right femoral artery was dissected and an indwelling catheter (Pulsiocath 3F, Pulsion, Munich, Germany) placed to measure Cardiac output (PiCCO Plus, Pulsion), heart rate (HR), mean arterial blood pressure (MABP) and central temperature and to obtain arterial blood samples for gasometric and other analyses. Body temperature was maintained at 37.5-38.5 ºC by an air-warmed blanket (Bair Hugger. Agustine Medical Inc. Eden Prairie. MN. USA). Once instrumentation was finished, a continuous infusion of propofol (14 mg/kg/h) and vecuronium (0.6 mg/kg/h) was started and maintained throughout the entire experimental period to obtain paralyzed sedation.

- Respiratory management and monitoring: Then, the piglet was disconnected from the inhaled anaesthetic device and connected to a mechanical ventilator (Evita 4. Dräger.Lubeck, Germany) with the following starting parameters: inspired oxygen fraction (FiO_2) 21%, inspiratory time 0.5 sec, breath rate 30 rpm, PEEP 5, and PIP enough for a tidal volume (Vt) of 6 ml/kg. Airway resistance, lung compliance and Vt were monitorized by the ventilator-integrated pneumotacography. End-tidal CO_2 ($etCO_2$) and transcutaneous arterial SO_2 ($tcSO_2$) was monitorized by an integrated device (Ohmeda 5250 RGM. Louisville. CO. USA). The $etCO_2$ probe was attached to the proximal end of the endotracheal tube whereas the pulsioximeter probe was attached to one leg. Ventilatory parameters were then modified as needed to maintain $tcSO_2$ 92-98% and $etCO_2$ 35-55 mmHg; arterial blood gases were checked hourly throughout the experimental period.

-Metabolic monitoring: Glycemia and serum levels of cations (Na^+, K^+) were checked out in blood samples every 3 h, and corrected if needed by dextrose 50% or ClNa 1M boluses.

- Brain activity monitoring: Finally, stainless steel wires were placed into the piglet head scalp to continuously monitorize brain activity by amplitude-integrated EEG (aEEG) (BRM3, BrainZInstruments, Auckland, New Zealand). Quantitative changes in aEEG amplitude were registered whereas aEEG background was qualitatively assessed by a neural activity score (4: continuous normal voltage; 3: discontinuous normal voltage; 2: burst suppression; 1: continuous low voltage; 0: Inactive, isoelectric pattern).

Hypoxic-ischemic insult: After completion of the surgical procedure, piglets were allowed to achieve haemodynamic stability (variation lesser than 10% in all haemodynamic and metabolic parameters monitorized) for at least 30 min. The end of this period of stabilisation was considered the t0 point for hypoxia-ischemia (HI). Then, piglets underwent a cerebral HI insult: carotid blood flow was interrupted by pulling out the carotid bands and FiO_2 was reduced to 10%, for 30 min. HI was confirmed by the suppression of brain activity in aEEG. If severe systemic hypotension (MABP <30 mm Hg) or bradycardia (HR<60) developed, FiO_2 was minimally increased to improve hemodynamic parameters but preventing aEEG recovery. If hemodynamic deterioration progressed and at least 25 min of HI had been completed, HI was ended. At the end of the period of HI, carotid flow was restored and FiO_2 was increased to 21%. If needed, resuscitation was then carried out by adrenaline infusion, chest compression and FiO_2 increase. Then, and throughout the entire experiment, appropriate changes in ventilatory parameters were done to regain normal $tcSO_2$, $etCO_2$ and gasometric values. If needed, sodium bicarbonate was infused to correct acidosis. If needed,

dopamine infusion (5-20 mcg/kg/min) was started to maintain appropriate MABP (50-70 mmHg).

Treatment: Thirty minutes after HI, piglets were randomly assigned to receive 10 ml i.v. of vehicle (HV, n=9) or CBD (HC, 1 mg/Kg IV) (n=11), alone or with the antagonist of CB2 receptors AM630 (1 mg/Kg) (CBD + AM630: HCA, n=6) or the antagonist of serotonin 5HT1A receptors WAY100635 (1 mg/Kg) (CBD + WAY100635: HCW, n=6).

CBD was prepared in a 5 mg/ml formulation of ethanol:solutol:saline at a ratio of 2:1:17. AM630 or WAY 100635 were administered 15 min before CBD and dissolved in the same vehicle.

After drug administration, piglets remained sedated and ventilated for 6 h. During this period hemodynamic and ventilatory parameters were recorded hourly and aEEG continuously monitorized. Blood samples were obtained hourly after drug administration, to check gasometric and metabolic parameters and to study CBD pharmacokinetics (PK).

Sample collection:

- Blood samples: Both at the beginning and at the end of the experiment 0.5 ml blood sample was obtained and placed in a container with EDTA, then centrifuged at 1500 rpm for 15 min at 4º C and then serum and plasma separated and stored at -80 ºC until use.

- Brain samples: Then, cold heparinized saline was infused through carotid arteries indwelling catheters to wash out blood from the brain; jugular veins were sectioned to facilitate this procedure. Then piglet was killed by 10 ml 1M KCl infusion, and brain removed. Brain hemispheres were separated. The left hemisphere was divided into 4 pieces that were placed into 4% formaldehyde and embedded in paraffin for histological and immunohistochemical studies. Three samples from parietoccipital cortex and three samples

from hipoccampus were obtained from the right hemisphere and immediately frozen in isopentane and conserved at -80 ºC for biochemical studies. Piglets similarly managed but with neither HI nor drug treatment (SHM) served as controls.

1.1.2. 72 H-LONG FOLLOW UP (72HFU)

***Animal preparation and instrumentation*:** piglets were anesthetized, intubated and ventilated and each carotid artery dissected, exposed and surrounded by an elastic band similarly to 6HFU piglets. Then, right jugular veins were dissected and an indwelling catheter (NutrilineTwinflo 2F, Vygon, Valencia, Spain) placed to continuously infusing drugs. The extravascular segment of the catheter was secured with several skin stitches with the end of the catheter placed and secured on the piglet back, to guarantee a venous access for medication and blood sampling throughout the entire experimental period. Then, 4 mg/Kg/min of dextrose were infused intravenously.

HR was monitorized using external electrodes. Central temperature was monitorized using a rectal probe. Body temperature was maintained at 37.5-38.5 ºC using the air-warmed blanket. A pulsioximeter probe was attached to one leg. Finally, stainless steel wires were placed into the piglet head scalp to continuously monitorize brain activity by aEEG. Once instrumentation was finished the piglet were kept anaesthetized under 2% sevoflurane.

Glycemia, serum levels of cations and blood gases were checked out until stabilization before starting the HI insult. Appropriate corrections on ventilator settings and/or dextrose or cations infusion were made until normalized parameters were obtained.

Hypoxic-ischemic insult: this was carried out similarly to that explained for 6HFU excepting that the insult lasted for 20 min. At the end of the period of HI, carotid flow was restored and FiO_2 was increased to 21%. Then, the piglets were disconnected from the anaesthetic device and connected to the Evita ventilator with the same parameters. Once spontaneous breathing movements were observed the ventilator setting was changed to CPAP at 6 cmH_2O and the lower FiO_2 needed for SO_2>92%. Once spontaneous breathing became regular the piglets were extubated. Afterwards, 100% O_2 was administered at 4 lpm through nasal prongs as long as this was needed to maintain SO_2>92%.

Treatment: Fifteen minutes after HI the piglets received acetaminophen 15 mg/Kg i.v. (for analgesia) and cefotaxime 50 mg/Kg i.v. (to prevent surgical infections). Both drugs were then administered every 12 h throughout the experimental period.

Thirty minutes after HI, piglets were randomly assigned to receive 10 ml i.v. of vehicle (HV, n=9) or CBD 1 mg/Kg. In some piglets CBD was administered single dose (HC1, n=5) whereas in other piglets CBD was administered once a day for 3 days (HC3, n=6). Drugs were administered alone or with WAY100635 (1 mg/Kg) every 12 h. In piglets receiving CBD single dose WAY was then administered just the first day (HCW1, n=4) whereas in piglets receiving CBD once a day for 3 days WAY was administered every 12 h during the three days (HCW3, n=4).(Table 2).

	Hour	0.5	12	24	36	48	60
SHM	VEH	x		x		x	
	CBD						
	WAY						
HV	VEH	x		x		x	
	CBD						
	WAY						
HC1	VEH						
	CBD	x					
	WAY						
HC3	VEH						
	CBD	x		x		x	
	WAY						
HCW1	VEH						
	CBD	x					
	WAY	x	x				
HCW3	VEH						
	CBD	x		x		x	
	WAY	x	x	x	x	x	x

Table 2. Scheme of drug administration

After drug administration, piglets were moved toan air heater-warmed cage once spontaneous breathing became stable. During the first 24 h after HI piglets remained with nothing per os receiving 4 mg/Kg/min of dextrose i.v. Then, up to 40 ml of milk formula suitable for newborns was offered every 2-3 h from 8 a.m. to 8 p.m. Milk formula was administered through a tube attached to a researcher's finger and connected to a 50 ml syringe; once the piglet started sucking the finger, the syringe was gently pushed to deliver milk as synchronized to suckling as possible.

Piglets similarly managed but with neither HI nor drug treatment (SHM) served as controls.

Neurobehavioral assessment:

-Functional brain assessment: 24, 48 and 72 h after HI a 10 min-long aEEG trace was recorded to register changes in aEEG amplitude.Electrographic seizures were defined as repetitive,rhythmic waveforms with a distinct beginning and end with a duration >10 s.

- Anxiety response: based on the stress response of mammals upon restrain, the time spent by piglets on fighting against restrain during the aEEG record was obtained as a surrogate of anxiety.

- Playful activity: since piglets start to interact with the environment and live beings (either pigs or humans) from the second day of life, playful activity with an object (a sheet) or with the researchers were videorecorded for 10 min at 48 and 72 h after HI. Later on, a researcher blinded to the experimental group quantified the percentage of that time spent on object or social playfulness.

- Eating behaviour: Swollen milk amount as well as suckling appropriateness was recorded each time piglets were fed.

- Motor performance: based on a standardized piglet neurobehavioral test (Schubert *et al.*, 2005; Lafuente *et al.*, 2011), piglet motor performance was assessed 24, 48 and 72 h (Table 3).

Mental status	Coma Stupor Letargy Awake	0 1 3 4
Behavior	None Weak Aggressive Normal	0 1 3 4
Pupils	Non reactive Slow, assimetric Normal	1 2 3
Vestibulo-ocular Reflex	Absent Nistagmus Normal	1 2 3
Stepping	None Just fore/hind paws Normal	1 2 3
Righting	Absent Present	1 2
Muscle tone	Atonic/Hypertonic Partially atonic Partially hypertonic Normal	1 2 3 4
Standing	No Paresis Unsteady Normal	1 2 3 4
Walking	No Paresis Falling Normal	1 2 3 4
Feeding	No suckling reflex Weak reflex, tube feeding No appetite Brief suckling Normal	1 2 3 4 5

Table 3. Neurobehavioral test

Sample collection:

- Blood samples: Both at the beginning and at the end of the experiment 0.5 ml blood sample was obtained and managed similarly to that described for 6HFU.

- Brain samples: Brains were obtained and managed at the end of the experiment similarly to that described for 6HFU.

1.2. MICE MODEL

Animals: NewbornC57/BL6mice (aged 9 to 10 days old) were bred in the animal facilities of the Rikshospitalet (Oslo, Norway). This model was selected to study the temporary therapeutic window of CBD treatment.

Animal preparation and instrumentation: Before surgery, pups were handled to avoid rejection by the dam. Firstly, the dam was placed into a cage besides the pup cage. After flushing gloves with 1% clorhexidine (Cristalmina®), pups were touched several times. Immediately after the dam was returned to the pup cage.

Fifteen minutes later, the dam was placed into a cage with free access to food and water and moved to another room and pups were touched again with clorhexidine-flushed gloves. Then, after 15 minutes the dam was once more returned to the pup cage. Fifteen minutes later the dam was placed into a cage with free access to food and water and moved to another room. Pups were then ready for surgery.

Hypoxic-ischemic insult: Pups in the cage were kept warm by an air heater. Pup cage was divided into two sections to separate operated from non-operated pups. The pup to be operated was anaesthetized with sevoflurane 5%. Then, pup was placed upright with both forepaw gently attached to a 1 ml syringe and the neck in gently hyperextension. Deep anaesthesia was confirmed by absent response to tail compression. Then, neck left side was cleaned with clorhexidine and a 1 cm-wide incision was made with a scalpel, starting in the middle point between clavicles and going then craneo-laterally oriented to pup left. Subcutaneous, fat and muscular planes were dissected by forceps and scissors. The vasculoneural plane was dissected by forceps. Then, sevoflurane was lowered to 2,5%. Once the left carotid artery was exposed, it was carefully dissected and separated from vein and

nerve. Using the L-shaped forceps, the left carotid artery was brought up and carefully electrocoagulated until it became divided into two parts. A drop of saline was poured in to gently separate both electrocoagulated arterial ends from forceps.

Finally, the wound was stitched up with two knots using 5-0 Vycril® and further cleaned with clorhexidine. Still under anaesthesia, the pup was identified by an ear-punch/scissor. Pups were allowed to awake and when moving by itself is placed into the cage, under the warmed air.

Six pups from each litter underwent the carotid electrocoagulation (HI group). The remaining ones just underwent scalpel incision, stitching and clorhexidine cleaning up (Sham group). In case it lasted more than 5 min to find the carotid artery surgery was finished and that pup was considered as Sham. In case bleeding appeared with no stop after gentle pressure by a cotton bud or if carotid artery was accidentally broken, the pup was killed by cervical dislocation under anaesthesia.

After surgery was completed, the dam was returned to the pup cage, which was placed in a quiet environment for at least 3 h. After that recovery period, the dam was placed into a cage with free access to food and water and moved to another room. Three randomly selected pups were weighted to obtain the mean pup weight.

Then, HI pups were placed in groups of three into 500-ml jars maintained at 37ºC by a warm water bath and exposed to a 10% O_2 mixture for 90 min. In the meanwhile, Sham(SHM, n=30)mice were maintained in the cage under warmed air.

After the end of HI, pups were resuscitated by tactile stimulation at room air. Freely moving pups were then placed into the cage under warmed air and animals were randomly assigned to receive s.c. injections of 0.1 ml of vehicle (ethanol:solutol:saline 2:1:17) (HV, n=60) or CBD (1 mg/Kg) was administered s.c. 15 min, or 1, 3, 6, 12 or 24 h after the end of

the HI insult (HC0.15 n=10; HC1, n=10; HC3, n=10; HC6, n=10; HC12, n=10; HC24, n=10, respectively).The CBD was prepared from the 5 mg/ml formulation of ethanol:solutol:saline (2:1:17).

Sacrifice: Seven days later (P16), the mice were sacrificed by a lethal i.p. injection of diazepam + ketamine. After opening the chest, left heart ventricle was cannulated with a 25 G needle and saline and then 4% paraformaldehyde (PFH) were transcardially perfused at 4 ml/min. Then, the skull was opened, brain removed and placed in Falcon tubes containing 4% paraformaldehyde. The brains were used to asses brain damage by Magnetic Resonance Imaging (MRI) and subsequently processed to histological studies.

2. IMAGING ANALYSIS

In mice model MRI was used to determine the volume of HI brain damage.The MRI scan of the brains was carried out in the MRI Unit of the Instituto Pluridisciplinar, (Universidad Complutense, Madrid) on a BIOSPEC BMT 47/40 (Bruker-Medical, Ettlingen, Germany) operating at 4.7 T, equipped with an actively shielded gradient insert with an 11.2-cm bore, a maximal gradient strength of 200 mT/m, an 80-ms rise time, and a homemade 4-cm surface coil. T2WI were acquired with multislice rapid acquisition (TR ¼ 3.4 s, RARE factor ¼ 8, interecho interval ¼ 30 ms, TEeff ¼ 120 ms; matrix size ¼ 256 _ 256(pixel dimensions 117_117 mm), field of view (FOV) ¼ 3 cm2). The slice package consisted of 26 consecutive 0.5-mm-thick slices in the axial plane with an interslice gap of 0.1 mm to image the entire brain. Brains were placed in Fluorinert FC-40 (3 M, Minnesota, USA) for the MRI scan and then replaced in PFH.

Volumetric analyses of the MRI slices were performed using ImageJ 1.43u software (U.S. National Institutes of Health). In the selected slice, the area of brain parenchyma was manually outlined, and the size of the selected area was calculated by the software program.

3. HISTOLOGICAL STUDIES

Once the tissues were fixed in 4% paraformaldehyde, they were dehydrated and embedded in paraffin to be cut on a Leica microtome. Consecutive sections (4μm) of each animal were cut and mounted on a glass slides for histological techniques.

3.1. NISSL STAINING: Sections were stained with Toluidine Blue (0.25%) in distilled water for 1 minute. Then sections were successively washed in 96% ethanol and mounted to analyse in an optical microscope.

Piglet model analysis: Consecutive coronal sections were analyzed. Areas of 1 mm^2 in the central three lobes of the parietal cortex at 3 mm in the posterior plane, as shown in a stereotaxic atlas of pig brain (Félix *et al.*, 1999), were examined, focusing on layers II-III, by an investigator blinded to the experimental group using an optical microscope and a grid of 50 compartments; the mean of three compartments was calculated. Apparently normal neurons were identified by the presence of typical nuclei with clear nucleoplasm and a distinct nucleolus surrounded by purple-stained cytoplasm. Neurons were defined as damaged when no distinction could be made between the nucleus and cytoplasm (pyknotic or necrotic).

Mice model analysis: Three consecutive sections corresponding to the plate 35 of the rat brain atlas (Paxinos & Watson, 1997) were selected for analysis by an examiner blinded

to the experimental group of the animal. The degree of brain damage in the ipsilateral hemisphere was scored as follows: 0 = normal, 1 = few neurons damaged (1-5%), 2 = several neurons damaged (6-25%), 3 =moderate number of neurons damaged (26-50%), 4 = more than half of neurons damaged (51-75%), 5 = majority of neurons damaged (>75%). The mean of 3 sections from each animal was determined.

3.2. IMMUNOHISTOCHEMISTRY: The basic protocol for immunohistochemistry assays is described below. Coronal sections were submitted to antigen retrieval process, washed in 0.1 M PBS (Phosphate Buffer Saline) and incubated with the corresponding primary antibody (Table 4) over night at 4ºC. After that, tissue sections were incubated with secondary antibody for 2 hours at 37ºC (1:200, Alexa; Molecular Probes). Finally, cell nucleuses were counterstaining with TO-PRO-3 (1:500, Molecular Probes) and mounted in aqueous medium Vectashield (Vector Laboratories, Burlingame, United Kingdom). Visualization and photography of the samples was carried out with a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with 10xHCX PL APO (0.4numerical aperture), 20x HCX PL APO (0.7 numerical aperture) and oil-immersion optics: 40× HCX PL APO (1.25 numerical aperture) and 63xHCX PL APO (1.4 numerical aperture).

Table 4. Primary Antibodies used for immunohistochemistry

Antibody	Company	Dilution
Myelin basic protein	Sigma-Aldrich	1:500
GFAP-Cy3	Sigma-Aldrich	1:1000

In piglets histological studies were done in the three medial crests of parietooccipital cortex and hippocampus; MBP immunohistochemistry was done in the subcortical area of the aforementioned crests (Fig. 7A), In mice, immunohistochemical studies were carried out in a Region of Interest (R.O.I) involving the parietooccipital cortex at the penumbral perilesional area (Fig. 7B).

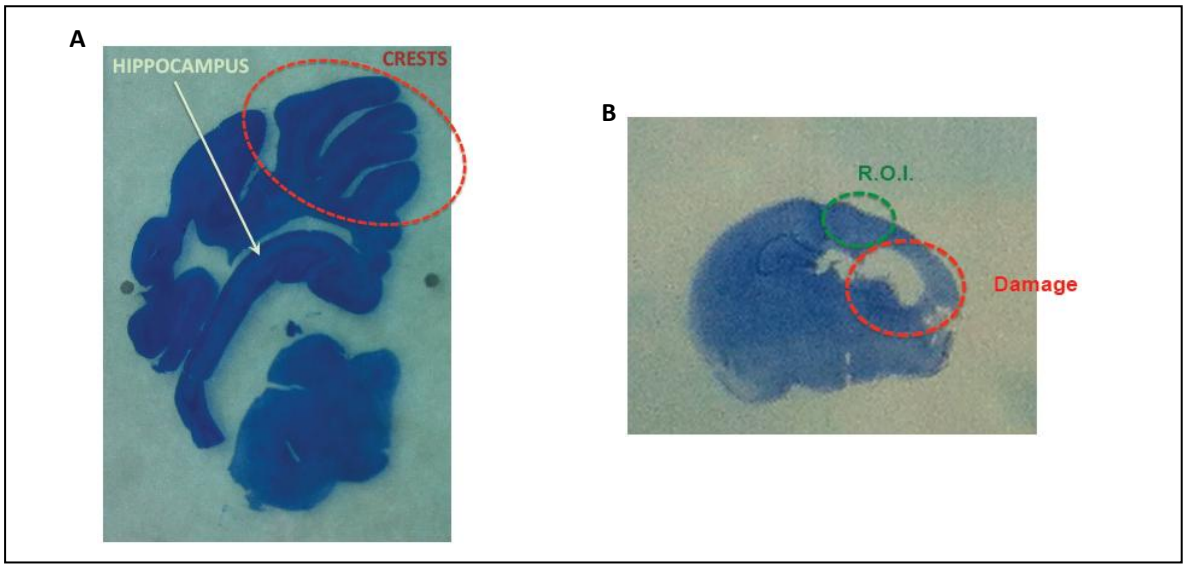

Figure 7. A) Parietoccipital crests and hippocampus for histological studies in piglets. **B)** Region of interest (R.O.I.) for immunohistochemical studies in newborn mice.

3.3. TUNEL ASSAY: Tunel assay was performed in mice brains to evaluate cell apoptosis in different groups. The fragmented DNA of the apoptotic cells was labelled using DeadEnd Colorimetric TUNEL System (Promega, Spain). As manufacturer instructions, after rehydration slides were treated with 20 mg/ml proteinase K for 10 min at room temperature, washed with PBS and re-fixed in 4% paraformaldehyde. Then slides were incubated with rTdT enzyme and the nucleotide mix at 37Cº for 60 min and the reaction was stopped by incubation in 2x SSC for 15 min. After washing with deionized water, the slides

were counterstaining with TO-PRO-3 (1:500, Molecular Probes) and covered with glass coverslips in an aqueous medium (Vectashield; Vector Laboratories, Burlingame, United Kingdom). Visualization and photography of the samples was carried out with a confocal TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Both GFAP immunohistochemistry and TUNEL assay images were analyzed using the ImageJ 1.43u software (U.S. National Institutes of Health). The number of cell positive was quantified in each selected area. For MBP immunohistochemistry, the number of positive pixels in the selected area was determined by using the ImageJ software.

4. BIOCHEMICAL STUDIES

4.1. WESTERN-BLOT: Protein analysis and quantifications were conducted through the use of western-blotting. Following the company instructions 0.2 grams of brain tissue from piglets were homogenated in 500 ml of T-PER Reagent (Thermo Scientific) and 5 ml of Protease Inhibitor Cocktail Kit (Thermo Scientific). The samples are quickly centrifuged at 4ºC at 10000G. The supernatant is collected and store at -80ºC. Previously to perform the western-blot, protein content was measured for each sample Pierce BCA Protein Assay Kit (Thermo Scientific).

IL-1beta quantification: Samples were heated for 5 minutes at 95ºC and quickly put on ice to be subjected to electrophoresis on a SDS-polyacrylamide gel for 1.5 h at 90V (constant voltage). Proteins thus separated were transferred to a PVDF (polyvinylidenedifluoride membrane) membrane (Amersham Hybond-P, GE Healthcare), at 60V for 1.5 h.

After transference, the non-specific sites were blocked by incubating the membrane with blocking solution (5% non-fat dried milk in PBS-T) for 1 hour at room temperature (RT).

Then membrane was incubated with primary antibody (1:200; Thermo Scientific), diluted in blocking solution overnight at 4ºC with gently shaking. After 3 washes of 10 minutes with TBS-T, the membrane is incubated with secondary antibody diluted 1:7000 (GE Healthcare; UK) in blocking solution for about 1h at room temperature with gently shaking. In order to normalize the data, beta-actin quantification is used for each sample. Chemiluminescence detection is performed using the ECL Western-blotting Analysis System (GE Healthcare; UK) and impressed in an autoradiographic film. Data analysis is made by Quantity One software (Bio-Rad) measuring the average of intensity for each band.

Oxyblot:OxyBlot protein oxidation detection kit (Millipore Iberica; Madrid) was used to quantify the presence of protein carbonyl groups in brain tissue. 15 mg of total protein were subjected to the derivatization reaction with 2,4-dinitrophenylhydrazine and processed for Western blot analysis. According to the manufacturer's protocol, the corresponding negative controls were used at the same time. Then, samples were electrophoresed in a 12% sodium dodecyl-sulfate-polyacrylamide gel (SDS-PAGE). DNP-BSA Standards (Millipore Iberica; Madrid) were included on each gel.

Proteins were electro-blotted onto PVDF membranes (GE Healthcare, UK) in Tris/glycine/methanol transfer buffer at 4ºC under constant voltage (2 h at 250 mA). The resultant blots were blocked in PBS-Tween (PBST) containing 5% nonfat dried milk at 4ºC by overnight incubation. Primary antibody incubation was carried out at 1:150 dilution in PBST containing 5% nonfat dried milk for 1 h at RT. After washing with PBST, the membranes were incubated with the secondary antibody (1:300) for 1 h at RT. Finally, the peroxidase reaction was developed with an ECL Kit (GE Healthcare; UK). Films were scanned and analyzed with ImageJ software. The levels of protein oxidation were quantified by means of densitometric

analysis and normalized by total protein loading (Red Ponceau staining) and expressed by the OxyBlot/Red Ponceau ratio.

4.2. PROTON MAGNETIC RESONANCE SPECTROSCOPY (H^+-MRS): H^+-MRS was performed in the MRI Unit of the InstitutoPluridisciplinar, (Universidad Complutense, Madrid, Spain) at 500.13 MHz using a Bruker AMX500 spectrometer 11.7 T operating at 4ºC on frozen cortex samples (5-10 mg weight) placed within a 50 μl zirconium oxide rotor with cylindrical insert and spun at 4000 Hz spinning rate. Standard solvent suppressed spectra were acquired into 16 k data points, averaged over 256 acquisitions, total acquisition ~14 min using a sequence based on the first increment of the NOESY pulse sequence to effect suppression of the water resonance and limit the effect of B0 and B1 inhomogeneities in the spectra (relaxation delay-90º-t1-90º-tm-90º-acquire free induction decay (FID)) in which a secondary radio frequency irradiation field is applied at the water resonance frequency during the relaxation delay of 2s and during the mixing period (tm = 150ms), with t1 fixed at 3 μs. A spectral width of 8333.33 Hz was used. All spectra were processed using TOPSPIN software, version 1.3 (Bruker Rheinstetten, Germany). Prior to Fourier transformation, the FIDs were multiplied by an exponential weight function corresponding to a line broadening of 0.3 Hz. Spectra were phased, baseline-corrected and referenced to the sodium (3-trimethylsilyl)-2,2,3,3-tetradeuteriopropionate singlet at δ 0ppm.

By using the 3.1.7.0 version of the SpinWorks software (University of Manitoba, Canada) curve fitting was performed and several ratios were calculated, including: the lactate/N-acetylaspartate (Lac/NAA), the N-acetylaspartate/choline (NAA/Cho), the glutamate/N-acetylaspartate (Glu/NAA) and the reduced gluthation/creatine (GSH/Cr).

4.3. DETERMINATION OF BRAIN ENDOCANNABINOID LEVELS: These studies were carried out in the Prof. Cecilia Hillard's laboratory, at the Neuroscience Research Center of the Medical College of Wisconsin (USA). Thus, tissue samples were weighed and placed into borosilicate glass culture tubes containing two ml of acetonitrile with 84 pmol of [2H8]anandamide and 186 pmol of [2H8]2-AG. Tissue was homogenized with a glass rod and sonicated for 30 min. Samples were incubated overnight at −20°C to precipitate proteins, then centrifuged at 1,500 × g to remove particulates. The supernatants were removed to a new glass tube and evaporated to dryness under N2 gas. The samples were resuspended in 300 µL of methanol to recapture any lipids adhering to the glass tube, and dried again under N2 gas. Final lipid extracts were suspended in 20 µL of methanol, and stored at −80°C until analysis. The contents of arachidonylethanolamide (AEA), 2-arachidonoylglycerol (2-AG), oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) were determined in the lipid extracts using isotope-dilution, liquid chromatography-mass spectrometry.

5. CBD PHARMACOKINETIC STUDY

Blood samples from piglets (6HFU) were obtained every 30 min after CBD injection. Plasma was immediately separated by centrifugation at 4 ºC and 7000 rpm for 15 min, and stored at -80 ºC. Then, plasmatic CBD concentration was determined by gas chromatography-mass spectrometry. Successive CBD concentrations defined the time-concentration curve, allowing the performance of the pharmacokinetic study by determining:

- Plasma half life($T_{1/2}$): the time necessary to halve the plasma concentration. It is useful to determine the frequency of administration of CBD. Allows to calculate the Elimination Rate Constant ($k_{el}=0.693/T_{1/2}$)

- Cmax: maximum CBD concentration after administration.

- Tmax: time after administration when Cmax is reached.

- Area under the curve (AUC): expressed in ng/h/ml. It is useful to study CBD bioavailability and total clearance.

In addition, samples (10 mg weight) from frozen 6HFU HC brains were homogenized in MeOH:water (10:90, v:v) added in a 3:1 solvent:brain ratio (1 g of brain tissue was taken to equal 1 ml). CBD was extracted from brain tissue homogenate using liquid-liquid extraction with 5% IPA (hexane), and CBD levels were quantitatively determined using LC-MS/MS at Quotient Bioresearch Ltd. (Fordham, UK).

RESULTS

RESULTS

1. NEUROPROTECTIVE EFFECT OF CBD

1.1. PIGLET MODEL

1.1.1. 6HFU

No significant differences were found between the distinct treatment groups in terms of age or weight (Table 5). Of a total of 38 animals, only two piglets (5.3%) died in the 90 min following the HI insult (one assigned to the HV and the other to the HC group).

Table 5. Piglet age and weight.

	SHM	HV	HC	HCA	HCW
Age (days)	1.8 ± 0.1	1.8 ± 0.1,	1.9 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
Weight (kg)	1.9 ± 0.1	1.7 ± 0.1	1.9 ± 0.1	1.8 ± 0.1	1.9 ± 0.1

Cardiopulmonary parameters

There were no differences in CO (cardiac output) among the piglets of the different groups, with the exception made for HCW piglets in which CO fell throughout the experimental period (Table 6). HI insult was associated with a progressive fall of MABP (mean arterial blood pressure) in HV-treated animals (Table 6), so that a half of HV piglets needed inotropic drug support (dopamine, mean dose 13±4 μg/Kg/min). Such a fall of MABP was not observed in HC, with no piglet from this group needing inotropic support. By contrast, in piglets receiving CBD plus the CB_2 or the $5HT_{1A}$ receptor antagonist MABP dropped 15-20 mmHg throughout the experimental period. The effect was more dramatic for HCW (Table 6) so that 5 out of 6 piglets from this group needed inotropic drug support (dopamine, mean dose 11.2±1.2 μg/Kg/min). Brain HI was associated with a drop in pH throughout the experimental period in all HI groups, although such a drop was more severe

in HCA or HCW (Table 6). There were no differences in CO_2 levels among the piglets of the different groups.

Table 6. Cardiorrespiratory parameters. B: Basal. D: Drug. E: End. *CO*: cardiac output (mL/min/kg). *MBP*: mean blood pressure (mmHg). (*) p<0.05 vs SHM. (#) p<0.05 vs B. (§) p<0.05 vs. HC.

		SHM	HV	HC	HCA	HCW
CO	B	31.8(2.9)	33.9(3.5)	35.7(2.5)	34.4(3.9)	34.4(2.8)
	D	35.5(2.7)	36.9(4.1)	33.4(4.1)	33.2(2.2)	36.2(2.3)
	E	35.0(3.6)	34.4(4.2)	33.6(4.3)	32.0(3.6)	29.6(2.1)*,#,§
MBP	B	79.3(5.0)	80.1(3.3)	82.5(2.6)	83.1(5.1)	76.9(3.1)
	D	90.5(5.8)	86.0(7.1)	91.4(7.8)	95.4(4.5)	85.1(5.3)
	E	78.8(5.1)	59.6(3.1)*,#,§	78.8(2.5)	68.4(2.5)*,#,§	56.4(7.1)*,#,§
pH	B	7.34(0.02)	7.32(0.02)	7.35(0.02)	7.32(0.01)	7.35(0.01)
	D	7.38(0.01)	7.21(0.03)*,#	7.20(0.05)*,#	7.18(0.04)*,#	7.23(0.04)*,#
	E	7.39(0.01)#	7.32(0.03)*	7.32(0.02)*	7.25(0.03)*,#,§	7.26(0.03)*,#,§
pCO_2	B	41.4(3.3)	39.4(1.9)	40.7(2.6)	38.8(1.6)	39.9(1.8)
	D	39.4(2.3)	42.4(2.2)	43.4(2.3)	41.2(3.1)	40.1(1.6)
	E	38.8(1.1)	42.2(2.9)	42.3(1.6)	42.5(2.7)	39.9(2.5)

CBD recovered brain activity

The benefits of CBD treatment in the piglet HI model were quantitatively assessed by determining the aEEG amplitude and qualitatively using a neural activity score (Tichauer *et al.*, 2009). Continued sedo-analgesia determined that aEEG amplitude dropped slightly in SHM animals throughout the experiment (Fig. 8A). When analyzing the neural activity score, however, it was apparent that such a drop was not associated with an impairment of background activity and/or pattern (Fig. 8B). HI led to a severe fall of brain activity (Fig. 8A) together with a severe impairment of background pattern, which were not recovered during the following 6 hours (Fig. 8B). CBD administration led both to a progressive recovery of brain activity (59.3±9% basal activity at 6 h) (Fig. 8A) and of the background pattern (Fig. 8B).

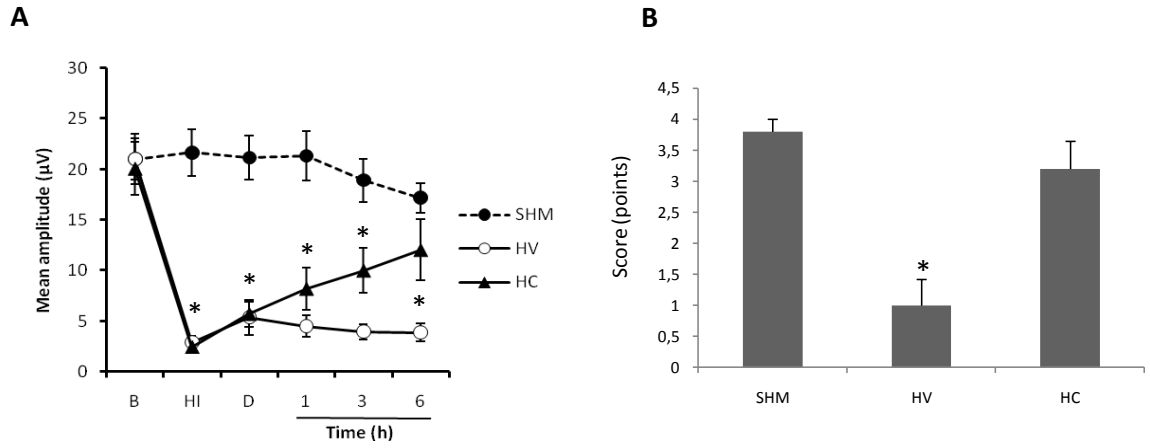

Figure 8. A) Quantitative CBD-induced recovery of brain activity after HI. Changes of mean amplitude of aEEG trace recorded in 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD (HC). Results are expressed as means ± SEM of 6-10 animals. (*) p<0.05 vs. SHM. (§) p<0.05 vs HC. **B)** Qualitative CBD-induced recovery of brain activity. Qualitative assessment of aEEG background activity by a neurological activity score in 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD (HC). (*) p<0.05 vs. SHM.

CBD protected neurons

HI insult led to a dramatic increase in the number of necrotic neurons in the cortex, as witnessed by Nissl staining of this tissue 6 hours after insult (Fig. 9A), although this increase was blunted by CBD administration (Fig. 9B).

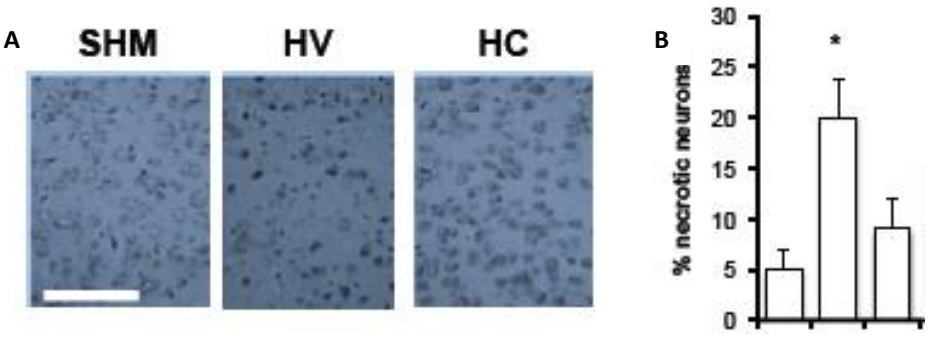

Figure 9. CBD-induced prevention of neuronal death after HI. **A)** Representative light microphotographs of Nissl stained brain sections, obtained from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV), or CBD (HC). **B)** In brain from HV there is an increase in number of pyknotic cells and a decrease in number of viable neurons. Administration of CBD reduced the presence of pyknotic cells. Original magnification x200, bar: 100 µm. Results are expressed as means ± SEM of 6-10 animals. (*) p<0.05 vs. SHM.

CBD increased the number of astrocytes

In the HV group, no reduction in the number of GFAP+ cells was evident in the cortex after HI insult (Fig. 10). By contrast, CBD administration led to a significant increase in the number of GFAP+ cells in the HC group.

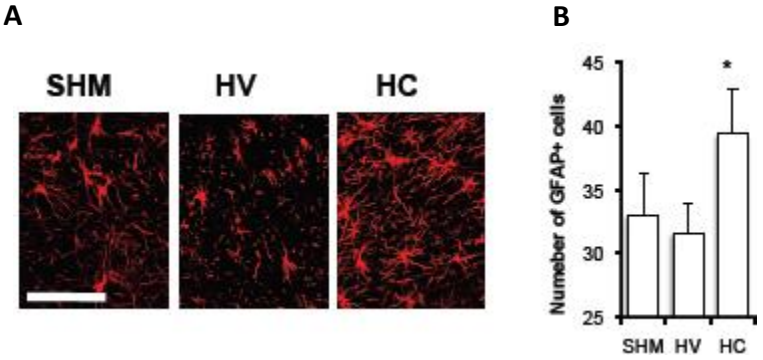

Figure 10. CBD-induced prevention of astroglial death after HI. **A)** Representative fluorescence microphotographs of GFAP stained brain sections, obtained from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV), or CBD (HC). **B)** Administration of CBD increased the presence of GFAP+ cells. Original magnification x200, bar: 100 µm. Results are mean ± SEM of 6-10 animals. (*) $p<0.05$ vs. SHM.

CBD improved H^+-MRS prognostic markers

Lac/NAA and NAA/Cho ratios were used as prognostic markers. Although Lac/NAA ratio increased and NAA/Cho decreased in cortex after the HI insult (Fig. 11B), these changes were not observed in HI piglets treated with CBD (Fig. 11B).

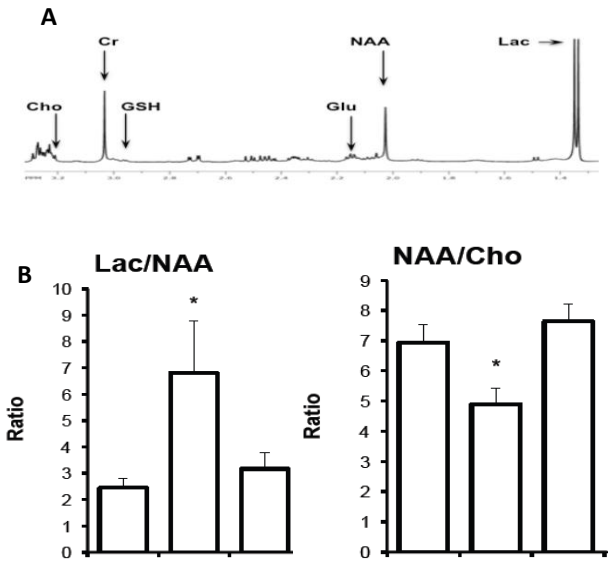

Figure 11. CBD-induced improvement of H^+-MRS biomarkers after HI. **A)** representative brain H^+-MRS spectrum from a normal piglet, showing the peaks of the different metabolites studied. **B)** Bars represent the results, expressed as means ± SEM, of different metabolite ratios obtained from studies performed in brain samples from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD (HC). Cho: choline; Lac: lactate; NAA: N-acetylaspartate. (*) $p<0.05$ vs. SHM.

1.1.2. 72HFU

All piglets were 1 day-old. No significant differences were found between the distinct treatment groups in weight (Table 7). Of a total of 39 animals, eight piglets (20.5%) died in the 24h following the HI insult (three assigned to the HV, two to the HC1, two to the HC3 and one to the HCW3 group).

Table 7. Piglet weight

	SHM	HV	HC1	HC3	HCW1	HCW3
Weight (kg)	1.5 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1

CBD recovered brain activity

The benefits of CBD treatment were quantitatively assessed by determining the aEEG amplitude (Fig. 12). HI insult led to a decrease of aEEG amplitude in all the experimental groups. In HV piglets, a mild recovery was observed at 72 h after HI but this was not enough to regain normal amplitude. From 48 h after HI, however, aEEG amplitude improved in animals treated with CBD, reaching normal values by 72 h after HI. There were no differences between HC1 and HC3.

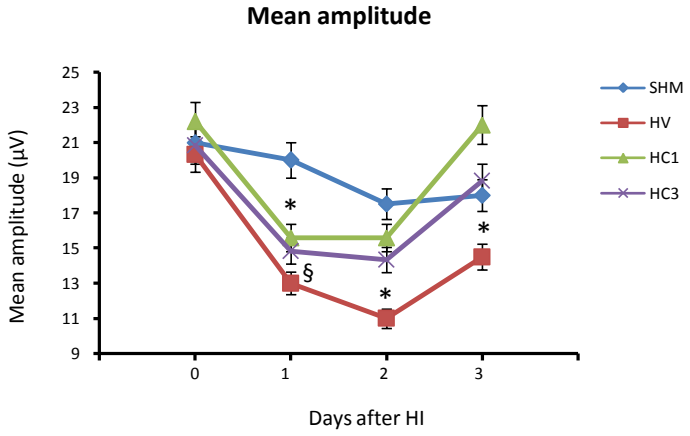

Figure 12. CBD-induced recovery of brain activity after HI. Changes of mean amplitude of aEEG trace recorded in piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD single dose (HC1) or in 3 doses (HC3). Results are expressed as means ± SEM of 6-10 animals. (*) $p<0.05$ vs. SHM. (§) $p<0.05$ vs HC.

CBD prevented seizures

Electrographic seizures were observed in on third of HV piglets 24 h after HI. This was worsening in the following days so that at the third day more than a half of HV piglets showed electrographic seizures at the aEEG record (Table 8). Only one piglet treated with CBD showed seizures in the aEEG 24 h after HI. CBD showed a robust antiepileptic effect afterwards, so that no piglet treated with CBD showed electrographic seizures 72 h after HI.

Table 8. Electrographic seizures. (*) Pearson's X^2 $p<0.05$

Group	24 h post HI	72 h post HI
SHM	0/4	0/4
HV	3/9	5/9*
HC	1/10	0/10

CBD protected neurons

HI insult led to a decrease of the number of neurones and an increase of the proportion of death neurones, as witnessed by Nissl staining in brain parietoccipital cortex 72 hours after insult (Fig. 13), although this increase was blunted by CBD administration (Fig. 13).There were no differences between HC1 and HC3.

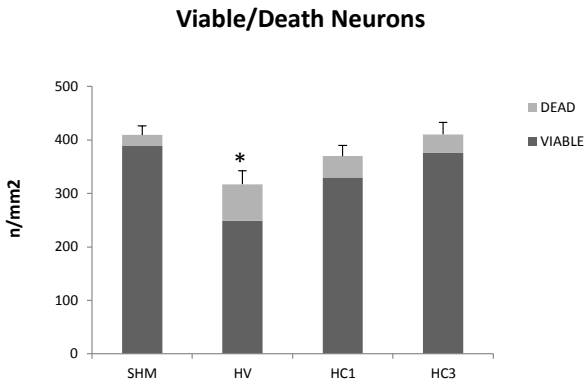

Figure 13. CBD-induced prevention of neuronal death after HI. Changes of neuronal density and proportion of death neurons in cortex as observed in Nissl stained brain sections, obtained from piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD single dose (HC1) or in 3 doses (HC3). Results are expressed as means ± SEM of 6-10 animals. (*) $p<0.05$ vs. SHM.

CBD increased myelin in brain

HI led to a decrease of the myelin density in the External Capsule, as observed after MBP immunohistochemistry of brain slices 72 h after HI (Fig. 14). In this case, CBD administered single dose did not modify such an effect of HI. CBD administered once a day for 3 days, however, did increase MBP density to levels similar to SHM.

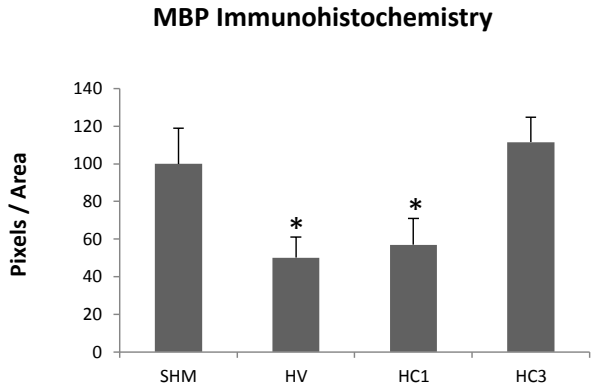

Figure 14. CBD-induced prevention of hypomyelinization after HI. Changes in Myelin Basic Protein density in brain sections obtained from piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV)or CBD single dose (HC1) or in 3 doses (HC3). Results are expressed as means ± SEM of 6-10 animals. (*) p<0.05 vs. SHM.

CBD improved H^+-MRS prognostic markers

Lac/NAA ratio increased in cortex after the HI insult (Fig. 15). This change was not observed in HI piglets treated with CBD (Fig. 16), with no differences between HC1 and HC3.

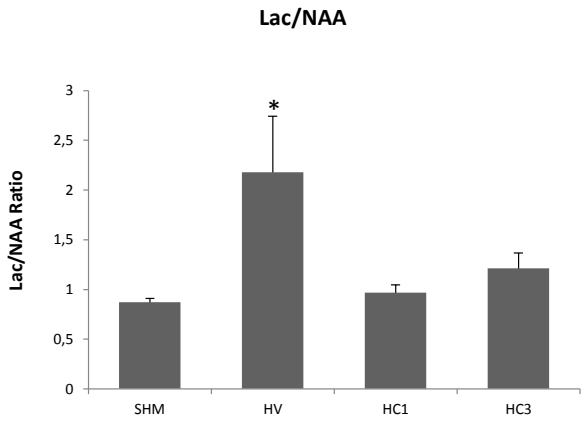

Figure 15. CBD-induced improvement of H+-MRS biomarkers after HI. Bars represent the results, expressed as means ± SEM, of the metabolite ratio obtained from studies performed in brain samples from piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD single dose (HC1) or in 3 doses (HC3). Lac: lactate; NAA: N-acetylaspartate. (*) p<0.05 vs. SHM.

CBD improved neurobehavioral performance

HI led to neurobehavioral impairment as reflected by the decrease of neurobehavioral scoring by near 30% 24 h after HI (Fig. 16). Later on there was a small and non-significant improvement so that at 72 h after HI NBS was still 20% lower than in SHM. NBS impairment was due mainly to behaviour as well as to muscle tone impairment (Table 9).

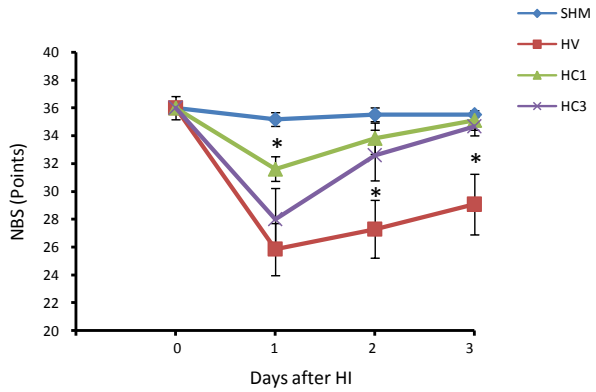

Figure 16. CBD prevented HI-induced neurobehavioral impairment. Neurobehavioral score (NBS) carried out on from piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD single dose (HC1) or in 3 doses (HC3). (*) p<0.05 vs. SHM.

By contrast, HI piglets treated with CBD performed better in the NBS, in particular from 48 h after HI when CBD treated piglets show similar NBS than SHM. CBD treatment improved NBS in all items (Table 9). There were no differences between HC1 and HC3.

Table 9. Neurobehavioral score 72 h after HI. Results as mean (SEM). (*): p<0.05 vs. SHM

	SHM	HV	HC1	HC3
Alertness	4(0)	3.8(0.1)	4 (0)	4(0)
Behavior	3.9(0.1)	2.6(0.3)*	3.7(0.2)	3.9(0.2)
Stepping	3(0)	2.5(0.2)*	3(0.2)	2.8(0.1)
Righting	2(0)	1.7(0.1)	2(0)	2(0)
MuscleTone	4(0)	2.8(0.3)*	4(0.1)	3.7(0.2)
Standing	4(0)	3.1(0.3)*	3.8(0.1)	3.7(0.2)
Walking	4(0)	3.1 (0.4)*	3.9(0.2)	3.8 (0.2)

CBD improved feeding behaviour

Looking particularly at feeding behaviour, HI impaired feeding both qualitatively (Fig. 17A) and quantitatively (Fig. 117B). Thus, HI piglets treated with vehicle scored worse in feeding items of the NBS throughout the experimental period (Fig. 17A). In addition, HV piglets ate 22% less milk by the end of the experimental period than SHM (Fig. 17B). By contrast, HI piglets treated with CBD show normal feeding behaviour from 48 h after HI (Fig. 17A), restoring the volume of ate milk 72 h after HI (Fig.17B).There were no differences between HC1 and HC3.

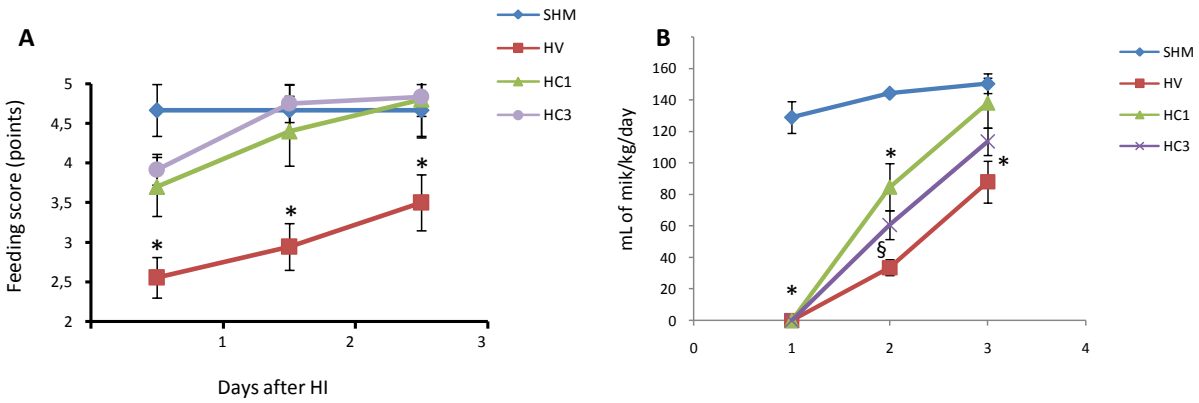

Figure 17. A) CBD prevented HI-induced impairment of feeding behaviour. Scoring of feeding behaviour in the NBS carried out on from piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD single dose (HC1) or in 3 doses (HC3). (*) $p<0.05$ vs. SHM. **B)** CBD prevented HI-induced impairment of feeding. Volume of milk ate by piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD single dose (HC1) or in 3 doses (HC3). (*) $p<0.05$ vs. SHM; (§) $p<0.05$ vs HC..

CBD had an anxiolytic effect

The HI insult led to an increase of anxiety in piglets, as reflected by the 50% increase from 48 to 72 h after HI of the time needed to become calm during restrain for aEEG recording (Fig. 18). By contrast, HI piglets treated with CBD showed no increase of anxiety

during the experimental period; with results similar to those observed in SHM (Fig. 20).There were no differences between HC1 and HC3.

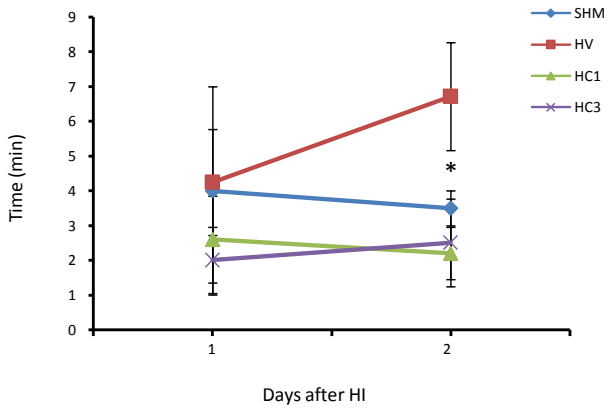

Figure 18. CBD prevented the HI-induced increase of anxiety. Changes in anxiety, quantified as the time needed to become calm during restrain for aEEG recording in piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD single dose (HC1) or in 3 doses (HC3). (*) p<0.05 vs. SHM.

CBD improved playful activity: SHM piglets show a significant playful activity, spending about 70% of time on it (Fig. 19). 77% playfulness time was devoted to interact with the researchers and 33% to explore objects. HI led to a dramatic decrease of playfulness activity, which was roughly a 40% shorter than in SHM; in addition, the time spent on social playfulness was proportionally shorter (Fig. 19). By contrast, HI piglets receiving CBD spent a normal time on playful activities, with the proportion devoted to social interaction being similar to SHM (Fig. 19). There were no differences between HC1 and HC3.

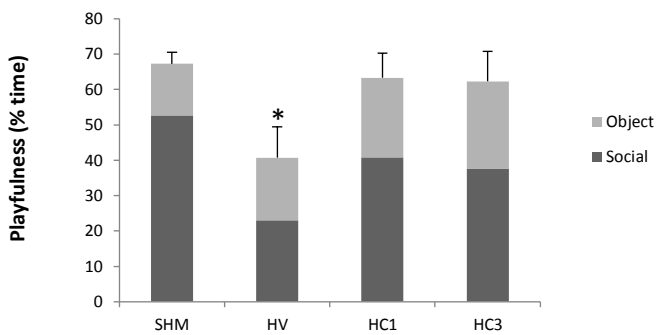

Figure 19. CBD prevented the HI-induced decrease of playfulness. Playful activity related to objects or researchers, referred to a 10 min-long videorecording of piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD single dose (HC1) or in 3 doses (HC3). (*) p<0.05 vs. SHM.

1.2. MICE MODEL

CBD reduced the volume of damage

HI insult led to the loss of 13.1 ± 1.3 % ipsilateral hemisphere volume, as determined by MRI 7 days after the HI insult (Fig. 20). Administration of CBD 15 min (0.25 h) after the HI reduced the loss of brain volume by 60% (Fig. 20).

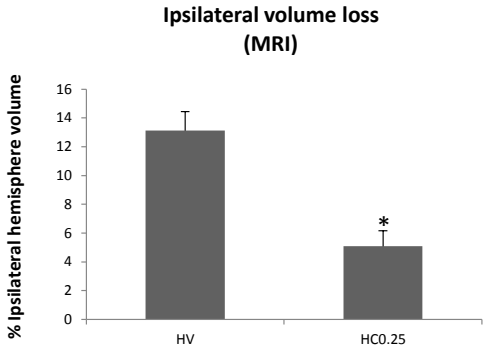

Figure 20. CBD reduced the HI-induced loss of brain volume. Ipsilateral hemisphere volume loss as determined by MRI in brains from C57BL6 mice seven days after HI and treatment with vehicle (HV) or CBD (HC) administered 15 min (0.25 h) after HI. Results are expressed as means ± SEM of 10-20 animals. (*) $p<0.05$ vs. HV.

CBD prevented brain tissue damage

HI led to moderate brain tissue damage in the ipsilateral hemisphere (Fig. 21), as determined using a neuropathological score after studying Nissl stained brain slices. Administration of CBD 15 min after the HI insult prevented such a damage to occur (Fig. 21).

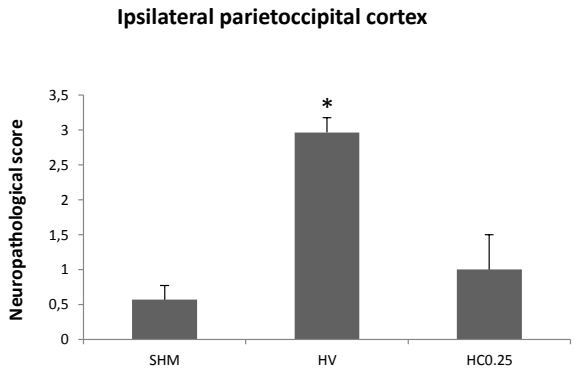

Figure 21. CBD prevented brain tissue damage. Brain tissue damage assessed using a neuropathological score in Nissl stained slices of brains obtained from C57BL6 mice seven days after HI and treatment with vehicle (HV) or CBD (HC) administered 15 min (0.25 h) after HI. Results are expressed as means ± SEM of 10-20 animals. (*) $p<0.05$ vs. HV.

CBD prevented apoptosis

HI increased apoptosis, as assessed by TUNEL staining of the perilesional tissue in the ipsilateral hemisphere (Fig. 22). This effect was not observed when CBD was administered 15 min after the HI insult (Fig. 22).

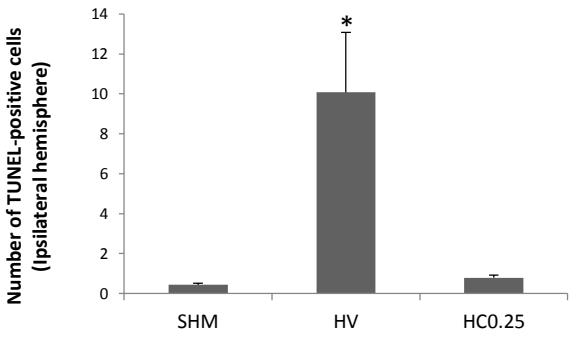

Figure 22. CBD prevented apoptosis. Apoptosis assessed using TUNEL staining in slices of brains obtained from C57BL6 mice seven days after HI and treatment with vehicle (HV) or CBD (HC) administered 15 min (0.25 h) after HI. Results are expressed as means ± SEM of 10-20 animals. (*) $p<0.05$ vs. HV.

CBD protected astrocytes

HI insult led to a decrease of the number of astrocytesas assessed by GFAP immunohistochemistry in the perilesional tissue of the ipsilateral hemisphere (Fig. 23). This effect was not observed when CBD was administered 15 min after the HI insult (Fig. 23).

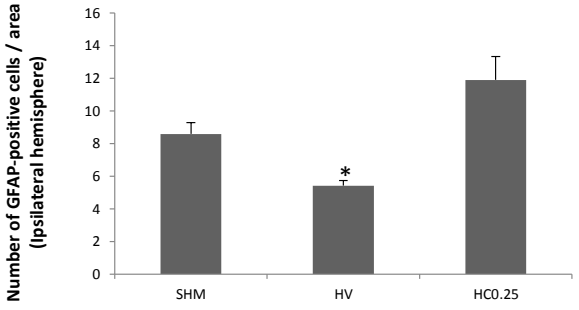

Figure 23. CBD prevented HI-induced reduction of astrocyte number.Astrocyte density assessed using GFAP immunohistochemistry in slices of brains obtained from C57BL6 mice seven days after HI and treatment with vehicle (HV) or CBD (HC) administered 15 min (0.25 h) after HI. Results are expressed as means ± SEM of 10-20 animals. (*) $p<0.05$ vs. SHM.

2. MECHANISMS OF CBD NEUROPROTECTION (PIGLET MODEL)

2.1. EXCITOTOXICITY

CBD reduced glutamate release

HI led to an increase of glutamate release in brain, as showed by the increase of Glu/NAA ratio after H^+-MRS of frozen piglet brain samples (Fig. 24). Administration of CBD blunted Glu/NAA increase (Fig. 24).

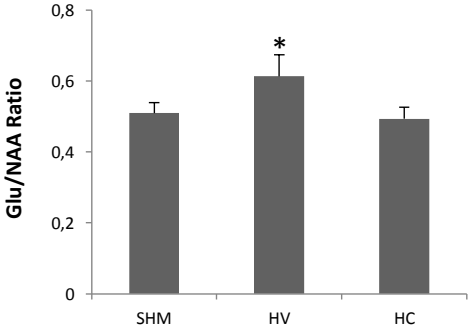

Figure 24. CBD reduced HI-induced excitotoxicity. Glutamate release quantified as Glu/NAA ratio by H^+-MRS of brain samples from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD (HC). Glu: glutamate; NAA: N-acetylaspartate. (*) $p<0.05$ vs. SHM.

2.2. OXIDATIVE STRESS

CBD prevented reduced glutathione consumption

The HI insult led to increased oxidative stress, as reflected by the consumption of reduced glutathione (GSH), which was quantified by the GSH/Cr ratio obtained by H^+-MRS of frozen piglet brain samples (Fig. 25). CBD exerted an antioxidant effect, preventing the HI-induced decrease of GSH/Cr (Fig. 25).

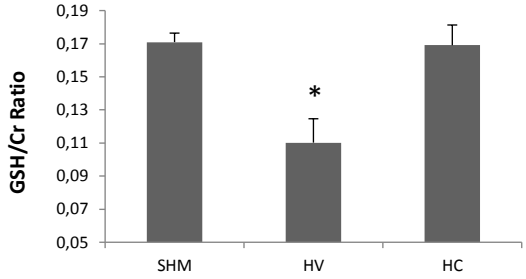

Figure 25. CBD reduced HI-induced oxidative stress. Changes in GSH/Cr ratio obtained by H^+-MRS of brain samples from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD (HC). GSH: reduced gluthation. Cr: creatine. (*) $p<0.05$ vs. SHM.

CBD reduced protein carbonylation

OxyBlot studies revealed that HI increased the protein carbonylation in the brain (Fig. 26). CBD administration after HI had a significant antioxidant effect, blunting the increase in protein carbonylation (Fig. 26).

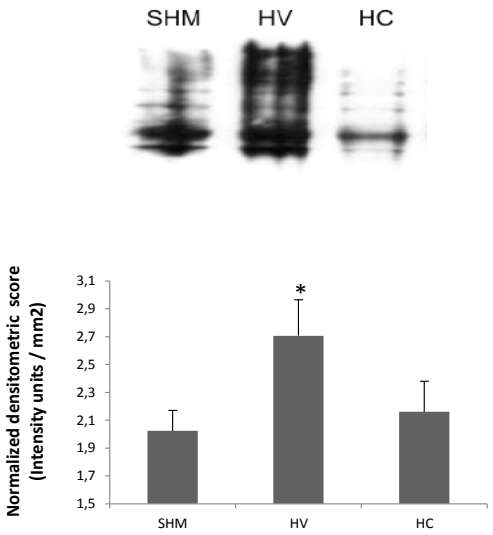

Figure 26. CBD-induced reduction of brain protein carbonylation. *Top:* representative Western blot probed with antibody to derived protein carbonyl side groups (OxyBlot), carried out in brain samples from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD (HC). *Bottom:* densitometric analysis of relative protein carbonyl contents. The levels of protein oxidation were normalized by total protein loading (Red Ponceau staining) and expressed by the OxyBlot/Red Ponceau ratio. Bars represent the mean ± SEM of 6-8 experiments. (*) p<0.05 vs. SHM.

2.3. INFLAMMATION

CBD reduced cytokine release

The HI insult led to the increase of interleukin (IL)-1 concentration in brain tissue (Fig. 27). Administration of CBD after the HI insult had an anti-inflammatory effect, blunting the HI-induced increase of IL-1 concentration in brain (Fig. 27).

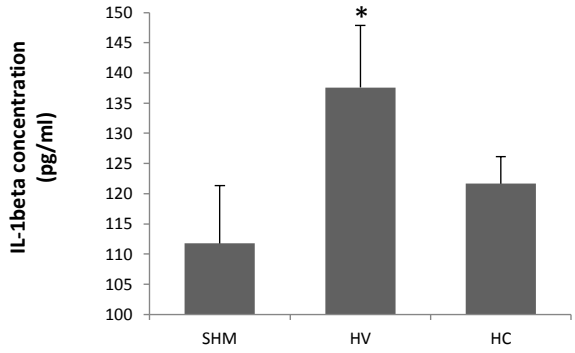

Figure 27. CBD-induced reduction of IL-1 production after HI**.** Brain concentration of IL-1 quantified by microarrays in samples from1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD (HC). Bars represent the mean ± SEM of 6-8 experiments. (*) p<0.05 vs. SHM.

2.4. INVOLVEMENT OF CB_2 AND $5HT_{1A}$ RECEPTORS

2.4.1. 6HFU

Effect of CB_2 or $5HT_{1A}$ antagonists on CBD-induced recovery of brain activity

The effects of CBD on aEEG amplitude (Fig.28A) or background (Fig. 28B) were abolished by co-administration with either AM630 or WAY100635 The effect of HI on aEEG was not different in animals receiving AM630 or WAY100635 alone than in those receiving vehicle (31.2 ± 9% and 28.1 ± 6% of basal activity at 6 h for AM630 and WAY100635, respectively, p>0.05 vs. HV).

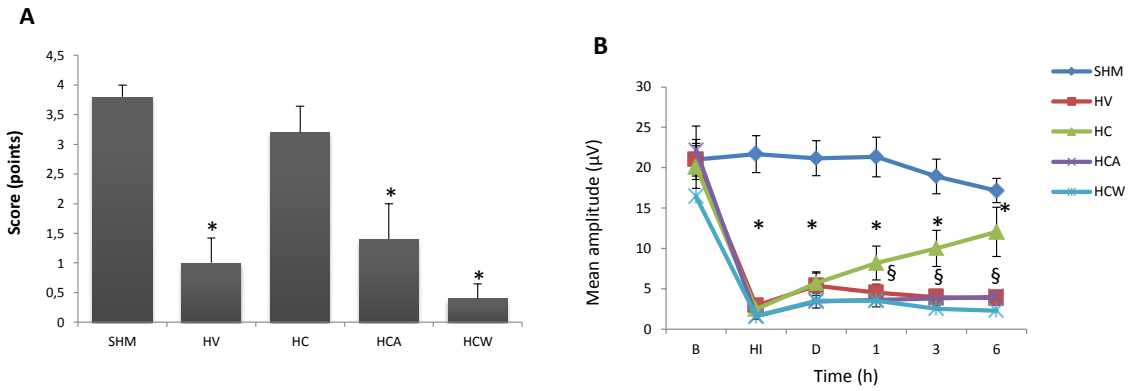

Figure 28. A) Quantitative CBD-induced recovery of brain activity after HI was reversed by 5-HT_{1A} or CB_2 receptor antagonists. Changes of mean amplitude of aEEG trace recorded in 1-to-2 day-old piglets after sham operation (SHM) or after hypoxic-ischemic (HI) insult and treatment with vehicle (HV), CBD (HC), CBD + AM630 (HCA) or CBD + WAY100630 (HCW). *Top*: throughout the experiment. Results are expressed as means ± SEM of 6-10 animals. (*) p<0.05 vs. SHM. (§) p<0.05 vs HC. **B)** Qualitative CBD-induced recovery of brain activity after HI was reversed by 5-HT_{1A} or CB_2 receptor antagonists. Qualitative assessment of aEEG background activity by a neurological activity scorein 1-to-2 day-old piglets after sham operation (SHM) or after hypoxic-ischemic (HI) insult and treatment with vehicle (HV), CBD (HC), CBD + AM630 (HCA) or CBD + WAY100630 (HCW). Results are expressed as means ± SEM of 6-10 animals. (*) p<0.05 vs. SHM. (§) p<0.05 vs HC.

Effect of CB_2 or $5HT_{1A}$ antagonists on CBD-induced neuronal protection

HI insult led to a dramatic increase in the number of necrotic neurons in the cortex, as witnessed by Nissl staining of this tissue 6 hours after insult (Fig. 29), although this increase was blunted by CBD administration (Fig. 29). The beneficial effect of CBD disappeared when it was administered along with either CB_2 or $5HT_{1A}$ antagonists (Fig. 29). The effect of HI on neuronal death was not different in animals receiving AM630 or WAY100635 alone than in those receiving vehicle (necrotic neurons: 16.2 ± 2.4% and 18.2 ± 3.2% for AM630 and WAY100635, respectively, $p>0.05$ vs. HV).

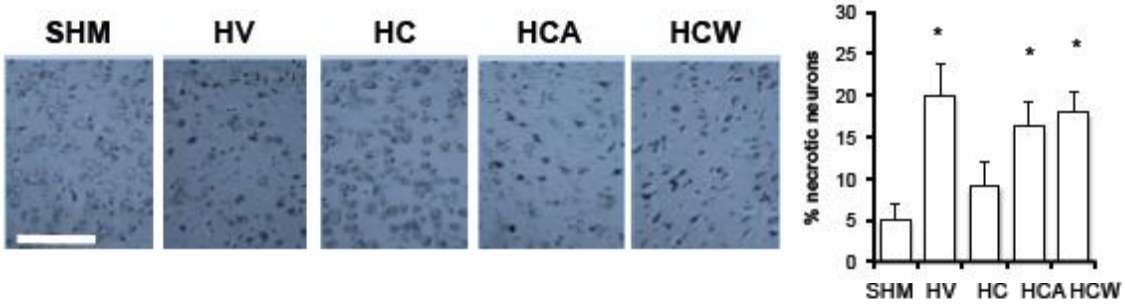

Figure 29. CBD-induced prevention of neuronal death after HI was abolished by 5-HT_{1A} or CB_2 receptor antagonists. Representative light microphotographs of Nissl stained brain sections, obtained after from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV), CBD (HC), CBD + AM630 (HCA) or CBD + WAY100630 (HCW). Original magnification x200, bar: 100 µm. Results are expressed as means ± SEM of 6-10 animals. (*) $p<0.05$ vs. SHM.

Effect of CB_2 or $5HT_{1A}$ antagonists on CBD-induced astrocyte protection

In the HV group, no reduction in the number of GFAP+ cells was evident in the cortex after HI insult (Fig. 30). By contrast, CBD administration led to a significant increase in the number of GFAP+ cells in the HC group, an effect that was prevented by co-administration of AM630 or WAY100635.

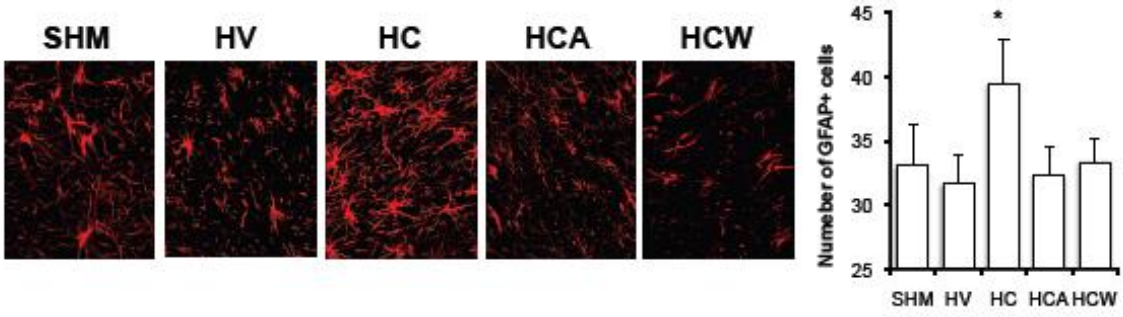

Figure 30. CBD-induced prevention of astroglial death after HI was abolished by 5-HT_{1A} or CB_2 receptor antagonists. Representative light microphotographs of GFAP stained brain sections, obtained after from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV), CBD (HC), CBD + AM630 (HCA) or CBD + WAY100630 (HCW). Original magnification x200. Results are expressed as means ± SEM of 6-10 animals. (*) $p<0.05$ vs. SHM.

Changes by CB_2 or $5HT_{1A}$ antagonists of CBD effects on H^+-MRS biomarkers

Although the Lac/NAA and NAA/Cho ratios increased and decreased, respectively, after HI insult these changes were not observed in the cortex of HI piglets that received CBD. The effects of CBD were reversed when CBD was co-administered with AM630 or WAY100635 (Fig. 31). The effect of HI on H+-MRS biomarkers was not different in animals receiving AM630 or WAY100635 alone than in those receiving vehicle (Lac/NAA: 6.5 ± 1.5 and 6.7 ± 0.9; NAA/Cho: 4.8 ± 0.6 and 4.6 ± 0.6; for AM630 and WAY100635, respectively, $p>0.05$ vs. HV).

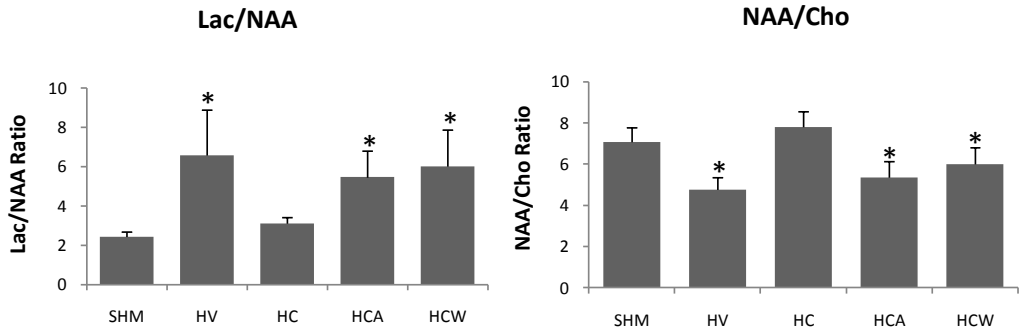

Figure 31. CBD-induced improvement of H^+-MRS biomarkers after HI was reversed by 5-HT_{1A} or CB_2 receptor antagonists. Bars represent the results, expressed as means ± SEM, of different metabolite ratios obtained from studies performed in brain samples from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV), CBD (HC), CBD + AM630 (HCA) or CBD + WAY100630 (HCW). Cho: choline; Lac: lactate; NAA: N-acetylaspartate. (*) $p<0.05$ vs. SHM.

Effect of CB_2 or $5HT_{1A}$ antagonists on CBD modulation of excitotoxicity

The increase in the Glu/NAA ratio in the HV group indicated that HI augmented the excitotoxicity in the cortex, an effect that was not observed in HC animals. The Normal Glu/NAA ratios were restored following CBD administration, but not when it was administered along with a CB_2 or $5HT_{1A}$ antagonist (Fig. 32). The effect of HI on H^+-MRS biomarkers was not different in animals receiving AM630 or WAY100635 alone than in those receiving vehicle (Glu/NAA: 0.69 ± 0.02 and 0.64 ± 0.04; for AM630 and WAY100635, respectively, $p>0.05$ vs. HV).

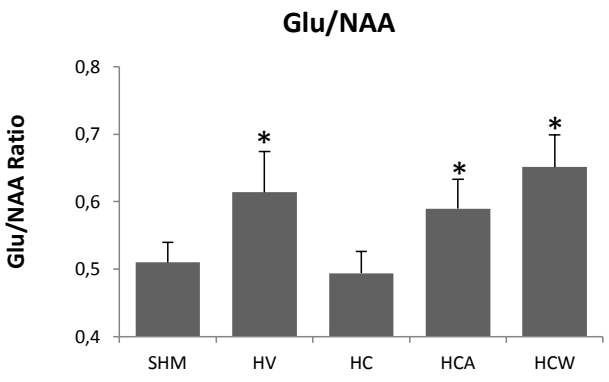

Figure 32. CBD-induced reduction of glutamate release after HI was reversed by 5-HT_{1A} or CB_2 receptor antagonists. Bars represent the results, expressed as means ± SEM, of different metabolite ratios obtained from studies performed in brain samples from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV), CBD (HC), CBD + AM630 (HCA) or CBD + WAY100630 (HCW). Glu: glutamate; NAA: N-acetylaspartate. (*) $p<0.05$ vs. SHM.

Effect of CB_2 or $5HT_{1A}$ antagonists on CBD modulation of oxidative stress

HI-induced increases in oxidative stress were analyzed by measuring the GSH/Cr ratio and the levels of protein carbonylation. HI insult diminished the brain GSH/Cr ratio determined by H^+-MRS and additionally, OxyBlot studies revealed that HI increased the protein carbonylation in the brain. CBD administration after HI had a significant antioxidant effect, blunting both the decrease in the GSH/Cr ratio (Fig. 33) and the increase in protein carbonylation.

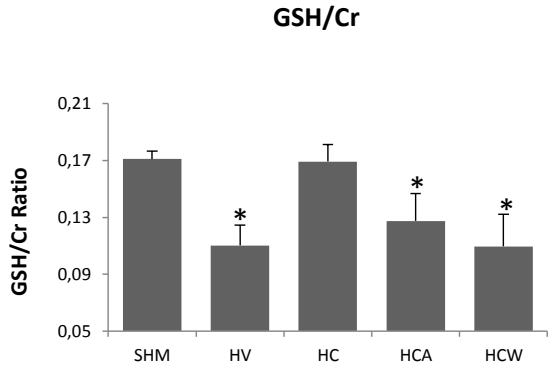

Figure 33. CBD-induced reduction of oxidative stress after HI was reversed by 5-HT_{1A} or CB_2 receptor antagonists. Bars represent the results, expressed as means ± SEM, of GSH/Cr ratio obtained from studies performed in brain samples from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV), CBD (HC), CBD + AM630 (HCA) or CBD + WAY100630 (HCW). GSH: reduced gluthation; Cr: creatine. (*) $p<0.05$ vs. SHM.

However, the antioxidant effect of CBD was lost when it was administered in combination with either AM630 or WAY100635 (Figs. 33 and 34). The effect of HI on oxidative stress was not different in animals receiving AM630 or WAY100635 alone than in those receiving vehicle (GSH/Cr: 0.13 ± 0.02 and 0.11 ± 0.04 for AM630 and WAY100635, respectively, $p>0.05$ vs. HV).

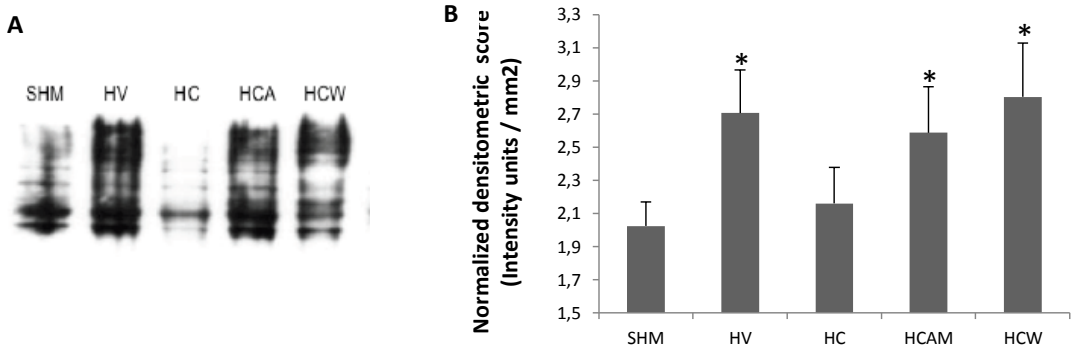

Figure 34. CBD-induced reduction of brain protein carbonylation after HI was reversed by 5-HT_{1A} or CB_2 receptor antagonists. **A)** representative Western blot probed with antibody to derived protein carbonyl side groups (OxyBlot), carried out in brain samples from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV), CBD (HC), CBD + AM630 (HCA) or CBD + WAY100630 (HCW). **B)** densitometric analysis of relative protein carbonyl contents. The levels of protein oxidation were normalized by total protein loading (Red Ponceau staining) and expressed by the OxyBlot/Red Ponceau ratio. Bars represent the mean ± SEM of 6-8 experiments. (*) p<0.05 vs. SHM.

Effect of CB_2 or $5HT_{1A}$ antagonists on CBD modulation of inflammation

HI was accompanied by a significant increase in IL-1 levels in the cortex. In accordance with its anti inflammatory effects, CBD reduced IL-1 levels in lesioned animals, although this effect was prevented when it was administered along with a CB_2 or $5HT_{1A}$ antagonist (Fig. 35). The effect of HI on inflammation was not different in animals receiving AM630 or WAY100635 alone than in those receiving vehicle (IL-1: 160 ± 25 and 140 ± 15 pg/ml for AM630 and WAY100635, respectively, p>0.05 vs. HV).

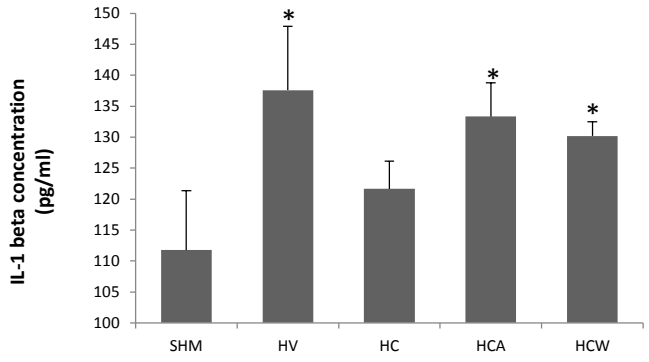

Figure 35. CBD-induced reduction of brain IL-1 production after HI was reversed by 5-HT_{1A} or CB_2 receptor antagonists. Brain concentration of IL-1 quantified by microarrays in brain samples from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV), CBD (HC), CBD + AM630 (HCA) or CBD + WAY100630 (HCW) Bars represent the mean ± SEM of 6-8 experiments. (*) p<0.05 vs. SHM.

2.4.2. 72HFU.

Since $5HT_{1A}$ receptors are involved in the behavioural and psychiatric effects of CBD (Russo *et al*, 2005; Campos *et al*, 2012), the effects of coadministeringWAY100635with CBD were studied in the 72HFU piglet model. Since in that model no significant differences were obtained between HC1 and HC3, in this case only HI piglets receiving CBD single constituted the HC group.

Effects of $5HT_{1A}$ antagonism on CBD-induced improvement of neurobehavioral performance

CBD prevention of HI-induced neurobehavioral impairment was lost when the single dose of CBD was administered together with WAY100635 (HCW1) (Fig. 36 and table 10). However, when CBD was administered for three days the beneficial effect of CBD was preserved despite WAY100635 was co-administered for the same period (HCW3) (Fig. 36 and table 10).

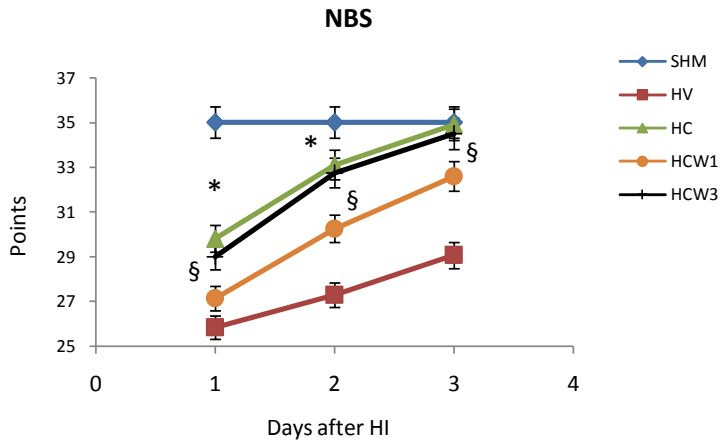

Figure 36. 5-HT_{1A} receptor antagonism reversed the prevention of HI-induced neurobehavioral by CBD single dose but not by CBD three doses. Neurobehavioral score (NBS) carried out on from piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or WAY100630 co-administered with CBD single dose (HCW1) or in 3 doses (HCW3). (*) $p<0.05$ vs. SHM.

Table 10. Neurobehavioral score 72 h after HI. Results as mean (SEM). (*): p<0.05 vs. SHM

	SHM	HV	HC	HCW1	HCW3
Alertness	4(0)	3.8(0.1)	4 (0)	4 (0)	4(0)
Behavior	3.9(0.1)	2.6(0.3)*	3.7(0.2)	3 (0.2)*	3.7(0.2)
Stepping	3(0)	2.5(0.2)*	3(0.2)	2.8(0.1)	2.8(0.1)
Righting	2(0)	1.7(0.1)	2(0)	2 (0)	2(0)
MuscleTone	4(0)	2.8(0.3)*	4(0.1)	3.6(0.1)*	3.9(0.1)
Standing	4(0)	3.1(0.3)*	3.8(0.1)	3.8(0.1)	3.9(0.1)
Walking	4(0)	3.1 (0.4)*	3.9(0.2)	3.6(0.1)*	3.8 (0.1)

Effects of 5HT$_{1A}$ antagonism on CBD-induced improvement of feeding behaviour

The beneficial effect of CBD on feeding behaviour and the volume of milk was lost when the single dose of CBD was administered together with WAY100635 (HCW1) (Figs. 37 and 38). However, when CBD was administered for three days the beneficial effect of CBD was preserved despite WAY100635 was co-administered for the same period (HCW3) (Figs. 37 and 38).

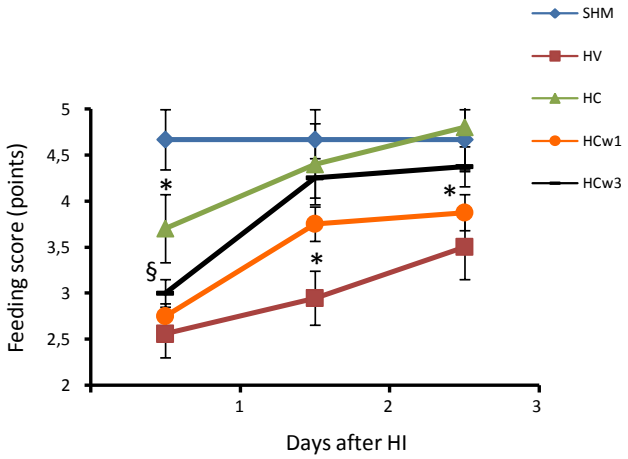

Figure 37. 5-HT$_{1A}$ receptor antagonism reversed CBD prevention of HI-induced impairment of feeding behaviour. Scoring of feeding behaviour in the NBS carried out on from piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) orWAY100630 co-administered with CBD single dose (HCW1) or in 3 doses (HCW3). (*) p<0.05 vs. SHM.

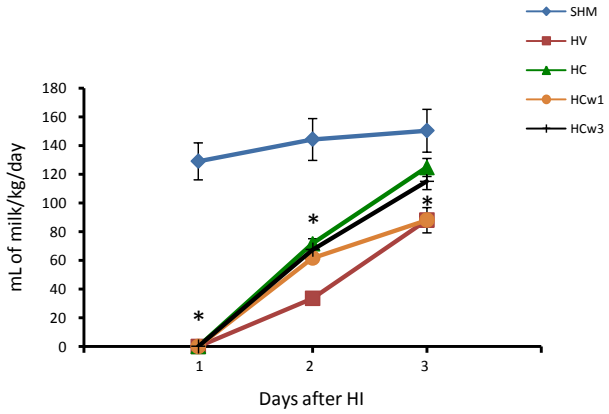

Figure 38. 5-HT_{1A} receptor antagonism reversed CBD prevention of HI-induced impairment of feeding. Volume of milk ate by piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) orWAY100630 co-administered with CBD single dose (HCW1) or in 3 doses (HCW3). (*) p<0.05 vs. SHM.

Effects of 5HT_{1A} antagonism on CBD-induced anxiolytic effect

The anxiolytic effect of CBD after HI was lost when the single dose of CBD was administered together with WAY100635 (HCW1) (Fig. 39). In this case, the anxiolytic effect of CBD after HI was lost when CBD was administered for three days too (HCW3) (Fig. 39).

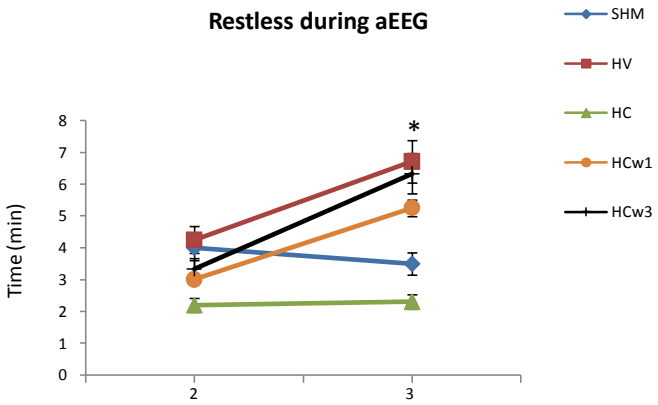

Figure 39. 5-HT_{1A} receptor antagonism reversed the anxiolytic effect of CBD. Changes in anxiety, quantified as the time needed to become calm during restrain for aEEG recording in piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) orWAY100630 co-administered with CBD single dose (HCW1) or in 3 doses (HCW3). (*) p<0.05 vs. SHM.

CBD improved playful activity

CBD prevention of HI-induced impairment of playfulness was lost when CBD was co-administered with WAY100630, no matter this was administered after CBD single dose (HCW1) or in three doses (HCW3) (Fig.40).

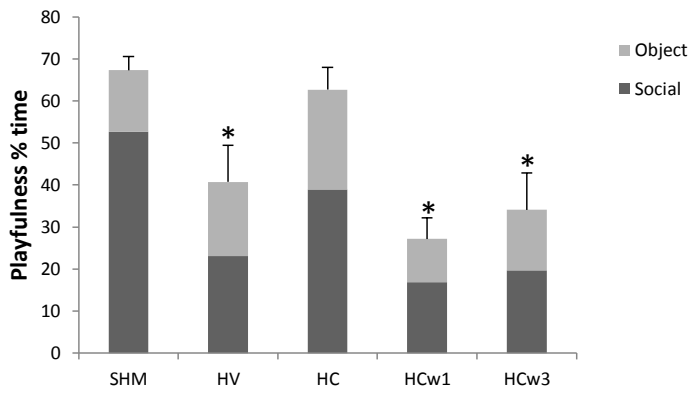

Figure 40. 5-HT_{1A} receptor antagonism reversed CBD prevention of HI-induced decrease of playfulness. Playful activity related to objects or researchers, referred to a 10 min-long video recording of piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) orWAY100630 co-administered with CBD single dose (HCW1) or in 3 doses (HCW3).(*) $p<0.05$ vs. SHM.

2.5. EFFECT OF CBD ON ENDOCANNABINOID BRAIN CONCENTRATION

As CBD was reported to reduce anadamide (AEA) uptake and/or degradation *in vitro* (Pertwee, 2004; Mechoulam et al, 2007), whether the contribution of CB_2 receptors to the effects of CBD was due to a CBD-induced increase in brain endocannabinoid levels was investigated in piglet brain 6 h after the HI insult. HI increased the levels of AEA, 2-AG, PEA and OEA in the brain, evident in brain tissue taken from HV animals (Fig. 41). However, similar levels of those endocannabinoids were detected in CBD-treated and SHM animals (Fig. 41).

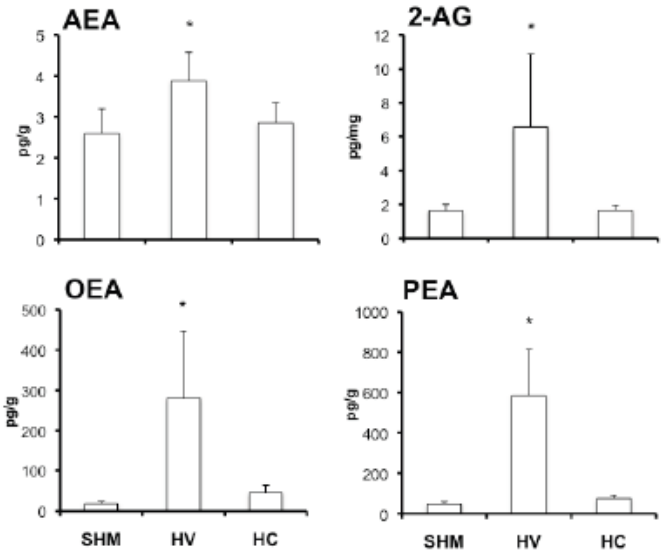

Figure 41. HI-induced increase of brain endocannabinoid levels was prevented by CBD. Brain concentration of endocannabinoidleves was quantified by liquid chromatography–mass spectrometry in samples from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia (HI) and treatment with vehicle (HV) or CBD (HC). See *2.7. Determination of brain endocannabinoid levels* for details. Bars represent the mean ± SEM of 6-8 experiments. AEA: arachinodoylethanolamide; 2-AG: 2-arachidonoylglycerol; OEA: oleylethanolamide; PEA: palmitoylethanolamide. (*) p<0.05 vs. SHM.

3. TEMPORARY THERAPEUTIC WINDOW OF CBD

Time to loss the CBD-induced reduction of the volume of damage

Administration of CBD 15 min (0.25 h) after the HI reduced the loss of brain volume by 60% (Fig. 42). Delaying the administration of CBD up to 12 h after HI did not significantly modify such an effect. When CBD administration was delayed 24 h after HI, however, the protective effect of CBD was lost (Fig. 42)

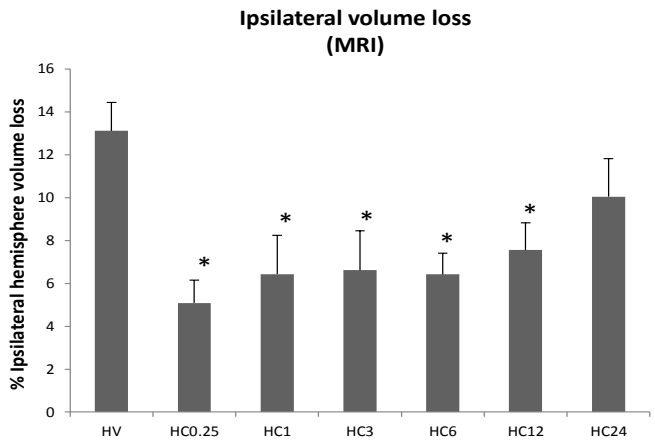

Figure 42. CBD reduction of the HI-induced loss of brain volume was lost when CBD administration was delayed for 24 h. Ipsilateral hemisphere volume loss as determined by MRI in brains from C57BL6 mice seven days after HI and treatment with vehicle (HV) or CBD (HC) administered form 15 min (HC0.25) to 24 h (HC24)after HI. Results are expressed as means ± SEM of 10-20 animals. (*) p<0.05 vs. HV.

Time to loss the CBD-induced prevention of brain tissue damage

Administration of CBD 15 min after the HI insult prevented HI-induced brain damage to occur (Fig. 43).Delaying the administration of CBD up to 1 h after HI did not significantly modify such an effect. When CBD administration was delayed from 3 to 12 h after HI, the protective effect of CBD was reduced, although the NPS was still lower than in HV (Fig. 43). When CBD administration was delayed 24 h after HI, however, the protective effect of CBD was totally lost (Fig. 43).

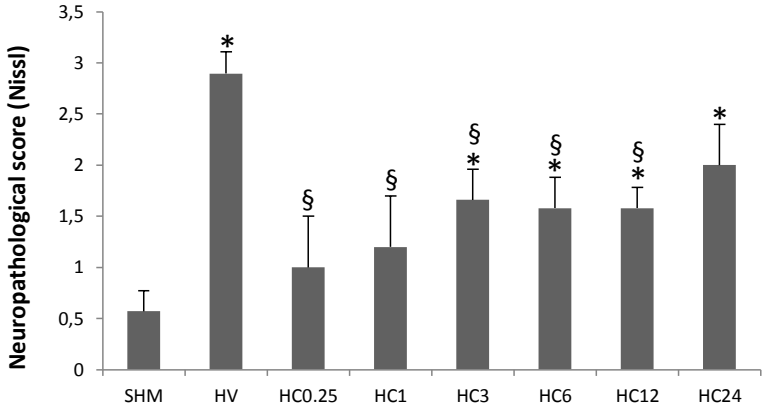

Figure 43. CBD prevention of brain tissue damagewas lost when CBD administration was delayed for 24 h. Brain tissue damage assessed using a neuropathological score in Nissl stained slices of brains obtained from C57BL6 mice seven days after HI and treatment with vehicle (HV) or CBD (HC) administered from 15 min (HC0.25) to 24 h (HC24) after HI. Results are expressed as means ± SEM of 10-20 animals. (*) $p<0.05$ vs. SHM.(§): $p<0.05$ vs. HV.

Time to loss the CBD-induced prevention of apoptosis

Administration of CBD 15 min after the insult prevented HI-induced apoptosis (Fig. 44). When CBD administration was delayed from 1 to 12 h after HI, the protective effect of CBD was reduced, although the number of TUNEL+ cells was still lower than in HV (Fig. 44). When CBD administration was delayed 24 h after HI, however, the protective effect of CBD was lost (Fig. 44).

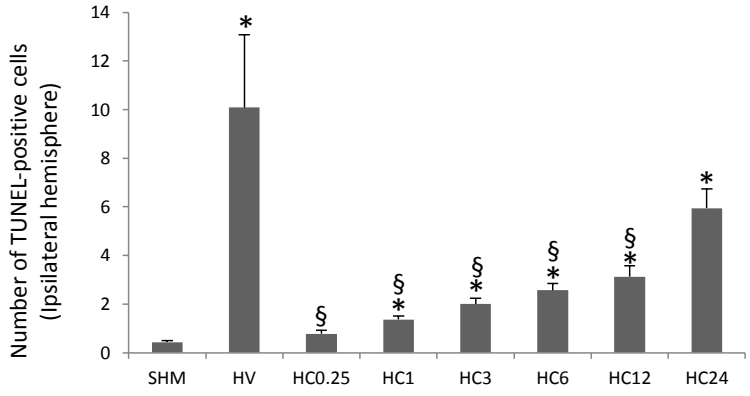

Figure 44. CBD prevention of apoptosis was lost when CBD administration was delayed for 24 h.Apoptosis assessed using TUNEL staining in slices of brains obtained from C57BL6 mice seven days after HI and treatment with vehicle (HV) or CBD (HC) administered from 15 min (HC0.25) to 24 h (HC24) after HI. Results are expressed as means ± SEM of 10-20 animals. (*) p<0.05 vs. SHM. (§): p<0.05 vs. HV.

Time to loss the CBD-induced protection of astrocytes

Administration of CBD 15 min after the insult prevented the HI-induced reduction of the density of GFAP+ cells. This protective effect on astrocytes was not modified when CBD administration was delayed from 1 to 12 h after HI (Fig. 45). When CBD administration was delayed 24 h after HI, however, the protective effect of CBD was lost (Fig. 45).

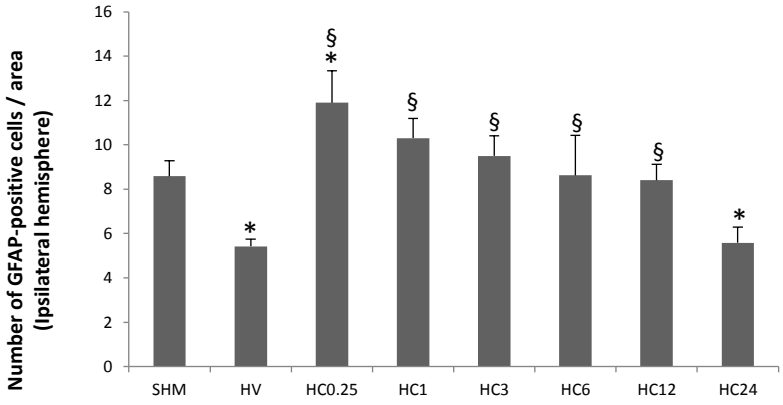

Figure 45. CBD prevention of HI-induced reduction of astrocyte number was lost when CBD administration was delayed for 24 h. Astrocyte density assessed using GFAP immunohistochemistry in slices of brains obtained from C57BL6 mice seven days after HI and treatment with vehicle (HV) or CBD (HC) administered from 15 min (HC0.25) to 24 h (HC24) after HI. Results are expressed as means ± SEM of 10-20 animals. (*) p<0.05 vs. SHM. (§): p<0.05 vs. HV.

4. PHARMACOKINETIC STUDIES

PK curve and parameters in blood

The study of CBD concentration in blood at different times after administering 1 mg/Kg i.v. to 6HFU piglets indicated that CBD concentration followed an exponential curve (Fig. 46) after the formula: $y = 64.951x^{-0.841}$

Where "x" is time after injection (in houyrs) and "y" CBD concentration.

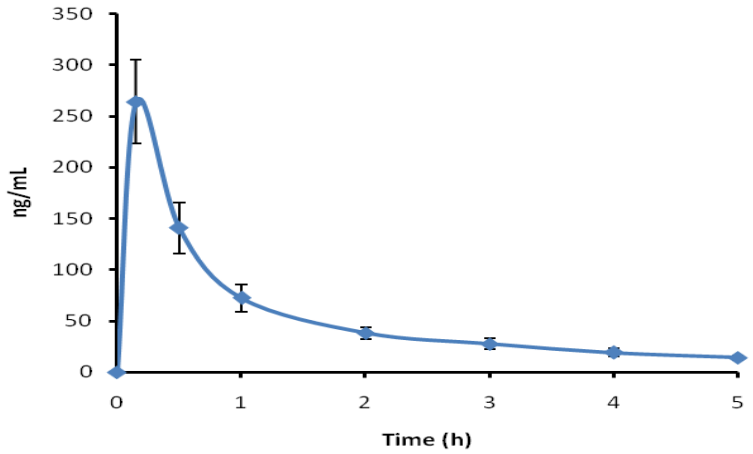

Figure 46. Time course of CBD concentration in blood. CBD concentration was measured in blood from 1-to-2 day-old piglets after receiving CBD 1 mg/Kg i.v.

The calculated PK parameters were:

- Tmax: 0.15 h

- Cmax: 263.8 ± 101.9 ng/mL

- Plasma half life ($T_{1/2}$): 1.98 ± 0.67 h

- Area under the curve (AUC):305.21 ± 107 ng/h/mL

CBD concentration in brain

Six hours after the administration of CBD (1 mg/kg) in the used formulation, the CBD concentration in brain tissue was 58 ± 14 ng/g.

DISCUSSION

DISCUSSION

1. NEUROPROTECTIVE EFFECT OF CBD

Post-insult administration of CBD to two different species of newborn animals successfully prevented HI-induced brain damage. These results agree with previous studies demonstrating the neuroprotective effect of CBD in different models of NHIE.

In *in vitro* studies using newborn mice forebrain slices exposed to OGD (Castillo *et al.*, 2010) CBD 100 µM reduces cell death, as shown by the CBD-induced prevention of post-OGD increase of LDH efflux. In this model, CBD also blunts apoptotic pathways, reducing the production of caspase-9 in brain.

In *in vivo* studies carried out on newborn piglets the administration of CBD 0.1 (Alvarez *et al.*, 2008; Lafuente *et al.*, 2011) i.v. after an HI insult (hypoxia and carotid occlusion) had neuroprotective effects as shown by aEEG studies, reporting a significant recovery of cerebral activity, the reduction of post-insult brain oedema (as reflected by the reduction of impedance increase in EEG) and the reduction of electrical seizure incidence. In addition, near-infrared spectroscopy studies report a significant improvement of brain metabolic activity, as reflected by the reduction of fractional tissue oxygen extraction fall, as well as the reduction of cerebral hemodynamic impairment, as reflected by the normalized tissue hemoglobin index (Alvarez *et al.*, 2008; Lafuente *et al.*, 2011). In those experiments, CBD 0.1 mg/Kg reduces HI-induced brain damage as reflected by the increase of the number viable cell loss and the decrease of that of degenerating neurons (fluorojade B stained). In agreement, CBD blunts the HI-induced increase of Neuronal Specific Enolase (reflecting neuronal damage) levels, as shown by ELISA studies on cerebrospinal fluid (CSF) 3 h after HI (Lafuente *et al.*, 2011). In addition to its neuronal protective effect, CBD administration

reduces astrocyte damage, blunting the HI-induced reduction of astrocyte number and size as observed by GFAP immunohistochemistry 6 h post-HI as well as the increase of S-100β protein (reflecting astrocyte damage) levels as shown by ELISA studies on cerebrospinal fluid (CSF) 3 h after HI (Lafuente *et al.*, 2011).

In *in vivo* studies on newborn rats undergoing a HI insult at P7 (Rice-Vannucci model) and then receiving CBD 1 mg/Kg or vehicle (Pazos *et al.*, 2012), CBD 1 mg/Kg s.c. reduces by 17% the volume of brain infarct as assessed by magnetic resonance imaging at P37. This effect correlates with the reduction of the extent of the histological damage: the mean neuropathological score was 1 point lower in CBD than in vehicle-treated animals. In those experiments, proton magnetic resonance spectroscopy (H^+-MRS) studies support the neuroprotective effect as reflected by the CBD-induced prevention of Lac/NAA increase (Pazos *et al.*, 2012). Those neuroprotective effects are associated with a neurofunctional restore: CBD treated animals score similarly to control animals in different neurobehavioral tests assessing motor (Rotarod, assessing motor coordination, and Cylinder Rear Test, assessing unilateral motor deficits) and cognitive (Novel Object Recognition, assessing work memory) whereas vehicle-treated animals show permanent deficits as scored in all tests (Pazos *et al.*, 2012). Altogether these data demonstrate a remarkable protective effect from a functional point of view, exceeding that expected by the mild histological protective effect.

The current work aimed then to test the later dose tested, CBD 1 mg/Kg, in two additional different species. It is mandatory to demonstrate the efficacy of a given new therapeutic approach in different species in order to aiming a future clinical trial in humans (Hamrick & Ferriero, 2003). For instance minocycline, which has demonstrated neuroprotective effects in HI newborn rats (Gonzalez & Ferriero, 2008) do worse HI brain

damage in newborn mice (Tsuji *et al.*, 2004). Thus, we aimed to check out if this therapy at that dose was also effective in piglets and mice.

1.1. CBD NEUROPROTECTION IN PIGLETS

1.1.1. 6HFU

Models in piglets have a strong translational nature. Pigs can fill the gap between preclinical models in rodents and clinical trials, because pig physiology and pathophysiology is closer to humans than rodents; in particular, pig brain is gyrencephalic as in humans instead of lisencephalic like in rodents (Ioroi *et al.*, 2002; Gileing *et al.*, 2011). The 6HFU model allow a close surveillance of cerebral and extracerebral changes in the first hours after HI, during the so-called "latent phase", which spreads up to 6-15 h after the HI insult when most of the brain damage mechanisms start (Johnston *et al.*, 2011; Drury *et al.*, 2014; Juul & Ferriero, 2014). In fact, substantial histological brain damage can be observed in the piglet brain 6 h after an HI insult (Alvarez *et al.*, 2008; Pazos *et al.*, 2013).

CBD was protective for HI piglets as soon as 6 h after HI

Our findings indicate that CBD administration after a hypoxic ischemic insult provides neuroprotection in newborn pigs.

As early as 6 h after HI insult, the background pattern and amplitude of the aEEG are good predictors of outcome after HI brain damage in newborn infants (Tao & Mathur, 2010). The more intense disruption of the aEEG parameters seen here as opposed to previous reports (Tichauer *et al.*, 2009) suggests that the HI insult applied in our study was very

severe. Nonetheless, administration of CBD 1 mg/Kg enhanced the mean amplitude and restored the neural activity. The effect was stronger than that reported for CBD 0.1 mg/Kg: in the experiments by Alvarez *et al.* (2008), aEEG amplitude 6 h after 20 min-long HI and treatment with CBD 0.1 mg/kg i.v. was about a half of that of SHM. In our work, despite a more severe HI insult, lasting for 30 min, aEEG amplitude 6 h after HI and treatment with CBD 1 mg/Kg i.v. was about two thirds of that of SHM.

The Lac/NAA ratio calculated by H^+-MRS is thought to be the most predictive early biomarker of a poor outcome to infant HI and a surrogate endpoint used to evaluate neuroprotective strategies (Thayyil *et al.*, 2010). CBD prevented the increase in Lac/NAA induced by HI, as previously reported for other neuroprotective treatments such as xenon and/or hypothermia in a similar experimental model (Faulkner *et al.*, 2011). CBD also prevented the HI-induced decrease in the NAA/Cho ratio, which is inversely correlated with the severity of neuronal damage in HI piglets (Li *et al.*, 2010).

The results from the aEEG and H^+-MRS analyses correlate with those of histological studies (Faulkner *et al.*, 2011; Tichauer *et al.*, 2009). Thus, the HI insult resulted in a 4-fold increase in the proportion of necrotic neurons in the cortex. CBD treatment reduced the density of necrotic neurons to values similar to those of SHM animals and notably, the beneficial effects of CBD were not limited to neurons but they were also observed in astrocytes (reflected by the increase in GFAP+ cells in the cortex of HC animals 6 h after HI insult). While late astrogliosis correlates with the extent of brain damage (as astrocytes are involved in post-necrotic scar formation), increased astrocyte proliferation soon after HI is correlated with a smaller infarct size and better functional recovery (Barreto *et al.*, 2011). Astrocytes support the neurons that survive the immediate effects of HI, modulating oxidative stress and glutamate excitotoxicity, and releasing neurotrophic factors, as well as

maintaining the integrity of the blood-brain barrier and thereby limiting brain invasion by inflammatory cells during reperfusion (Barreto *et al.*, 2011; Mehta *et al.*, 2007). Accordingly, protecting astrocytes from HI injury is now considered a critical component of neuroprotective strategies (Barreto *et al.*, 2011).

CBD show no side effects in HI piglets

Remarkably, and consistent with previous studies (Alvarez *et al.*, 2008; Lafuente *et al.*, 2011), CBD caused no extra-cerebral alterations in HI piglets that could limit its potential therapeutic use in HI infants, yet it resulted in extra-cerebral benefits. For instance, CBD prevented the HI-induced decrease in MABP. It is unlikely that this decrease could influence the development of HI brain damage because in all animals MABP was over the limit below which cerebral blood flow is affected (Laptook *et al.*, 1982).

1.1.2. 72HFU

CBD restored brain activity

Brain activity, as reflected by aEEG amplitude, felt by 30% during the following 24 h to the HI insult, similarly to that previously reported in a similar model (Lafuente *et al.*, 2011). However, in the work by Lafuente *et al.* (2011) some modest increase of aEEG amplitude was observed from 24 to 48 h post-HI whereas in our present work aEEG amplitude did fall again to become 50% lower than in SHM at 48 h post-HI. This feature suggests that in the present experiments the HI insult was more severe. In fact, piglet mortality in 72HFU experiments accounted for 20.5%, which was very similar to the actual mortality of HI insults in human newborns (Volpe, 2001). This supports the value if this model as a representation

of the actual human condition. There are no data on experimental mortality in the work by Lafuente *et al.* (2011). By the end of the experiment aEEG amplitude in HV piglets was about 30% of the baseline.

In CBD treated animals the fall of aEEG amplitude during the first 24 h post-HI was similar to that observed in HV, suggesting that HI severity was similar. However, aEEG did not further decrease, starting a recovery from 48 h after HI to regain normal amplitude by the end of the experiment. This effect was more robust than that observed after CBD 0.1 mg/kg, since in the work by Lafuente *et al.* (2011) aEEG did not regain amplitude similar to sham animals.

CBD prevented seizures

Since subtle seizures are hardly apparent in piglets just severe epileptic forms are easy to detect by physical examination (Björkman *et al.*, 2010). Therefore, in the present study aEEG records were obtained daily in piglets to detect electrographic seizures. More than a half of HV piglets showed electrographic seizures 72 h after HI. Seizures are a frequent complication of HI insults in newborns (Volpe, 2001). In HI piglets subclinical (electrographic) seizures are associated with increased severity as assessed by histology, MRI or H^+-MRS (Björkman *et al.*, 2010).

However, none of the HI piglets treated with CBD showed electrographic seizures at 72 h after HI. CBD is a substance with a well-known anticonvulsant effects by mechanisms not fully understood yet (Jones *et al.*, 2010). In HI piglets CBD reduced the incidence of seizures as assessed by aEEG during the 6 h following to HI (Alvarez *et al.*, 2008).

CBD prevented neuronal death

The HI insult led to reduction of the number of neurones observable in the parietooccipital cortex. In particular, the number of viable neurones was 40% lower in HV than in SHM. In addition, the proportion of death neurones observable in cortex was increased, accounting for 21% of the total number on visible neurones. In addition, the sum of viable and death neurones was significantly lower than that observable in SHM animals, suggesting that many death neurones had disappeared by the moment the histological analysis was done. Thus, the actual proportion of death neurones must be greater than 21%. These figures are similar to that reported by Lafuente *et al.* (2011).

Administration of CBD 1 mg/Kg after the HI insult, no matter it was single dose or in three doses, reduced the HI-induced loss of neurones. Thus, the total number of neurones observables in cortex as well as the proportion of viable or death ones were similar to SHM. Such a strong protective effect was similar to that observed after administering CBD 0.1 mg/kg (Lafuente *et al.*, 2011). The effect of CBD 0.1 mg/Kg preventing HI-induced neuronal death was so strong (Lafuente *et al.*, 2011) that in this case further improve by increasing the dose was unconceivable.

CBD increased myelinization

The HI insult led to a decrease of the myelin density in the External Capsule. It is well known that immature oligodendrocyte are particularly susceptible to HI damage due to its high metabolism and iron content which made them particularly susceptible to oxidative stress and inflammation (Volpe, 2011). Thus, late oligodendrocyte progenitors, which are the predominant oligodendrocyte lineage cells in the newborn brain, show massive apoptotic

death after a HI insult resulting in impaired remyelinization (Back *et al.*, 2002). Therefore, oligodendroglial damage during HI insults has a paramount importance determining the risk of development of Cerebral Palsy (Volpe, 2011). The finding of a decrease of myelin density supports this point since MBP is produced by mature oligodendrocytes, deriving from surviving late oligodendrocyte progenitors.

Noteworthy, CBD administration after the HI prevented such a decrease of myelin density, in particular when CBD was administered once a day for three days. This finding is of great interest since it opens new perspectives for using CBD in different paediatric conditions in which demyelinization could take part.

CBD improved H^+-MRS prognostic markers

Similarly to that described for the 6HFU model, the HI insult led to an increase of Lac/NAA ratio. The increase of Lac/NAA is observed very early after a HI insult, lasting for several days in humans (Thayyil *et al.*, 2010) and piglets (Faulkner *et al.*, 2011).

Administration of CBD 1 mg/Kg after the HI insult, no matter it was single dose or in three doses, prevented the HI-induced increase of Lac/NAA, supporting the neuroprotective effect of this treatment.

1.2. NEUROBEHAVIORAL EFFECTS OF CBD

Piglet behaviour assessment

Most reports on the behavioural effects of perinatal HI and neuroprotective treatments come from studies carried out in rodents (Gieling *et al.*, 2011). However, the domestic pig (*Sus scrofa*) is an animal with complex and rich behaviour.

Reports on the neurobehavioral effects of HI on piglets have usually been focused on the motor performance, describing hindquarter diplegia, ataxia or weakness 48-72 h after HI (Leblanc *et al.*, 1991; Thoresen *et al.*, 1996; McCulloch *et al.*, 2005; Schubert *et al.*, 2005; Björkman *et al.*, 2010; Lafuente *et al.*, 2011). LeBlanc *et al.* (1991) adapted a usual neurological examination to piglets, with several items related to reflexes, muscle tone and movement scored from 1 to 4 to a maximum of 36. Thoresen *et al.* (1996) changed the sore to a maximum of 2 per item and included seizure categorization: 2: absence of pathologic movements; 1: occasional cycling movements or jerks; 0: sustained clonic movements or persistent tonic postures (Thoresen *et al.*, 1996). This scheme, with some modifications, has been largely used in further reports on the neurobehavioral consequences of HI in piglets (Schubert *et al.*, 2005; Björkman *et al.*, 2010; Lafuente *et al.*, 2011). The one used in the present work was the one reported by Lafuente *et al.* (2011).

In the aforementioned piglet evaluations (LeBlanc *et al.*, 1991; Thoresen *et al.*, 1996; McCulloch *et al.*, 2005; Schubert *et al.*, 2005; Björkman *et al.*, 2010; Lafuente *et al.*, 2011) the items devoted to pure behaviour are scant and fairly unspecific. Scoring just the level of alertness at the beginning (LeBlanc *et al.*, 1991), motivation to explore the surroundings (Thoresen *et al.*, 1996) and aggressiveness (Björkman *et al.*, 2010) were then included. However, piglet behaviour is fairly more complex.

Just after birth piglets start a playful behaviour, firstly related with sow, becoming one or two days later curious about the surrounding world including objects and peers (social) (Blackshaw *et al.*, 1997; Park *et al.*, 2010). Thus, piglets develop a rich behaviour performance that can be categorized as (Cox & Cooper, 2001):

- Social interactions: Pigs are social species. Social behaviour included nudging, nuzzling, body contact or pushing other animals or people (Blackshaw *et al.*, 1997; Park *et al.*, 2010). Social playfulness is related to neocortex activity, time devoted to it increasing in parallel with maturation (Park *et al.*, 2010).

-Maintenance activities: Those are foraging activities, mostly devoted to eating. Eating, that is suckling in newborn piglets, is considered as a key part of behaviour assessment since eating is essential for survival (Park *et al.*, 2010). In addition, chewing is a feeding-motivated behaviour typically used by piglets to explore novel objects (Kittawornrat & Zimmerman, 2010).

- Environmental interactions: Object playful behaviour included pulling strings or pushing, rooting, biting or sniffing an inanimate object (Litten *et al.*, 2003).

These categories are explored in the present studies. Social interactions were assessed by determining the time spent by piglets interacting with the researchers. Maintenance activities were assessed by qualifying piglet suckling of the researcher's finger during feeding as well as by quantifying the volume of swollen milk. And environmental interactions were assessed by determining the time spent by piglets interacting with an object (sheet).

Piglets spend about 40% of time in active behaviour, mostly early morning and early evening (Park *et al.*, 2010). From this, about 20% is devoted to object play and about 30% to social play (Blackshaw *et al.*, 1997). The proportion of time devoted to social play was

increased in piglets in the present study, accounting for near 70% of time. Piglets prefer bright spaces (Kittawornrat & Zimmerman, 2010).

Pigs perform with efficacy on learning and memory tests and several tests originally designed for rodents (open field, novel object recognition, T maze, even water maze) have been adapted to pigs (Gileing *et al.*, 2011).

Restrain is a usual stress-induced manoeuvre in animals; in piglets, back restrain prompt an escaping behaviour (Kittawornrat & Zimmerman, 2010). Piglets with increased levels of stress tend to show increased locomotion, grunting, chewing, nosing and drinking, early and stronger escape behaviour, increased aggressiveness and more superficial exploratory behaviour (Parrott *et al.*, 2000; Kittawornrat & Zimmerman, 2010).

It is important to have the piglet weight into account when piglet behaviour is analysed. Just after birth piglets form the same litter set hierarchies by setting teat order so that the more active and heavier piglets gets the first pairs of teats; since those teats have more milk the more active piglets remain being larger and heavier (Litten *et al.*, 2003; Park *et al.*, 2010). Thus, smaller piglets spend less time in object play than heavier piglets (Litten *et al.*, 2003). Since in the present experiments no significant differences were observed among the different group piglets, the differences in playful behaviour among groups cannot be attributed to this factor.

Effects of HI on piglet neurobehaviour

Piglets suffering from a HI insult treated with vehicle showed neurobehavioral impairment.

From a motor point of view, HI led to a dramatic decrease of NBS, which scored 28% lower 24 h after HI than SHM animals. Later on there was a modest and non-significant improvement so that by the end of the experiment HV piglets performed 22% worse than normal piglets. The more affected items were related with movement and hind limb muscle tone. This outcome was similar to that reported for similar experiments in piglets (LeBlanc *et al.*, 1991; Schubert *et al.*, 2005; Lafuente *et al.*, 2011) and support the significant brain damage inflicted by this approach in comparison for instance with other based on prolonged hypoxia (McCulloch *et al.*, 2005). HI compromised the ability for sucking too, with HV scoring worse than SHM in eating behaviour throughout the experimental period, as reported (Lafuente *et al.*, 2011). In addition, the amount of milk drunk was significantly lower than in SHM. This feature pointed to a severe neurological impairment, because eating has to be preserved as long as survival is aimed (Park *et al.*, 2010). This feature was even more significant having into account that HV piglets showed increased anxiety (see after), and anxiety leads to increased feeding in piglets (Parrott *et al.*, 2000).

Time spent in fighting or escape behaviour during restrain for aEEG recording was used to quantify anxiety. HI increased anxiety as reflected by the more than 50% increase of fighting time from 48 to 72 h after HI.

From a "pure" behavioural point of view, HI reduced by near 50% the time spent in exploring the environment in piglets treated with vehicle. This reduction was mainly due to the decrease of social playful time, which was so reduced that the time spent in social play was slight lower than that spent in object play. This finding is of great relevance since pig is a social species (Park *et al.*, 2010) usually devoting twice as much time in social than in object

play (Blackshaw *et al.*, 1997), as observed on SHM animals in the present work. The reduction of social playfulness might be related with the increased anxiety.

CBD prevented HI -induced impairment of piglet neurobehaviour

Administration of CBD 1 mg/Kg after HI restored NBS as soon as 48 h post-insult. This effect was stronger than that observed in piglets receiving CBD 0.1 mg/Kg, in which NBS was non-significantly lower than sham animals just 72 h after HI (Lafuente *et al.*, 2011). The effect also was stronger than that observed for other neuroprotective treatments now tested in clinical trials as topiramate, which improves but not restores NBS in HI piglets (Schubert *et al.*, 205). Normalization of the neurological assessment in the first days after HI is one of the best predictors of good outcome in asphyxiated newborns (Volpe, 2001).

The beneficial effect of CBD was extended to eating behaviour, which scored similar to SHM just 48 h after HI. In addition, the amount of milk drunk was increased so that at the third day after HI the volume of milk was similar in HC piglets than in SHM. This effect can be only attributed to a neurological improvement since CBD has no activity on CB_1 receptors being thus void of effects on appetite (Pertwee, 2004; Mechoulam *et al.*, 2007). Besides, CBD treated animals showed less anxiety (see after). Therefore, an anxiety-induced enhancement of eating behaviour or the amount of milk swollen can be ruled out.

Remarkably, CBD treatment abolished HI-induced increase of anxiety. In fact, HC piglets spent lees time fighting against restrain than SHM animals, although this difference did not reach statistical significance. CBD show a well-known anxiolytic effect (Pertwee, 2004; Mechoulam *et al.*, 2007), which prompted the study of CBD in the treatment of anxiety-related psychiatric conditions (Campos *et al.*, 2012). In rats, CBD was shown to

decrease defensive behaviours evoked by predator exposure (Campos *et al.*, 2012). Interestingly, CBD can interfere in learning and/or memory of aversive events in animals, (Campos *et al.*, 2012), which can explain why fighting time against restrain was even shorter 72 h than 48 h after HI.

CBD treatment restored the amount of time spent in playful activities. Although the recovery of time devoted to social play was not complete, in these animals social play time was significantly longer than object play time. Effects of CBD on social behaviour, which is a key component of psychosis, are controversial. In SHR rats CBD reduces anxiety but does not modify social behaviour although it does increase social interaction in Wistar rats (Almeida *et al.*, 2013). In a rat model of Alzheimer chronic administration of CBD decreased anxiety and improved social behaviour (Cheng *et al.*, 2014). The latter results might indicate that CBD could more effective recovering social behaviour in neurodegenerative conditions.

1.3. CBD NEUROPROTECTION IN MICE

CBD reduced the volume of damage

HI led to 12% reduction of ipsilateral hemisphere volume as assessed by MRI seven days after HI. This was remarkably lower than that reported for newborn rats (Pazos *et al.*, 2012), which may be related to a greater resistance to HI of mice than rats.

Post-insult administration of CBD to newborn mice dramatically reduced HI-induced loss of brain volume. In this case, CBD effect was stronger than that reported for newborn rats since 7 day after CBD-treated HI newborn Wistar rats show similar volume of damage than vehicle-treated ones (Pazos *et al.*, 2012). This might represent a greater sensitivity to CBD in newborn mice. CBD fully prevent necrotic damage in newborn mice forebrain slices

exposed to OGD (Castillo *et al.*, 2010). CBD reduces hypoxic-ischemic brain damage *in vivo* in adult mice too (Schiavon *et al.*, 2014).

CBD reduced the brain histological damage

HI led to moderate brain tissue damage, as assessed by a NPS. This was fully prevented by CBD administration just after the HI insult. Once more this point to a greater sensitivity to the protective effects of CBD in mice than rats, since 7 days after HI immature Wistar rats receiving CBD show a lower NPS than HV rats but higher than control rats (Pazos *et al.*, 2012). CBD has proven to reduce necrotic brain cell damage in mice as reflected by the CBD-induced reduction of LDH release in mice forebrain slices exposed to OGD (Castillo *et al.*, 2010).

The beneficial effect of CBD was extended to the apoptotic processes. In vehicle-treated mice HI led to a dramatic increase of the number of TUNEL+ cells as observed in brain 7 days after HI. Such an increase was fully prevented by CBD. A similar effect was observed in piglets 3 days after HI (Lafuente *et al.*, 2011). In mice forebrain slices exposed to OGD, CBD prevented the OGD-induced increase of concentration of caspase 9 (Castillo *et al.*, 2010). Apoptosis is a key process in HI-induced immature brain damage, spreading injury spatially and temporary (Volpe, 2001).

CBD protected astrocytes

As observed in piglets, CBD administration prevented the HI-induced reduction of the number of GFAP+ cells. The importance of preserving astrocyte from HI damage has been stressed before.

2. MECHANISMS OF CBD NEUROPROTECTION

As deduced from piglet 6HFU model, CBD-mediated neuroprotection in HI piglets involved the modulation of excitotoxicity, inflammation and oxidative stress, confirming previous *in vitro* findings from the immature rodent brain (Castillo *et al.*, 2010) in a large mammal *in vivo*.

Excitotoxicity, inflammation and oxidative stress are the "deadly triad" leading to HI-induced brain damage (Johnston *et al.*, 2011; Drury *et al.*, 2014; Juul & Ferriero, 2014). Only pleiotropic therapies acting on all of those mechanisms are suitable for successful neuroprotection (Cilio & Ferriero, 2010; Johnston *et al.*, 2011; Juul & Ferriero, 2014).

CBD modulated excitotoxicity

The deleterious effect of glutamate excitotoxicity is greater in the immature brain (Johnston *et al.*, 2011; Mehta *et al.*, 2007) and consequently, the increase in the Glu/NAA ratio after HI in human newborns is proportional to the severity of encephalopathy (Groenendaal *et al.*, 2001). The increase in the Glu/NAA ratio observed in the piglet brain after HI was dampened by CBD administration. It has been reported that CBD reduces Glu release in vitro in newborn mice forebrain slices exposed to oxygen-glucose deprivation

(OGD) (Castillo *et al.*, 2010). In vivo in HI rats, CBD administration led to a decrease of Glu/NAA in the H^+-MRS studied carried out 7 days after HI (Pazos *et al.*, 2012). It is worth it that a neuroprotective substance reduces Glu release instead of blocking NMDA receptors since NMDA receptor blockade in immature brain has demonstrated to have deleterious effects (Hamrick & Ferriero, 2003).

CBD modulated oxidative stress

The deleterious effects of oxidative stress are magnified in the immature brain due to low levels of antioxidant activity and high iron content (Johnston *et al.*, 2011). The GSH/Cr ratio decreased here after HI, an effect that was prevented by CBD administration. GSH is the most abundant water-soluble antioxidant that is readily identified by H^+-MRS, and its reduction in the brain correlates with oxidative stress (Satoh & Yoshioka, 2006). CBD also prevented the HI-induced increase of protein carbonylation, which plays an important role in HI-induced neuronal death (Oikawa *et al.*, 2009). Indeed, CBD is a powerful antioxidant molecule (Hampson *et al.*, 1998) with proven beneficial effects in oxidative stress-related neurodegenerative processes (Hayakawa *et al.*, 2010). CBD is a strong antioxidant because of its molecular properties (Hampson *et al.*, 1998) and also because it modulates iNOS expression, a major source of free radicals after HI (Hamrick & Ferriero, 2003; Johnston *et al.*, 2011; Juul & Ferriero, 2014), as demonstrated in newborn mice forebrain slices after OGD (Castillo *et al.*, 2010). CBD administration did not modify the concentration of malondyaldehide (MDA) in CSF in asphyxiated piglets as compared with vehicle-treated animals (Lafuente *et al.*, 2011). However, MDA is a low sensitive marker of oxidative stress (Johnston *et al.*, 2011).

CBD modulated neuroinflammation

CBD prevented the HI-induced increase in IL-1 levels in piglets. Inflammation plays a key role in HI-induced damage in the immature brain (Johnston *et al.*, 2011). Among the different pro-inflammatory cytokines IL-1 is particularly important in the context of HI-induced brain damage (Allan & Rothwell 2001), IL-1 levels increasing in the CSF of HI infants in parallel with the severity of encephalopathy. Indeed, this cytokine better predicts HI brain injury than TNFα (Oygür *et al.*, 1998), suggesting that modulation of IL-1 may have neuroprotective effects (Allan & Rothwell 2001). CBD has a wide range of anti-inflammatory properties, modulating cytokine release and exhibiting anti-inflammatory effects both in vivo and in vitro (Pertwee, 2004; Mechoulam *et al.*, 2007). CBD has demonstrated to reduce the release of IL-6 and to inhibit COX-2 expression in newborn mice forebrain slices after OGD (Castillo *et al.*, 2010). In rats suffering a HI insult at P7 CBD blunts the HI-induced increase of TNFα concentration in brain tissue as determined seven days after HI (Pazos *et al.*, 2012). In aphyxiated piglets CBD prevents the HI-induced increase TNFα(+) cells as observed by flow cytometry of brain tissue (Lafuente *et al.*, 2011).

Role of CB_2 receptors in CBD effects

In HI piglets CBD neuroprotection was abolished when CBD was administered with AM630. In addition, the mechanisms involved in CBD neuroprotection (anti-excitotoxicity, anti-oxidation and anti-inflammation) were also affected by CB_2 antagonism. CB_2 receptors have been largely involved in the anti-inflammatory and iNOS expression modulatory effects of different cannabinoids (Pertwee *et al.*, 2010). It has been described a protective effect of

CB_2 activation in immature (Fernandez-Lopez *et al.,* 2006; Fernandez-Lopez *et al.,* 2007) and mature (Zhang *et al.,* 2007) rodent brain after HI.

It is generally assumed that CBD does not bind to CB_2 receptors (Pertwee *et al.*, 2010). In adult mice, CB_2 antagonists did not reverse CBD neuroprotection after HI *in vivo* (Hayakawa *et al.*, 2008). However, it has been reported that CB_2 receptor antagonism blocks the effects of CBD on cytokine release in cultured cells (Sacerdote *et al.*, 2005) and rat body weight gain (Ignatowska-Jankowska *et al.*, 2011). Besides, CBD has demonstrated to activate CB_2 receptors in poorly differentiated cells as tumoral cells (Ligresti *et al.,* 2006). Thus, it is possible that the CBD affinity to CB_2 receptors we observed in immature brain might be related to developmental variations in CB_2 physiology. In agreement, co-incubation with AM630 reverses the protective effects of CBD newborn mice forebrain slices exposed to OGD (Castillo *et al.*, 2010). Thus, the involvement of CB_2 receptors in some of the effects of CBD (Mechoulam *et al.*, 2007) cannot be completely ruled out.

Role of endocannabinoid levels in CBD neuroprotection

Since CBD inhibits the uptake and/or hydrolysis of several endocannabinoids, including anandamide (Pertwee, 2004), the involvement of CB_2 receptors might not be due to the direct action of CBD but rather to an increase in brain endocannabinoid levels induced by CBD. We observed an increase in brain endocannabinoid levels in the HV group, similar to those reported in adult rodents following ischemic events in the brain (Hillard, 2008). By contrast, endocannabinoid levels in HC animals were lower than in HV animals, and comparable with those of the SHM group. Interestingly, increased endocannabinoid levels immediately after brain HI are thought to contribute to the damage produced (Hillard, 2008)

and accordingly, preventing the HI-induced increase in brain endocannabinoids by administering CBD may be at least partially responsible for the neuroprotective effects of CBD. In any case, these observations rule out the possibility of CB_2 receptor activation by CBD through the increase in endocannabinoid levels.

Role of $5HT_{1A}$ receptors in CBD neuroprotection

CBD inhibits 5HT re-uptake and acts as an agonist of $5HT_{1A}$ receptors (Russo *et al.*, 2005; Rock *et al.*, 2010; Magen *et al.*, 2012). In HI piglets in the 6HFU model WAY100635 reversed the neuroprotective effects of CBD, including the CBD-mediated modulation of glutamate release, oxidative stress and inflammation. There are no precedent reports on the involvement of $5HT_{1A}$ receptors in CBD-mediated neuroprotection in the immature brain. Blockade of $5HT_{1A}$ receptors inhibits the neuroprotective effect of CBD in adult mice by reversing the increase in cerebral blood flow during ischemia induced by CBD (Hayakawa *et al.*, 2010). However, we cannot rule out the possibility that the beneficial effects of CBD on inflammation and the excito-oxidative cascade in piglets were the result of a non-specific neuroprotective effect due to increases in cerebral blood flow mediated by the $5HT_{1A}$ receptor. Indeed, the fact that WAY100635 reversed the beneficial systemic hemodynamic effects of CBD after HI supports this hypothesis.

Interestingly, our results demonstrate remarkably similar effects of WAY100635 and AM630. Heteromers of G-protein-coupled receptors can be found in neural cells (Casadó *et al.*, 2010; Ferré *et al.*, 2009; Pertwee *et al.*, 2010), possessing specific functions (other than those of the individual homomeric receptors), and they can be identified by cross-antagonism (Moreno *et al.*, 2011). As such, in heteromeric receptor complexes the

activation of one receptor can result in the engagement of the G protein coupled to the partner receptor, while antagonists of a partner receptor in the heterodimer can block the signalling mediated by the heteromer (Ferre *et al.*, 2009; Moreno *et al.*, 2011). Hence, it can be speculated that $5HT_{1A}$ and CB_2 receptors form heteromers and that such heteromers were at least in part responsible for the CB_2 involvement in CBD effects.

Role of $5HT_{1A}$ receptors in CBD neurobehavioral effects

Serotonin $5HT_{1A}$ receptors are involved in many of the behavioural effects of CBD (Russo *et al.*, 2005; Campos *et al.*, 2012).

In the present experiments, CBD prevention of HI-induced NBS impairment was reduced but not abolished by co-administration of WAY100635 only when CBD was administered single dose. Thus, in HCW1 muscle tone and walking were affected by HI despite CBD administration. However in HCW3, in which CBD was administered once a day during the three days of experiment, WAY100635 failed to modify CBD effects in spite of the fact that the $5HT_{1A}$ antagonist was administered during the three experimental days too. Similar results were obtained regarding eating behaviour. Altogether these results and the results from the 6HFU model suggest that $5HT_{1A}$ receptors were involved in but were not indispensable for CBD preservation of motor function, and that such involvement took place just in the first hours after HI. After 24 h post HI, however, CBD beneficial effects on neurological function were independent from $5HT_{1A}$ receptor activation. Since $5HT_{1A}$ receptor involvement on CBD neuroprotection is thought to be related with the increase of cerebral blood flow (Hayakawa *et al.* 2010), it can be speculated that this mechanism must be of great importance during and shortly after the ischemic episode. Later on, and in

particular when the secondary phase is established and different deleterious processes (excitotoxicity, inflammation, oxidative stress) have started, other receptor-independent properties of CBD (anti-inflammatory and anti-oxidant effects) (Pertwee, 2004; Mechoulam *et al.*, 2007) might gain importance.

Regarding anxiety, by contrast, WAY100635 blunted the anxiolytic effect of CBD no matter it was administered single dose or in three doses. A similar result were observed regarding playfulness, since the $5HT_{1A}$ receptor antagonist abolished CBD prevention of HI-induced decrease of play time both in HCW1 and HCW3. These data suggest that anxiolytic and social effects of CBD in HI piglets are largely dependent on $5HT_{1A}$ receptor activation. In agreement, WAY100635 reverses the anxiolytic effects of CBD in rat models of anxiety as predator exposure or intracerebral injection of DPAG (Campos *et al.*, 2012). Besides, CBD show a similar anxiolytic profile than a $5HT_{1A}$ receptor agonist, ipsapirone, in reducing anxiety to public speaking in naïve volunteers (Campos *et al.*, 2012).

3. CBD TEMPORARY THERAPEUTIC WINDOW

The protective effects of CBD against HI-induced brain damage in mice was still apparent even when CBD administration was delayed up to 12 h after HI but was lost when CBD administration was delayed for 24 h. Regarding the volume of brain damage as assessed by MRI, CBD administration led to the same result no matter CGD was administrated 15 min or 1, 3, 6 or 12 h after HI. Regarding the protection of neurones, however, CBD effect loosed some strength when the administration was delayed for 3 h, so that either the number of necrotic neurons or the number of TUNEL+ cells were greater in HC3 or later than in HC0.25 although those numbers were still significantly lower than in HV. Regarding astrocytes,

however, once again CBD remained similarly protective when administered 15 min or 12 h after HI. These data support the great protective effect of CBD on astrocyte, which at least in newborn mice seems to be stronger than neuronal protection. This feature is of paramount importance since protecting astrocyte not only reduces HI brain damage but also guarantees effective neuro-repair, supporting the newly created neurones (Takuma *et al.*, 2004).

The aforementioned data suggest that the temporary therapeutic window should be between 12 and 24 h. In a model of stroke in adult rats, CBD is able to reduce the number of TUNEL+ cells, prevent the decrease of GFAP+ cells and improve neurological function even when CBD administration started 3 days after the insult (Hayawa *et al.*, 2009). This difference may be due to the greater susceptibility of immature than mature brain to HI insults (Volpe, 2001; Ferriero, 2004). On the other hand, in the experiments on stroke adult rats CBD was administered 3 mg/Kg whereas in the present experiments CBD was 1 mg/Kg. In fact, in the present experiments the volume of lesion as well as the number of necrotic neurons or TUNEL+ cells was lower in HC24 than in HV although this difference was not statistically significative. Therefore, it cannot be ruled out the possibility that greater dose of CBD might lead to a longer temporary therapeutic window.

In any case, the therapeutic temporary window showed by CBD in the present experiments was longer than that reported for the gold standard of neuroprotection, hypothermia. The best results from hypothermia are obtained when this procedure is started during the first 6 h after HI (Laptook, 2009). In fact, recent experimental evidence indicates that hypothermia delayed for 12 h might be actually harmful (Sabir *et al.*, 2012). This data enhances the interest for CBD being part of the therapeutic package for asphyxiated infants since CBD might be of election when hypothermia was unfeasible.

Other therapeutic strategies under investigation show similar or even broader therapeutic temporary window to that described for CBD. For instance, erythropoietin has demonstrated some efficacy even when its administration is delayed for 48-72 h (Wu *et al.*, 2012). Melatonin, administered 24 or 48 h after HI to newborn rats reduces the histological damage and restores long term neurological examination (Carloni *et al.*, 2008). However other treatments as xenon (Thoresen *et al.*, 2009) are effective only when administered in the following two hours to the HI insult. In the case of topiramate the temporary therapeutic window is even shorter (Noh *et al.*, 2006)

4. PHARMACOLOGICAL ASPECTS

Administration route

In the present experiments we used a formulation suitable for i.v. administration. This formulation contained Solutol$^{(R)}$, a dissolvent already used in humans. Thus, this formulation is more appropriate for use in humans than the typical formulation of CBD in TWIN or cremophor (Alvarez *et al.*, 2008). Although CBD can be administered by oral route (Pertwee, 2004; Mechoulam *et al.*, 2007), the i.v. route is of great interest because asphyxiated infants candidates for neuroprotective treatments are under intensive care when oral route is forbidden. In addition, CBD has demonstrated better bioavailability when used by i.v. than oral route (Deiana *et al.*, 2012).

Dose

As described before, in the present experiments in piglets with CBD 1 mg/Kg the neuroprotective effects were stronger that those reported for CBD 0.1 mg/Kg (Alvarez *et al.*, 2008; Lafuente *et al.*, 2011), in spite of the fact that the HI insult was heavier. Since most results of CBD 1 mg/kg i.v. are hardly improvable because CBD restored activity or biomarker values, this dose and route seems to be appropriate for a future clinical trial. It remains to be determined, however, whether or not slightly greater doses (3 or 5 mg/Kg, for instance) might further improve the result of CBD.

Dose frequency

Experiments using the 72HFU model indicated that CBD efficacy was similar no matter CBD was administered in a single dose or in three doses once a day. The only exception was the effect of CBD on myelinization, since CBD administered once a day for three days showed better results than CBND single dose. This result together with the capability of CBD in three daily doses to overcome the blockade of $5HT_{1A}$ receptors makes more recommendable the alternative of CBD in daily doses for at least three days.

Pharmacokinetics

There are no precedents of PK studies of CBD administered i.v. to piglets. The shape of the plasma concentration curve was similar to that reported for adult dogs after i.v. administration of CBD 45 mg in ethanol 70% (Samara *et al.*, 1987). In dogs, the $T_{1/2}$ wasas

twice as longer than in newborn pigs, which might indicate a faster clearance of CBD from blood due to a faster brain entrance.

In adult rats, CBD dissolved in cremophor and administered i.p. at a dose 100 times higher than in our experiments show a Cmax only 7 times higher, a Tmax 10 times higher and a $T_{1/2}$ 50% longer (Deiana *et al.,* 2012). These data suggest that CBD by i.v. route, at the present formulation and in piglets have a greater bioavailability than by i.p. route in rats. Similarly, the brain concentration of CBD in the present experiments was as twice as higher than that reported for newborn rats after s.c. administration of the same dose (1 mg/Kg) (Pazos *et al.*, 2012). In addition the longer $T_{1/2}$ supports a faster clearance of CBD in newborn pigs than in adult rats.

CONCLUSIONS

CONCLUSIONS

1. Blocking carotid artery and reducing inhaled oxygen content to 10% for 30 min in newborn piglets is an efficient model of newborn hypoxic-ischemic brain damage, as demonstrated by functional, histological and biochemical studies. In this model:

1.1. Cannabidiol prevents the hypoxia-ischemia-induced depression of brain activity as assessed by amplitude-integrated EEG, restoring amplitude and background.

1.2. Cannabidiol prevents the hypoxia-ischemia-induced brain damage as assessed by histological studies:

1.2.1. Preventing neuronal death.

1.2.2. Increasing the survival of astrocytes.

1.3. Cannabidiol prevents the hypoxia-ischemia-induced impairment of magnetic resonance spectroscopy biomarkers, preventing the Lac/NAA increase and NAA/Cho decrease.

2. Blocking carotid artery and reducing inhaled oxygen content to 10% for 20 min allowing then the recovery of the piglets is an efficient model of newborn hypoxic-ischemic brain damage, as demonstrated by functional, histological, biochemical and neurobehavioral studies. In this model:

2.1. Cannabidiol prevents the hypoxia-ischemia-induced depression of brain activity as assessed by amplitude-integrated EEG, restoring amplitude and background 72 hours after the insult.

2.2. Cannabidiol prevents the hypoxia-ischemia-induced brain damage as assessed by histological studies 72 h after the insult:

2.2.1. Preventing the decrease of viable neurons as well as the increase of necrotic neurons.

2.2.2. Preventing the decrease of myelin content.

2.3. Cannabidiol prevents the hypoxia-ischemia-induced impairment of magnetic resonance spectroscopy biomarkers, restoring the Lac/NAA ratio 72 h after the insult.

2.4. Cannabidiol prevents the hypoxia-ischemia-induced impairment of neurological function:

2.4.1. Preventing the hypoxia-ischemia-induced impairment of muscle tone and locomotion.

2.4.2. Preventing the hypoxia-ischemia-induced impairment of eating behaviour and environment exploration.

2.4.3. Preventing the hypoxia-ischemia-induced increase of anxiety and social play impairment.

2.5. Cannabidiol exerts all these effects no matter it was administered single dose or once a day for three days, with the exception made for the effect on myelinization that was observed only after administration of three doses.

3. Regarding the mechanisms of cannabidiol neuroprotection:

3.1. Cannabidiol exerted antiexcitotoxic effects, preventing the hypoxia-ischemia-induced increase of glutamate release.

3.2. Cannabidiol exerted antoxidant effects, preventing the hypoxia-ischemia-induced consumption of reduced glutathione and increase of protein carbonylation.

3.3. Cannabidiol exerted antiinflammatory effects, preventing the hypoxia-ischemia-induced increase of interleukine 1 production.

3.4. Regarding the receptors involved in cannabidiol neuroprotection:

3.1.2. The coadministration of a CB_2 receptor antagonist reversed all the protective effects of cannabidiol, indicating the involvement of such receptors in cannabidiol neuroprotection.

3.1.3. The coadministration of a $5HT_{1A}$ receptor antagonist reversed all the protective effects of cannabidiol, indicating the involvement of such receptors in cannabidiol neuroprotection.

3.5. Endocannabinoid brain concentration was not increased by cannabidiol, ruling out the involvement of this mechanism on cannabidiol neuroprotection.

3.6. Regarding the involvement of $5HT_{1A}$ receptors in cannabidiol neurobehavioral effects:

3.6.1. The coadministrarton of a $5HT_{1A}$ receptor antagonist reversed all the protective effects of cannabidiol on muscle tone and locomotion only when cannabidiol was administered single dose but not when it was administered for three days, indicating that these receptors are involved in cannabidiol neuroprotection the first hours after the insult but not afterwards.

3.6.2. The coadministration of a $5HT_{1A}$ receptor antagonist reversed all the protective effects of cannabidiol on behaviour no matter how cannabidiol was

administered, indicating that these receptors are mediating the behavioral effects of cannabidiol.

4. Exposing newborn mice to 10% oxygen atmosphere after left carotid artery electrocoagulation is an efficient model of newborn hypoxic-ischemic brain damage, as demonstrated by neuroimaging and histological studies. In this model:

4.1. Cannabidiol prevents the hypoxia-ischemia-induced loss of ipsilateral brain hemisphere volume seven days after hypoxia-ischemia.

4.2. Cannabidiol prevents the hypoxia-ischemia-induced histological brain damage seven days after hypoxia-ischemia:

4.2.1. Preventing the increase of necrotic neurons.

4.2.2. Preventing the increase of apoptotic cells.

4.2.3. Preventing the decrease of astrocytes.

4.3. Cannabidiol shows a temporary therapeutic window lasting between 12 and 24 hours.

5. Regarding the pharmacological properties of cannabidiol:

5.1. Cannabidiol dissolved in a formulation containing ethanol, solutol and saline in a proportion of 2:1:17 is suitable for intravenous use.

5.2. The administration of cannabidiol 1 mg/Kg once a day for three days is the best schedule for treatment.

5.3. Intravenous administration of cannabidiol to newborn pigs shows better bioavailability and faster clearance to tissues than that observed in adult animals.

GENERAL CONCLUSION

Cannabidiol, administered at 1 mg/Kg up to 12 h after a hypoxic-ischemic insult, prevents the resulting brain damage, protecting neurons and astrocytes, protecting myelinization, restoring brain activity and function and preventing behavioural consequences. Cannabinoid CB_2 receptors are somehow involved in these effects. Serotonin $5HT_{1A}$ receptors are involved in the cannabidiol histological, biochemical and motor protective effects and mediate the behavioural effects of cannabidiol. Cannabidiol properties meet when not overtake those described for the gold standard of current treatment, hypothermia. Altogether these results confirm cannabidiol as a potent, effective, feasible and suitable candidate to be included in the treatment of asphyxiated infants.

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