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

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**Abbreviations:** AEA, arachidonylethanolamide; 2-AG, 2-arachidonoylglycerol; aEEG, amplitude-integrated EEG; CBD, cannabidiol; CD, cardiac output; HI, hypoxic–ischemic; NHIE, newborn HI encephalopathy; OEA, oleoylethanolamide; OGD, oxygen-glucose deprivation; PEA, palmitoylethanolamide.

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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 (1H-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/creatinine 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 mouse 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 (Sacerdote 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., 2005; Rock et al., 2012). In the immature brain, these receptors have not been studied extensively, although they are known to mediate some of the actions of CBD. Adenosine receptors are also involved 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 A2B 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 number 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 sedoanaesthesia 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 (MAPB) and central temperature were monitored (Picollo 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, all animals with an i.v. catheter in the OGD dose group were allocated into three groups (n = 9) or CBD (HC, 1 mg/kg) (CBD + HC) or control (HV, 1-2 day-old male piglets) (HV) (Castillo et al., 2010). Thirty minutes after Hl 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) (HC + AM630), HCA, n = 6) or serotonin 5HT1A receptors WAY100635 (1 mg/kg) (HC + WAY100635), HCW, 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 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 (Ferrington, 1999). 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 of the same hemisphere were stained by the Nissl method (Alvarez et al., 2008; Lafuente et al., 2011). Apparently normal areas of sections were analyzed by the presence of typical nuclei with clear nucleoplasm and a normal background (4: continuous background; 3: discontinuous normal voltage; 2: burst suppression; 1: continuous low voltage; 0: inactive, isoelectric pattern) (Tichauer et al., 2009). After a 2.5 min period of stabilization, piglets underwent a 30-min long cerebral HI insult, 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 21%. 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) (HC + AM630), HCA, n = 6) or serotonin 5HT1A receptors WAY100635 (1 mg/kg) (HC + WAY100635), HCW, 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 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 (Ferrington, 1999). Piglets similarly managed but with neither HI nor drug treatment, namely sham piglets (SHM, n = 6), served as controls.

2.4. Proton-magnetic-resonance-spectroscopy (1H–MRS)

H+MRS was performed in the MRI Unit of the Instituto Pluridisciplinar (Universidad Complutense, Madrid, Spain) at 500.13 MHz using a Bruker AMX500 spectrometer 11.7 T operating at 4 °C 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 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 C-terminal end of the receptor (5HT1AR-Rluc; CB2-R-YFP or DBA-R-YFP). Expression of constructs was tested by confocal microscopy and the receptor functionality by ERK1/2 activation pathway.

2.8.2. Cell cultures and transient transfection

Human embryonic kidney 293T (HEK-293T) cells were grown in DMEM supplemented with 2 mM-L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin/streptomycin, and 5% (v/v) heat-inactivated fetal bovine serum (FBS) (all supplements were from Invitrogen (Paisley, Scotland, UK)). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2, and were passaged when they were 70-80% confluent, i.e. approximately twice a week. HEK-293T cells were transiently transfected with the indicated fusion protein cDNA by the ramified PEI (Poly-Ethylentimine, Sigma, St. Louis, MO, USA) method. Cells were incubated (4 h) with the corresponding cDNA together with ramified PEI (5 mL of 10 mM PEI for each mg cDNA) and 150 mM NaCl in a serum-starved medium. After 4 h, the medium was changed to a fresh complete culture medium and cells were used 48 h after transfection. To control the cell number, sample protein concentration was determined using the Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin dilutions as standards.

2.8.3. 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 μM coelenterazine H (PerkinElmer, Wellesley, MA, USA). BRET is expressed as milli BRET units (mBU: 1000 arbitrary units), statistical analysis was performed after 10 min of adding 5 μM coelenterazine H. 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 (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 μM coelenterazine H 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 mili BRET units (mBU: 1000 × net BRET).

2.9. Chemicals and statistical analyses

CBD was a generous gift from GW Pharma (Leeds, UK). Solutis H515 was gifted by BASF Española SL (Barcelona, Spain). AM630 and WAY100635 were obtained from Biogen Científico SL (Madrid, Spain) and, unless otherwise stated, the other chemicals were obtained from Sigma (Madrid, Spain). SPSS 15.0.0 software was used for all statistical analyses. Median values have been used and compared using the Kruskal–Wallis analysis of ranks with the Dunn’s post test for multiple comparisons. A p value < 0.05 was considered to be significant. All data are presented as means ± standard error.

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 from this group required inotropic support. By contrast, the MABP decrease in MABP was not observed in the HC group, and no piglets treated with CBD in combination with either CB2 or 5HT1A receptor antagonists. This effect was more dramatic in HCW piglets (Table 1).
and 5 of the 6 piglets in this group required inotropic drug support (dopamine, mean dose 11.2 ± 1.2 μg/kg/min). A decrease in pH was observed during the experimental period in all HI groups, although this effect was more severe in the HCA and HCW groups (Table 1). No differences in CO₂ levels were observed among the piglets in the different groups.

### 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 ± 9% 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. 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 (Fig. 2).

The effect of HI on aEEG was not different in animals receiving AM630 or WAY100635 alone than in those receiving vehicle (31.2 ± 6% 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 ± 2.4% and 18.2 ± 3.2% for AM630 and WAY100635, respectively, p > 0.05 vs. HV).

### 3.3. CBD improved H⁺-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 of HI on neuronal death was not different in animals receiving AM630 or WAY100635 alone than in those receiving vehicle (31.2 ± 6% and 28.1 ± 6% of basal activity at 6 h for AM630 and WAY100635, respectively, p > 0.05 vs. HV).


#### Table 1

Cardiorespiratory parameters.

<table>
<thead>
<tr>
<th></th>
<th>SHM (n = 6)</th>
<th>HV (n = 8)</th>
<th>HC (n = 10)</th>
<th>HCA (n = 5)</th>
<th>HCW (n = 5)</th>
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<tr>
<td>CO</td>
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<td>33.9(3.5)</td>
<td>35.7(2.5)</td>
<td>34.4(3.9)</td>
<td>34.4(2.8)</td>
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<tr>
<td>D</td>
<td>35.5(2.7)</td>
<td>36.9(4.1)</td>
<td>33.4(4.1)</td>
<td>33.2(2.2)</td>
<td>36.2(2.3)</td>
</tr>
<tr>
<td>E</td>
<td>35.0(3.6)</td>
<td>34.4(4.2)</td>
<td>33.6(4.3)</td>
<td>32.0(3.6)</td>
<td>29.6(2.1)*</td>
</tr>
<tr>
<td>MBP</td>
<td>79.3(5.0)</td>
<td>80.1(3.3)</td>
<td>82.5(2.6)</td>
<td>83.1(5.1)</td>
<td>76.9(3.1)</td>
</tr>
<tr>
<td>P</td>
<td>90.5(5.8)</td>
<td>86.0(7.1)</td>
<td>91.4(7.8)</td>
<td>95.4(4.5)</td>
<td>85.1(5.3)</td>
</tr>
<tr>
<td>E</td>
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<td>59.6(3.1)</td>
<td>78.8(2.5)</td>
<td>68.4(2.5)</td>
<td>56.4(7.1)</td>
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<tr>
<td>pH</td>
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<td>7.32(0.02)</td>
<td>7.35(0.02)</td>
<td>7.32(0.01)</td>
<td>7.35(0.01)</td>
</tr>
<tr>
<td>E</td>
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<td>7.32(0.02)</td>
<td>7.32(0.02)</td>
<td>7.25(0.03)</td>
<td>7.23(0.04)</td>
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<tr>
<td>pCO₂</td>
<td>38.8(2.5)</td>
<td>42.2(2.2)</td>
<td>42.4(2.3)</td>
<td>42.1(3.1)</td>
<td>40.1(1.6)</td>
</tr>
<tr>
<td>E</td>
<td>38.8(1.1)</td>
<td>42.2(2.9)</td>
<td>42.3(1.6)</td>
<td>42.5(2.7)</td>
<td>39.9(2.5)</td>
</tr>
</tbody>
</table>

Values obtained from 1- to 2-day-old piglets after sham operation (SHM) or after hypoxia–ischemia and treatment with vehicle (HV), CBD (HC), CBD + AM630 (HCA) or CBD + WAY100635 (HCW).

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.

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**Fig. 1.** CBD-induced recovery of brain activity after HI was reversed by 5HT₁A or CB₂ receptor antagonists. Brain activity studied by continuous aEEG recording 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 + WAY100635 (HCW). Top: Changes of mean amplitude of aEEG trace throughout the experiment. Bottom: Qualitative assessment of aEEG background activity by a neurological activity score. Results are expressed as means ± SEM of 6–10 animals. (*) p < 0.05 vs SHM. (!) p < 0.05 vs HC. See Section 2.1 for details.
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 carbonylation. HI insult diminished the brain GSH/Cr ratio determined by H+-MRS (Fig. 3) and additionally, OxyBlot studies revealed that HI increased the protein carbonylation 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 carbonylation (Fig. 4A). However, the antioxidant effect of CBD was again lost when it was administered in combination with either AM630 or WAY100635 (Figs. 3 and 4A). Oxidative stress is often accompanied by neuroinflammation, evident through the increased levels of interleukins, and accordingly, HI was accompanied by a significant increase in IL-1 levels in the cortex (Fig. 4B). In accordance with its antioxidant effects, CBD reduced IL-1 levels in lesioned animals, although this effect was prevented when it was administered along 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 5HT1AR to form CB2R/5HT1AR 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; Martínez-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 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 effect of CBD was 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-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 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).

Fig. 4. CBD-induced reduction of brain protein carbonylation and IL-1 production after HI was reversed by SHT1a or CB2 receptor antagonists. (A) 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 hypoxic-ischemic (HI) insult and treatment with vehicle (HV), CBD (HC), CBD + AM630 (HCA) or CBD + WAY100630 (HCW). 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 (see Section 2.5 for details). (B) Brain concentration of IL-1 quantified by microarrays in samples from the aforementioned groups. Bars represent the mean ± SEM of 6–8 experiments. (*) p < 0.05 vs. SHM.

in newborn rats (Pazos et al., 2012), suggesting greater bioavailability in large mammals than in rodents. 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). However, we cannot rule out the possibility that brain damage was somehow mitigated in HV, HCA or HCW animals since MABP stabilization by dopamine infusion reduces brain damage in experimental meningitis in newborn pigs (Park et al., 2003).
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.

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. 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: Arachidonylthanolamide; 2-AG: 2-Arachidonoylglycerol; OEA: Oleylethanolamide; PEA: Palmitoylethanolamide. (*) p < 0.05 vs. SHM.

Fig. 6. 5HT1A receptors form heteromers with CB2 receptors in living cells. BRET saturation experiments showing CB2R/5HT1A heteromerization were performed using HEK-203 cells transfected with 0.5 μg of cDNA corresponding to 5HT1AR-Rluc and increasing amounts of cDNA (0–2 μg cDNA) corresponding to CB2R–YFP (circles). As negative control, cells transfected with cDNA corresponding to 5HT1AR-Rluc (0.5 μg) and to D4,2R–YFP (0–4 μg cDNA) were 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.
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 CB2 receptor activation 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 CBD (Mechoulam et al., 2007) cannot be completely ruled out. 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 heteromorphic 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 heteromer (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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