Mechanisms of cannabidiol neuroprotection in hypoxic–ischemic newborn pigs: Role of 5HT₁A and CB2 receptors

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Abstract

The mechanisms underlying the neuroprotective effects of cannabidiol (CBD) were studied in vivo using a hypoxic–ischemic (HI) brain injury model in newborn pigs. One- to two-day-old piglets were exposed to HI for 30 min by interrupting carotid blood flow and reducing the fraction of inspired oxygen to 10%. Thirty minutes after HI, the piglets were treated with vehicle (HV) or 1 mg/kg CBD, alone (HC) or in combination with 1 mg/kg of a CB2 receptor antagonist (AM630) or a serotonin 5HT₁A receptor antagonist (WAY100635). HI decreased the number of viable neurons and affected the amplitude-integrated EEG background activity as well as different prognostic proton-magnetic-resonance-spectroscopy (H⁺-MRS)-detectable biomarkers (lactate/N-acetylaspartate and N-acetylaspartate/choline ratios). HI brain damage was also associated with increases in excitotoxicity (increased glutamate/N-acetylaspartate ratio), oxidative stress (decreased glutathione/creatine ratio and increased protein carbonylation) and inflammation (increased brain IL-1 levels). CBD administration after HI prevented all these alterations, although this CBD-mediated neuroprotection was reversed by co-administration of either WAY100635 or AM630, suggesting the involvement of CB2 and 5HT₁A receptors. The involvement of CB2 receptors was not dependent on a CBD-mediated increase in endocannabinoids. Finally, bioluminescence resonance energy transfer studies indicated that CB2 and 5HT₁A receptors may form heteromers in living HEK-293T cells. In conclusion, our findings demonstrate that CBD exerts robust neuroprotective effects in vivo in HI piglets, modulating excitotoxicity, oxidative stress and inflammation, and that both CB2 and 5HT₁A receptors are implicated in these effects.

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1. Introduction

Hypoxic–ischemic (HI) brain damage is the most frequent acquired cause of neonatal encephalopathy (Johnston et al., 2011; Mehta et al., 2007). Hypothermia has recently emerged as a promising therapy for newborn HI encephalopathy (NHIE), reducing death and/or disability after HI (Cilio and Ferriero, 2010; Johnston et al., 2011). However, such benefits are not universal and a significant number of asphyxiated infants exhibit no improvements following hypothermia after HI, particularly in severe cases (Cilio and Ferriero, 2010). Thus, it is crucial to develop therapeutic
approaches that can be used synergistically with hypothermia to protect against the damage produced by HI (Cilio and Ferriero, 2010). Cannabinoids are promising candidates given their beneficial effects on many of the parameters associated with HI-induced brain damage, including excitotoxicity, inflammation and oxidative stress (Cilio and Ferriero, 2010; Martinez-Orgado et al., 2007).

It has been shown that the phytocannabinoid cannabidiol (CBD) exerts neuroprotective effects in different models of NHIE (Alvarez et al., 2008; Castillo et al., 2010; Lafuente et al., 2011; Pazos et al., 2012). In vitro, CBD (100 µM) reduces the necrotic and apoptotic damage in forebrain slices from newborn mice exposed to oxygen-glucose deprivation (OGD: Castillo et al., 2010), while administration of CBD to newborn pigs (0.1 mg/kg) in vivo after HI insult reduces the immediate brain damage by modulating cerebral hemodynamic impairment and metabolic derangement in the brain, thereby preventing the development of brain edema and seizures (Alvarez et al., 2008). In these animals neurobehavioral performance is restored 72 h post-HI (Lafuente et al., 2011). Similarly, administering CBD (1 mg/kg) to newborn rats after an HI insult provides long-lasting neuroprotection and restores neurobehavioral function one month after HI (Pazos et al., 2012). Despite these findings, the mechanisms underlying CBD-mediated neuroprotection under HI in the immature brain have scarcely been studied. The neuroprotective effects of CBD are associated with the modulation of excitotoxicity, oxidative stress and inflammation in immature mice brain slices exposed to OGD, where CBD modulates glutamate and cytokine release, as well as the induction of inducible nitric oxide synthase (iNOS) and type 2 cyclooxygenase (COX2: Castillo et al., 2010). CBD does not bind to CB1 receptors, which explains its lack of psychoactive effects (Mechoulam et al., 2007; Pertwee, 2004). However, it remains unclear whether some effects of CBD are mediated by CB2 receptors, although CB2 antagonists may reverse some of the effects of CBD in vitro and in vivo (Sacredote et al., 2005; Ignatowska-Jankowska et al., 2011), including its neuroprotective effect (Castillo et al., 2010). CBD is also thought to be an agonist of serotonin 5HT1A receptors (Russo et al., 2008; Rock et al., 2012; Magnen et al., 2010), 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).

As previous studies of the mechanisms underlying CBD neuroprotection have been carried out in vitro and/or in adult rodent models of HI, we investigated these mechanisms in vivo using the newborn pig model of NHIE. The proximity of this species to newborn pig model of NHIE (Sacerdote et al., 2005) or WAY100635: HCA, n = 6) or serotonin 5HT1A receptors WAY100635 (1 mg/kg) (CBD + WAY100635: HCA, n = 6) was prepared in a 5 mg/ml formulation of ethanol:saline at a ratio of 2:1.17. AM630 or WAY100635 were administered 15 min before CBD and dissolved in the same vehicle. Doses were selected following previous in vivo experiments by our group and others (Collinson and Dawson, 1997; Ignatowska-Jankowska et al., 2011; Pazos et al., 2012). After a further 6 h period of study piglets were killed by KCl infusion and their brains removed and sliced to be placed into 4% paraformaldehyde to perform histological and immunohistochemical studies (left hemisphere) or frozen in isopentane and conserved at −80 °C to perform spectroscopy and biochemical studies and to determine CBD concentration (right hemisphere). All the studies were carried out in the brain area corresponding to 1−5 mm in the posterior plane, as shown in a stereotaxic atlas of pig brain (Fordham et al., 1995).

Piglets similarly managed but with neither HI nor drug treatment, namely sham piglets (SHM, n = 6), served as controls.

2.2. Determination of brain CBD concentration

Samples (10 mg weight) from HC brains were stored at −20 °C until use. The 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).

2.3. Histological analysis

Fixed brain hemispheres were cut into sections (5 mm width) and embedded in paraffin. Coronal sections (4 µm thick) were cut and mounted on a glass slide for staining. To determine early neuronal necrosis, consecutive pairs of brain sections were subjected to OGD, in particular A2A receptors (Castillo et al., 2010).

As previous studies of the mechanisms underlying CBD neuroprotection have been carried out in vitro and/or in adult rodent models of HI, we investigated these mechanisms in vivo using the newborn pig model of NHIE. The proximity of this species to humans and the similar extent of HI damage induced in both species should provide us with data that are more directly relevant for clinical applications.

2. Materials and methods

The experimental protocol met European and Spanish regulations for protection of experimental animals (86/609/EEC and RD 1201/2005) and was 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 necessary to achieve statistical significance.

2.1. Experimental protocol

The protocol was based on the model extensively described elsewhere (Alvarez et al., 2008; Lafuente et al., 2011). Briefly, 1- to 2-day-old male piglets were intubated under 5% sevoflurane anesthesia and then mechanically ventilated (Evita, Dräger, Germany) under sedoanalgia and paralysis by continuous infusion of propofol 12 mg/kg/h and vecuronium 0.6 mg/kg/h through a cannulated ear vein. In each animal, each carotid artery was exposed and surrounded by an elastic band, and a right jugular vein indwelling catheter was placed to infuse dextrose 4 mg/kg/min. Cardiac output (CO), heart rate (HR), mean arterial blood pressure (MABP) and central temperature were monitored (PicCO Plus, Pulson) by a femoral artery indwelling catheter (Ominare CMS24, HP). Body temperature was maintained at 37.5−38 °C by an air-warmed blanket. Arterial blood gases and glycemia were monitored throughout the experimental period and kept between normal limits. Finally, animals were exposed to OGD by interrupting carotid blood flow by pulling out the carotid bands and by reducing inspired oxygen fraction (FiO2) to 10%. HI was confirmed by the suppression of brain activity in aEEG. At the end of the period of HI, carotid flow was restored and FiO2 increased to 11%. Thirty minutes after HI piglets were randomly assigned to receive i.v. vehicle (HV, n = 9) or CBD (HC, 1 mg/kg iv) (n = 11), alone or with the antagonists of CB2 receptors AM630 (1 mg/kg) (CBD + AM630: HCA, n = 6) or serotonin 5HT1A receptors WAY100635 (1 mg/kg) (CBD + WAY100635: HCA, n = 6). CBD was prepared in a 5 mg/ml formulation of ethanol:saline at a ratio of 2:1.17. AM630 or WAY100635 were administered 15 min before CBD and dissolved in the same vehicle. Doses were selected following previous in vivo experiments by our group and others (Collinson and Dawson, 1997; Ignatowska-Jankowska et al., 2011; Pazos et al., 2012). After a further 6 h period of study piglets were killed by KCl infusion and their brains removed and sliced to be placed into 4% paraformaldehyde to perform histological and immunohistochemical studies (left hemisphere) or frozen in isopentane and conserved at −80 °C to perform spectroscopy and biochemical studies and to determine CBD concentration (right hemisphere). All the studies were carried out in the brain area corresponding to 1−5 mm in the posterior plane, as shown in a stereotaxic atlas of pig brain (Fordham et al., 1995).

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to effect suppression of the water resistance and limit the effect of B0 and B1 inhomogeneities in the spectra (relaxation delay-90°; t1-90°; t2-90°) acquire free induction decay (FID) in which a secondary radio frequency irradiation field is applied at the Larmor resonance frequency during the relaxation delay of 2 s and during the mixing period (tM = 150 ms), with f1 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-tetraduteropropionate singlet at 0 ppm. By using the 3.170 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 glutathione/creatinine (GSH/ Cr).

2.5. Western blot studies

The protocol used was as previously described (Pazos et al., 2012), with slight modifications. Frozen brain tissue was homogenized in tissue protein extraction reagent (T-PER; 1 g of tissue/5 mL; Pierce Biotechnology, Rockford, IL) and after centrifugation at 10,000 × g for 5 min at 4 °C, the protein content was measured with a Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) using bovine serum albumin as the standard. OxyBlot protein oxidation detection kit (Millipore Iberica; Madrid, Spain) was used to quantify the presence of protein carbonyl groups in brain tissue. 15 μg 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, Spain) were included on each gel. Proteins were electroblotted onto PVDF membranes (GE Healthcare; Buchinghamshire, UK) in Tris/glycine/methanol transfer buffer at 4 °C under constant voltage (2 h at 250 mA). The resultant blots were blocked in PBS-Tween (PBST) containing 5% nonfat dried milk at 4 °C by overnight incubation. Primary antibody incubation was carried out at 1:150 dilution in PBST containing 5% nonfat dried milk for 1 h at room temperature (RT). After washing with PBST, the membranes were incubated with the secondary antibody (1:300) for 1 h at RT. Finally, the secondary reaction was developed with an Enhance Chemiluminescence (ECL) kit (GE Healthcare; Buchinghamshire, 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 (Libera et al., 2009).

2.6. Microarrays studies

Samples of frozen brain (30 mg) were obtained for determining IL-1 concentration. Tissue proteins were extracted by T-PER (Tissue Protein Extraction Reagent, Pierce Biotechnology, Rockford, IL, USA) and then quantified by BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA); protein concentration was measured in a 12% sodium dodecyl-sulfate–polyacrylamide gel (SDS-PAGE). DNP-BSA Standards (Millipore Iberica; Madrid, Spain) were included on each gel. Proteins were electroblotted onto PVDF membranes (GE Healthcare; Buchinghamshire, UK) in Tris/glycine/methanol transfer buffer at 4 °C under constant voltage (2 h at 250 mA). The resultant blots were blocked in PBS-Tween (PBST) containing 5% nonfat dried milk at 4 °C by overnight incubation. Primary antibody incubation was carried out at 1:150 dilution in PBST containing 5% nonfat dried milk for 1 h at room temperature (RT). After washing with PBST, the membranes were incubated with the secondary antibody (1:300) for 1 h at RT. Finally, the secondary reaction was developed with an Enhance Chemiluminescence (ECL) kit (GE Healthcare; Buchinghamshire, 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 (Libera et al., 2009).

2.7. Determination of brain endocannabinoid levels

Frozen brain samples were subjected to a lipid extraction process as described previously (Patel et al., 2005). Tissue samples were weighed and placed into boriculate glass culture tubes containing two ml of acetonitrile with 84 ml 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 1500 × 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), oleoylthanolamide (OEA) and palmitoylthanolamide (PEA) were determined in the lipid extracts using iso-tate dilution, liquid chromatography–mass spectrometry as described previously (Patel et al., 2005).

2.8. Bioluminescence resonance energy transfer (BRET) assays

The equivalent of 20 μg protein of transfected cell suspensions was distributed in 96-well microplates (white plates; Porvair, Leatherhead, UK) and 5 mM coenzyme A (H2K GMBH, Germany) was added. After 1 min of adding coenzyme A, the membranes were collected using 125 μl LB 940 equilibration buffer (Eppendorf, Germany), which allows the integration of the signals detected in the short-wavelength filter at 485 nm (440–500 nm) and in the long-wavelength filter at 530 nm (510–590 nm). To quantify receptor-Rluc expression luminescence readings were performed after 10 min of adding 5 mM coenzyme A. To quantify expression of YFP constructs, cells (20 μg protein) were distributed in 96-well microplates (black plates with a transparent bottom; Porvair, Leatherhead, UK) and fluorescence was read in a Mithras LB 940 using an excitation filter of 485 nm. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)] – C, where C corresponds to [(long-wavelength emission)/(short-wavelength emission)] in the absence of receptor-YFP expression. BRET curves were fitted by using a non-linear regression equation, assuming a single phase with GraphPad Prism software (San Diego, CA, USA). BRET is expressed as milli BRET units (mBU: 1000 × net BRET).

3. Results

No significant differences were found between the distinct treatment groups in terms of age (1.8 ± 0.1, 1.8 ± 0.1, 1.9 ± 0.1, 1.8 ± 0.1 and 1.8 ± 0.1 d for SHM, HV, HC, HCA and HCW, respectively) or weight (1.9 ± 0.1, 1.7 ± 0.1, 1.9 ± 0.1, 1.8 ± 0.1 and 1.9 ± 0.1 kg for SHM, HV, HC, HCA and HCW, respectively). Of a total of 38 animals, only two piglets died in the 90 min following the HI insult (one assigned to the HV and the other to the HC group). Six hours after the administration of CBD (1 mg/kg) in our current formulation, the CBD concentration in brain tissue was 58 ± 14 ng/g.

With the exception of the HCW group in which CO levels fell throughout the experimental period, no differences in CO levels were observed between the distinct groups (Table 1). The HI insult was associated with a progressive decrease in MABP in HV-treated animals (Table 1), such that half of the HV piglets required inotropic drug support (dopamine, mean dose 13 ± 4 μg/kg/min). This decrease in MABP was not observed in the HC group, and no piglets from this group required inotropic support. By contrast, the MABP dropped 15–20 mmHg during the experimental period in piglets treated with CBD in combination with either CB2 or 5HT1A receptor antagonists. This effect was more dramatic in HCW piglets (Table 1).
3.1. CBD treatment resulted in the recovery of brain activity

Continued sedoanalgesia determined that aEEG amplitude decreased slightly in SHM animals throughout the experiment (Fig. 1a), although this effect was not associated with an impairment of background EEG activity and/or the EEG pattern (Fig. 1b). HI led to a dramatic decrease in brain activity together with a severe disruption of the background EEG pattern, neither of which recovered during the following 6 h (Fig. 1). CBD administration led to the progressive recovery of both brain activity (59.3 ± 6% of basal activity at 6 h) and the background EEG pattern (Fig. 1). These effects of CBD were abolished by co-administration with either AM630 or WAY100635 (Fig. 1).

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).

3.2. CBD protected neurons and increased the number of astrocytes

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 h after insult (Fig. 2), although this increase was blunted by CBD administration (Fig. 2). The beneficial effect of CBD disappeared when it was administered along with either CB2 or 5HT1A antagonists (Fig. 2). In the HV group, no reduction in the number of GFAP+ cells was evident in the cortex after HI insult (Fig. 2). 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.

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 ± 3.5% and 18.2 ± 3.2% for AM630 and WAY100635, respectively, p > 0.05 vs. HV).

3.3. CBD improved H1-MRS prognostic markers and modulated excitotoxicity

Lac/NAA and NAA/Cho ratios are used as prognostic markers. 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 (Fig. 3). 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 (Fig. 3). The normal Glu/NAA ratios were restored following CBD administration, but not when it was administered along with a CB2 or 5HT1A antagonist (Fig. 3).

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; and Glu/NAA: 0.69 ± 0.02 and 0.64 ± 0.04; for AM630 and WAY100635, respectively, p > 0.05 vs. HV).

3.4. CBD modulated oxidative stress and neuroinflammation

HI-induced increases in oxidative stress were analyzed by measuring the GSH/Cr ratio and the levels of protein carboxylation. HI insult diminished the brain GSH/Cr ratio determined by H+–MRS (Fig. 3) and additionally, OxyBlot studies revealed that HI increased the protein carboxylation in the brain (Fig. 4A). CBD administration after HI had a significant antioxidant effect, blunting both the decrease in the GSH/Cr ratio (Fig. 3) and the increase in protein carboxylation (Fig. 4A). However, the antioxidant effect of CBD was again lost when it was administered in combination with a CB2 or 5HT1A antagonist (Fig. 4B).

The effect of HI on oxidative stress or inflammation 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; IL-1: 160 ± 25 and 140 ± 15 pg/mL; for AM630 and WAY100635, respectively, p > 0.05 vs. HV).

3.5. CBD administration did not increase endocannabinoid levels in the brain

HI increased the levels of AEA, 2-AG, PEA and OEA in the brain, evident in brain tissue taken from HV animals (Fig. 5). As CBD was reported to reduce AEA uptake and/or degradation in vitro (Pertwee, 2004; Mechoulam et al., 2007), we investigated whether the contribution of CB2 receptors to the effects of CBD was due to a CBD-induced increase in brain endocannabinoid levels following HI insult. Our results indicate that this is not the case and that although brain endocannabinoid levels augmented in HV animals, similar levels were detected in CBD-treated and SHM animals (Fig. 5).

3.6. CB2 and 5HT1A receptors form heterodimers in living cells

A receptor heteromer is a macromolecular complex composed of at least two (functional) receptor units with biochemical properties that are demonstrably different from those of its individual components (Ferré et al., 2009). Heteromers are often identified indirectly in natural tissues through the detection of a so-called biochemical fingerprint. One recently discovered biochemical fingerprint of heteromers is cross-antagonism, i.e., the blockade of an agonist’s activity by a selective antagonist of any of the receptors in the heteromer (Moreno et al., 2011). Accordingly, the cross-antagonism demonstrated in the aforementioned effects of CBD may reflect the formation of heteromers containing CB2 or 5HT1A receptors. BRET studies indicated strong energy transfer, i.e., close proximity of the donor and acceptor in the C-terminus of the two fusion proteins to generate a strong BRET signal (Fig. 6). The saturation of the BRET signal when the amount of the acceptor was increased (CB2R–YFP) indicated that the interaction between CB2R and 5HT1A R to form CB2R/5HT1A heteromers was specific. Fitting the data as described in Methods allowed the BRET parameters to be calculated: maximum BRET = 116 ± 6 mBU; BRET50 = 18 ± 3.
The linear relationship between the BRET and YFP/Rluc ratios obtained when using D4,2R YFP (4.2 isoform of the human dopamine D4 receptor) as a negative control also confirmed the specificity of the CB2R/5HT1AR interaction in live HEK-293 cells.

4. Discussion

The present findings indicate that CBD administration after a hypoxic ischemic insult provides neuroprotection in newborn pigs by modulating several key processes that promote damage in the immature brain, namely excitotoxicity, inflammation and oxidative stress (Cilio and Ferriero, 2010; Johnston et al., 2011; Martinez-Orgado et al., 2007; Mehta et al., 2007). In newborn rats, CBD brain concentration peaks 3–6 h post-administration of 1 mg/kg in ethanol:solutol:saline 2:1:17 (Pazos et al., 2012). In the current experiments, 6 h after the administration of CBD in the same dose and formulation, the levels of CBD in the brain were 58 ng/g, equivalent to 200 nM. This was two-fold higher than those detected...
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 and 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 relatively low doses of CBD (1 mg/kg) enhanced the mean amplitude and restored the neural activity. The Lac/NAA ratio calculated by $^1$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 $^1$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 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 infant 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-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. 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. The deleterious effects of oxidative stress are magnified in the immature brain due to low levels of antioxidant activity and a 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 $^1$H-MRS, and its reduction in the brain correlates with oxidative stress (Satoh and 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). Inflammation also plays a key role in HI-induced damage in the immature brain (Johnston et al., 2011) and CBD has a wide range of anti-inflammatory properties, modulating cytokine release and exhibiting anti-inflammatory effects both in vivo and in vitro (Mechoulam et al., 2007; Pertwee, 2004). Among the different pro-
inflammatory cytokines IL-1 is particularly important in the context of HI-induced brain damage (Allan and 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 (Oygur et al., 1998), suggesting that modulation of IL-1 may have neuroprotective effects (Allan and Rothwell, 2001). Significantly, we found that CBD prevented the HI-induced increase in IL-1 levels in piglets.

CBD inhibits 5HT re-uptake and acts as an agonist of 5HT1A receptors (Russo et al., 2005; Rock et al., 2012; Magen et al., 2010). In our work WAY100635 reversed the neuroprotective effects of CBD, including the CBD-mediated modulation of glutamate release, oxidative stress and inflammation. This is the first time that the involvement of 5HT1A receptors in CBD-mediated neuroprotection has been demonstrated in the immature brain. Blockade of 5HT1A 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 5HT1A receptor. Indeed, the fact that WAY100635 reversed the beneficial systemic hemodynamic effects of CBD after HI supports this hypothesis.

CBD inhibits SHT re-uptake and acts as an agonist of 5HT1A receptors (Russo et al., 2005; Rock et al., 2012; Magen et al., 2010). In our work WAY100635 reversed the neuroprotective effects of CBD, including the CBD-mediated modulation of glutamate release, oxidative stress and inflammation. This is the first time that the involvement of 5HT1A receptors in CBD-mediated neuroprotection has been demonstrated in the immature brain. Blockade of 5HT1A 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 5HT1A receptor. Indeed, the fact that WAY100635 reversed the beneficial systemic hemodynamic effects of CBD after HI supports this hypothesis.

Although it is generally assumed that CBD does not bind to CB2 receptors (Pertwee et al., 2010), CBD neuroprotection was abolished when it was administered with AM630. Since CBD inhibits the uptake and/or hydrolysis of several endocannabinoids, including anandamide (Pertwee, 2004), the involvement of CB2 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

![Fig. 5. HI-induced increase of brain endocannabinoid levels was prevented by CBD.](image)

**Fig. 5.** HI-induced increase of brain endocannabinoid levels was prevented by CBD. Brain concentration of endocannabinoid level was quantified by liquid chromatography–mass spectrometry in samples from 1- to 2-day-old piglets after sham operation (SHM) or after hypoxic-ischemic (HI) insult and treatment with vehicle (HV) or CBD (HC). See Section 2.7 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.

![Fig. 6. SHT1A receptors form heteromers with CB2 receptors in living cells. BRET saturation experiments showing CB2R/SHT1A heteromerization were performed using HEK-203 cells transfected with 0.5 µg of cDNA corresponding to SHT1A-Rhluc and increasing amounts of cDNA (0–2 µg cDNA) corresponding to CB2R-YFP (circles). As negative control, cells transfected with cDNA corresponding to SHT1A-Rhluc (0.5 µg) and to D4,2R-YFP (0–4 µg cDNA) were also used (squares). Both fluorescence and luminescence for each sample were measured before every experiment to confirm similar donor expressions (approximately 100,000 bioluminescence units) while monitoring the increase in acceptor expression (up to 70,000 net fluorescence units). The relative amount of BRET is given as the ratio between the net fluorescence of the acceptor (YFP) and the luciferase activity of the donor (Rluc). BRET data are expressed as means ± SEM. Of 4–8 different experiments grouped as a function of the amount of BRET acceptor. See Section 2.8 for details.](image)
ischemic events in the brain (Hillard, 2008). By contrast, endo-
cannabinoid 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 endo-
cannabinoids 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 CB2 receptor acti-
vation by CBD through the increase in endocannabinoid levels. CB2 receptor antagonism blocks the effects of CBD on cytokine release in cultured cells (Sacerdote et al., 2005), rat body weight gain (Ignatowska-Jankowska et al., 2011) and cell death in newborn forebrain slices exposed to OGD (Castillo et al., 2010). Thus, the involvement of CB2 receptors in some of the 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 signaling mediated by the hetero-
eromer (Ferré et al., 2009; Moreno et al., 2011). Hence, we propose that 5HT1A and CB2 receptors form heteromers. Our BRET data support the view that these two receptors form heteromers in living cells and moreover, the BRET50 value indicated a relatively high affinity of these receptors, indicating a high probability of heterodimer formation in cells co-expressing these two receptors.

5. Conclusions

In conclusion, CBD administration after HI in newborn piglets resulted in robust neuroprotection. The neuroprotective effects of CBD were not evident when it was co-administered with WAY100635, suggesting that 5HT1A receptors are involved in CBD-induced neuroprotection. Similarly, co-administration of AM630 also reversed the neuroprotective effects of CBD, implicating CB2 receptors in these effects. Finally, BRET analyses demonstrated that heteromers of CB2 and 5HT1A receptors are likely to form. Thus, together these data point to CB2/5HT1A heteromers as important targets for CBD-based therapies for HI brain injury, a condition for which no effective therapies currently exist.

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