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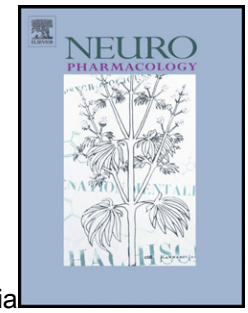

Mechanisms Of Cannabidiol Neuroprotection In Hypoxic-Ischemic Newborn Pigs: Role Of 5HT1A And CB2 Receptors

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Title: 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 minutes 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 CB_2 receptor antagonist (AM630) or a serotonin $5HT_{1A}$ 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 gluthatione/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 CB_2 and 5HT1A receptors. The involvement of CB_2 receptors was not dependent on a CBD-mediated increase in endocannabinoids. Finally, bioluminescence resonance energy transfer studies indicated that CB_2 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 CB_2 and $5HT_{1A}$ receptors are implicated in these effects.

Keywords: hypoxia-ischemia; brain; neuroprotection; cannabidiol; cannabinoids; serotonin; newborn; pigs

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 (Cillio & 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 (Cillio & 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 (Cillio & 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 (Cillio & 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, 2010; 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 a 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 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

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

induction of inducible nitric oxide synthase (iNOS) and type 2 cyclooxygenase (COX2: Castillo et al, 2010). CBD does not bind to CB_1 receptors, which explains its lack of psychoactive effects (Mechoulam 2008; Pertwee 2004). However, it remains unclear whether some effects of CBD are mediated by CB_2 receptors, although 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 $5HT_{1A}$ receptors (Russo et al, 2005; Rock et al, 2010; Magen et al, 2012), 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 A_{2A} 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, 2010). Briefly, one-to-two day-old male piglets were intubated under 5% sevoflurane anaesthesia and then mechanically ventilated (Evita, Dräger, Germany) under sedo-analgesia 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 monitorized (PiCCO Plus, Pulsion) by a femoral artery indwelling catheter (Ominare CMS24, HP). Body temperature was maintained at 37.5-38 ºC by an air-warmed blanket. Arterial blood gases and glycaemia were monitorized throughout the experimental period and kept between normal limits. Finally, stainless steel wires were placed into the piglet head scalp to continuously monitor brain activity by amplitude-integrated EEG (aEEG) (BRM3, BrainZ Instruments). 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) (Tichauer et al, 2009). After a 30 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) to10%. 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 min 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: 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. Doses were selected following previous *in vivo* experiments by our group and others (Collinson& Dawson, 1997; Ignatowska-Jankowska et al, 2010; 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 ºC 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 (Felix et al, 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 ºC 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 stained by the Nissl method (Alvarez et al, 2008; Lafuente et al, 2010). Areas of 1 mm2 in the central three lobes of the parietal cortex were examined, focusing on layers II-III, by an investigator blinded to the experimental group using an optical microscope (x 400) and a grid of 50 compartments; the mean of 3 compartments was calculated. Parietal cortex was selected to illustrate brain histological damage because this is the most vulnerable area to HI in piglets and damage seen in this area correlates with that observed in other vulnerable areas as hippocampus or striatum (Foster et al, 2001; Iwata et al, 2005; Alvarez et al, 2008). 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). Then, to asses glial activation Glial Fibrillary Acidic Protein (GFAP) positive cells (astrocytes) were identified in the same areas by inmunohistochemistry. Briefly, tissue sections were deparaffinizied and extensively washed in phosphate-buffered saline (PBS 0.1M) and incubated with the GFAP-Cy3 conjugated antibody (1:1000) at 4ºC overnight. After incubation with the primary antibody, sections were washed in distilled water and the nuclei were counterstained with TO-PRO (1:500, Life Technologies; Madrid, Spain). Finally, the slides were mounted in aqueous medium with Vectashield (Vector Laboratories; Burlingame, UK). Visualization and photography of the samples was carried out with an epifluorescence Optika B600TIFL microscope coupled to a Optikam Procool 3 camera (Optika, BG, Italy). By using the

ImageJ 1.43u software (NIH, Bethesda, USA), the glial cell density was calculated in three different areas (0.3 mm2) from the central three lobes of the parietal cortex.

2.4. Proton magnetic resonance spectroscopy (H^+-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 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).

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 10000 x g for 5 min at 4 ºC, 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; Buckinghamshire, UK) in Tris/glycine/methanol transfer buffer at 4 ℃ under constant voltage (2 h at 250mA). The resultant blots were blocked in PBS-Tween (PBST) containing 5% nonfat dried milk at 4 ºC by overnight incubation. Primary antibody incubation was carried out at 1:150 dilution in PBST containing 5% nonfat dried milk for 1 hr at room temperature (RT). After washing with PBST, the membranes were incubated with the secondary antibody (1:300) for 1 hr at RT. Finally, the peroxidase reaction was developed with an Enhance Chemiluminescence (ECL) Kit (GE Healthcare; Buckinghamshire, 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 adjusted to 500 µg/mL per well. Cytokin concentration was then measured by a microarray panel specific for swine tissue (Quantibody Porcine Cytokine Array, RayBiotech, Inc., Atlanta, GA, USA). Microarrays were scanned at the Parque Científico de Madrid (Spain).

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 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℃ 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℃ until analysis. The contents of ara chidonylethanolamide (AEA), 2-arachidonoylglycerol (2-AG), oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) were determined in the lipid extracts using isotope-dilution, liquid chromatography-mass spectrometry as described previously (Patel et al, 2005).

2.8. Bioluminescence resonance energy transfer (BRET)

2.8.1. Fusion proteins and expression vectors: The human cDNA for the CB_2, serotonin $5HT_{1A}R$ and dopamine $D_{4.2}$ receptors cloned in pcDNA3.1 were amplified without their stop codons using sense and antisense primers harbouring either unique EcoRI and BamH1 sites (CB_2R), EcoRI and BamH1 ($5HT_{1A}R$) or Xho1 and EcoR1 ($D_{4.2}R$). The fragments were then subcloned to be in-frame with Rluc into the EcoRI and BamH1 ($5HT_{1A}R$) restriction site of an Rluc-expressing vector (pRluc-N1, PerkinElmer, Wellesley, MA), or into the EcoRI and BamH1 (CB_2R) or Xho1 and EcoR1 ($D_{4.2}R$) restriction site of an EYFP expressing vector (EYFP-N1; enhanced yellow variant of GFP; Clontech, Heidelberg, Germany), to give the plasmids that express $5HT_{1A}$, CB_2 or $D_{4.2}$ receptor fused to Rluc or YFP on the C-terminal end of the receptor ($5HT_{1A}R$-Rluc, CB_2R-YFP or $D_{4.2}R$-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℃ in a humidified atmosphere of 5% CO2, and were passaged when they were 80-90% confluent, i.e. approximately twice a week. HEK-293T cells were transiently transfected with the indicated fusion protein cDNA

by the ramified PEI (PolyEthylenImine, 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 forty-eight hours 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 (PJK GMBH, Germany) was added. After 1 minute of adding coelenterazine H, readings were collected using a Mithras LB 940 equipment (Berthold, Bad Wildbad, 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 minutes 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)]-Cf where Cf 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 X net BRET).

2.9. Chemicals and statistical analyses

CBD was a generous gift from GW Pharma (Leeds, UK). Solutol HS15 was gifted by BASF Española SL (Barcelona, Spain). AM630 and WAY100635 were obtained from Biogen Cientifica 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 compared using the Kruskall-Wallis analysis of ranks with the Dunn's post hoc 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 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 CB_2 or $5HT_{1A}$ 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 CO_2 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 hours (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. 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 hours 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 CB_2 or $5HT_{1A}$ 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 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 CB_2 or $5HT_{1A}$ 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 CB_2 or $5HT_{1A}$ 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, 2008), we investigated whether the contribution of CB_2 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. CB_2 and $5HT_{1A}$ 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 CB_2 or $5HT_{1A}$ 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 (CB_2R-YFP) indicated that the interaction between CB_2R and $5HT_{1A}R$ to form $CB_2R/5HT_{1A}R$ heteromers was specific. Fitting the data as described in Methods allowed the BRET parameters to be calculated: maximum BRET = 116 ± 6 mBU; $BRET_{50}$ = 18 ± 3. The linear relationship between the BRET and YFP/Rluc ratios obtained when using $D_{4,2}R$-YFP (4.2 isoform of the human dopamine D_4 receptor) as a negative control also confirmed the specificity of the $CB_2R/5\text{-}HT_{1A}R$ 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 process that promote damage in the immature brain, namely excitotoxicity, inflammation and oxidative stress (Cillio & 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, six hours 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 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).

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 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 (Thayer 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-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 & 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 & 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). 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 $5HT_{1A}$ receptors (Russo et al 2005; Rock et al, 2010; Magen et al, 2012). 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 $5HT_{1A}$ receptors in CBD-mediated neuroprotection has been demonstrated 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.

Although it is generally assumed that CBD does not bind to CB_2 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 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. CB_2 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, 2010) and cell death in newborn 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. 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, 2009b; 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 heteromer (Ferre et al, 2009; Moreno et al, 2011). Hence, we propose that $5HT_{1A}$ and CB_2 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 $5HT_{1A}$ receptors are involved in CBD-induced neuroprotection. Similarly, co-administration of AM630 also reversed the neuroprotective effects of CBD, implicating CB_2 receptors in these effects. Finally, BRET analyses demonstrated that heteromers of CB_2 and $5HT_{1A}$ receptors are likely to form. Thus, together these data point to $CB_2/5HT_{1A}$ 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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Figure legends

Figure 1. CBD-induced recovery of brain activity after HI was reversed by 5-HT_{1A} or CB_2 receptor antagonists. Brain activity studied by continuous aEEG recording in 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). *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 *2.1. Experimental protocol* for details.

Figure 2. CBD-induced prevention of neuronal and astroglial death after HI was abolished by 5-HT_{1A} or CB_2 receptor antagonists. Representative light microphotographs of Nissl (*top*) or GFAP (*bottom*) 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). In brain from HV there is an increase in number of pyknotic cells and a decrease in number of viable neurons and GFAP+ cells (arrows). Administration of CBD reduced the presence of pyknotic cells and the loss of viable neurons and GFAP+ cells. Original magnification x200, bar: 100 µm. Results are expressed as means ± SEM of 6-10 animals. (*) $p<0.05$ vs. SHM.

Figure 3. CBD-induced improvement of H^+-MRS biomarkers after HI was reversed by 5-HT_{1A} or CB_2 receptor antagonists. *Top:* representative brain H^+-MRS spectrum from a normal piglet, showing the peaks of the different metabolites studied. 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; Cr: creatine; Lac: lactate; Glu: glutamate; GSH: reduced gluthation; NAA: N-acetylaspartate. (*) $p<0.05$ vs. SHM.

Figure 4. CBD-induced reduction of brain protein carbonylation and IL-1 production after HI was reversed by 5-HT$_{1A}$ or CB$_2$ 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 hypoxia-ischemia (HI) 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 *2.5. Western blot studies* 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.

Figure 5. HI-induced increase of brain endocannabinoid levels was prevented by CBD. Brain concentration of endocannabinoid leves 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.

Figure 6. **5-HT$_{1A}$ receptors form heteromers with CB$_2$ receptors in living cells**. BRET saturation experiments showing CB$_2$R/5-HT$_{1A}$R heteromerization were performed using HEK-203 cells transfected with 0.5 µg of cDNA corresponding to 5-HT$_{1A}$R-Rluc and increasing amounts of cDNA (0 to 2 µg cDNA) corresponding to CB$_2$R-YFP (circles). As negative control, cells transfected with cDNA corresponding to 5-HT$_{1A}$R-Rluc (0.5 µg) and to D$_{4,2}$R-YFP (0 to 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 100000 bioluminescence units) while monitoring the increase in acceptor expression (up to 70000 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 *2.8. Bioluminescence resonance energy transfer (BRET)* for details.

Table 1. Cardiorespiratory parameters.

		SHM (n=6)	HV (n=8)	HC (n=10)	HCA (n=5)	HCW (n=5)
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)

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 + WAY100630 (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

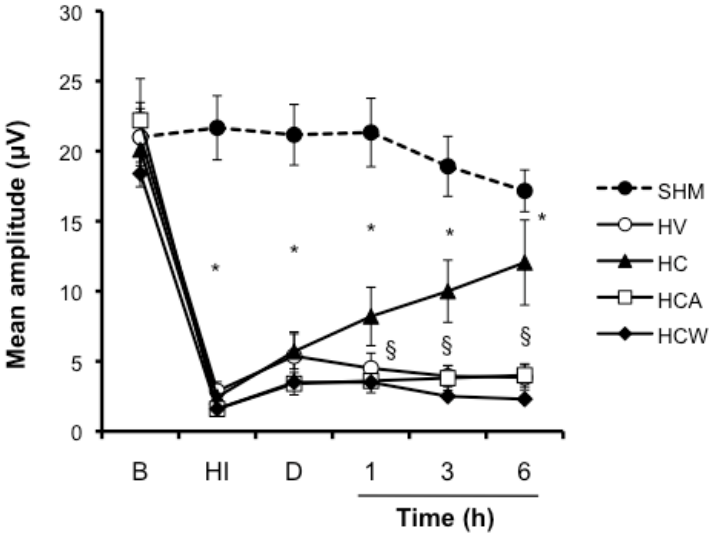
Highlights

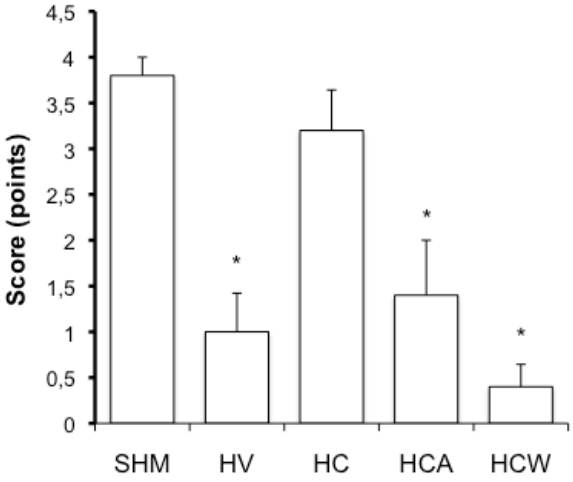
- CBD administration prevents neuron and astrocyte damage after hypoxia-ischemia.
- CBD modulates excitotoxicity, inflammation and oxidative stress.
- $5HT_{1A}$ and CB_2 receptors are involved in CBD neuroprotection.
- CB_2 involvement in CBD effects is not because of endocannabinoid levels enhancement
- $5\text{-}HT_{1A}$ receptors form heteromers with CB_2 receptors in living cells

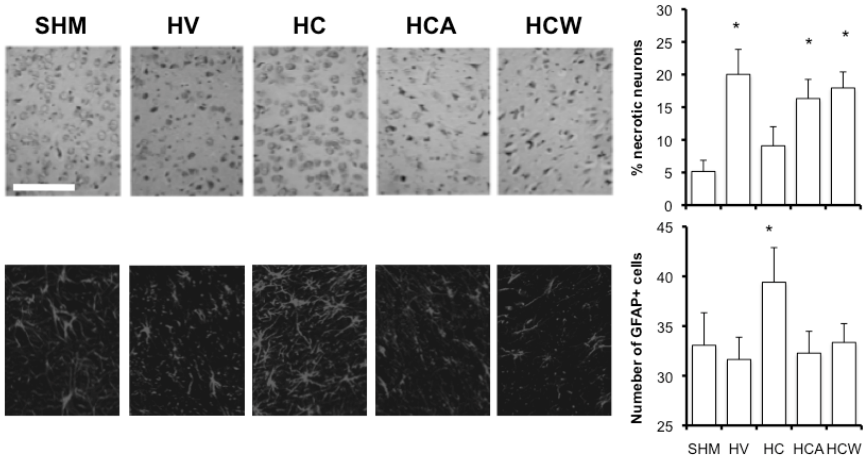
Table 1. Cardiorespiratory parameters.

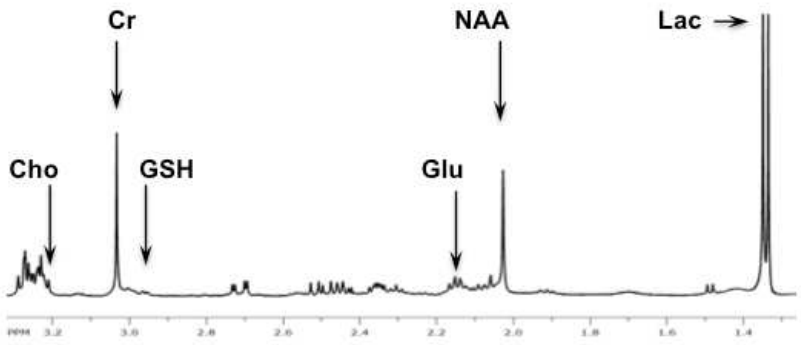
		SHM (n=6)	HV (n=8)	HC (n=10)	HCA (n=5)	HCW (n=5)
CO	B	31.8(2.9)	33.9(3.5)	35.7(2.5)	34.4(3.9)	34.4(2.8)
	D	35.5(2.7)	36.9(4.1)	33.4(4.1)	33.2(2.2)	36.2(2.3)
	E	35.0(3.6)	34.4(4.2)	33.6(4.3)	32.0(3.6)	29.6(2.1)*,#,§
MBP	B	79.3(5.0)	80.1(3.3)	82.5(2.6)	83.1(5.1)	76.9(3.1)
	D	90.5(5.8)	86.0(7.1)	91.4(7.8)	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)

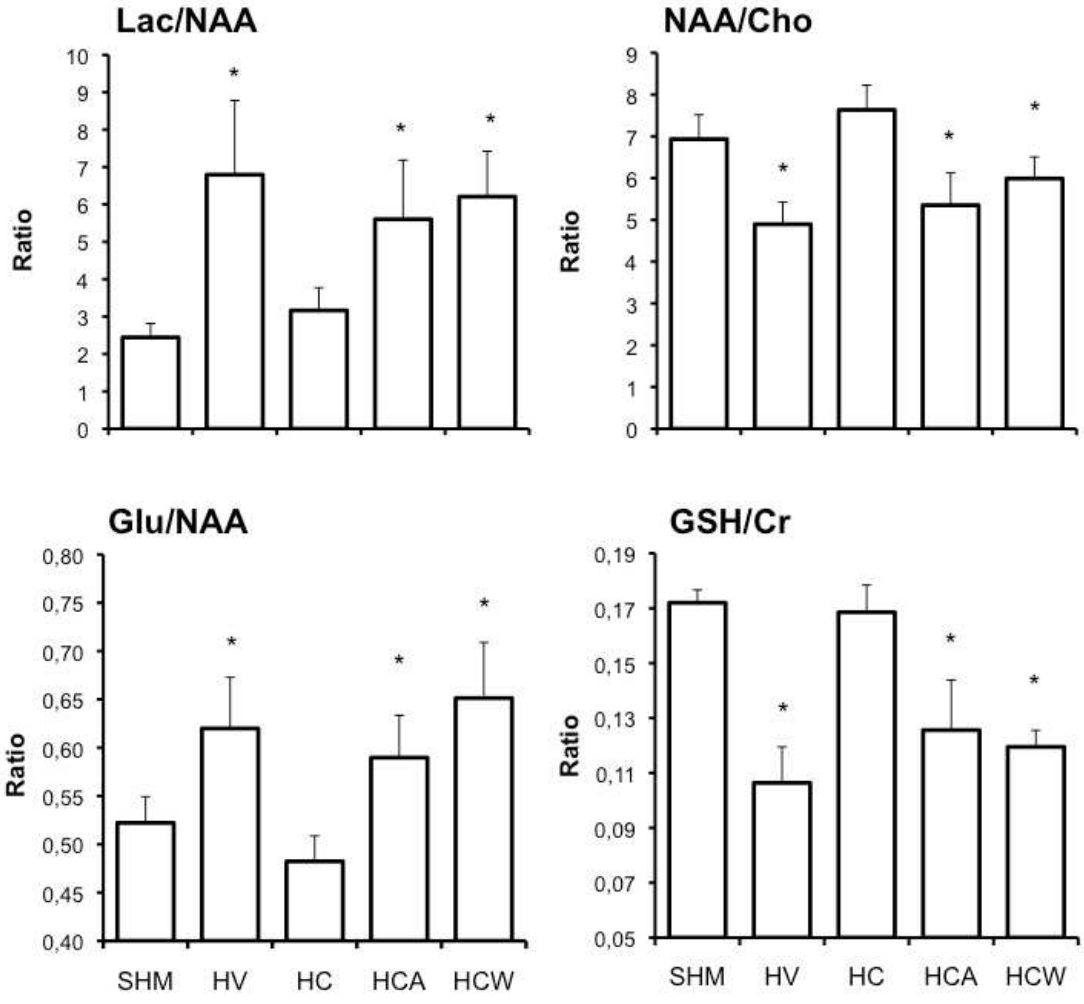
Values obtained from 1-to-2 day-old piglets after sham operation (SHM) or after hypoxia-ischemia and treatment with vehicle (HV), CBD (HC), CBD + AM630 (HCA) or CBD + WAY100630 (HCW)
B: Basal. D: Drug. E: End. *CO*: cardiac output (mL/min/kg). *MBP*: mean blood pressure (mmHg).
(*) p<0.05 vs SHM. (#) p<0.05 vs B. (§) p<0.05 vs. HC


Mean amplitude (µV)
30
25
20
15
10
5
0
B
HI
D
1
3
6
Time (h)
SHM
HV
HC
HCA
HCW
*
§

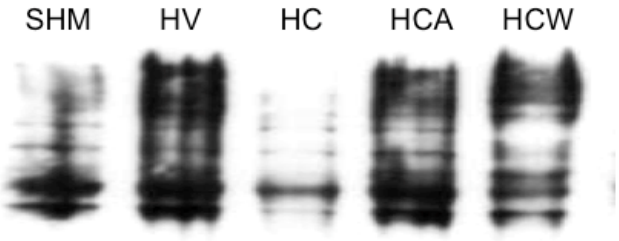

Score (points)
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
SHM
HV
HC
HCA
HCW
*

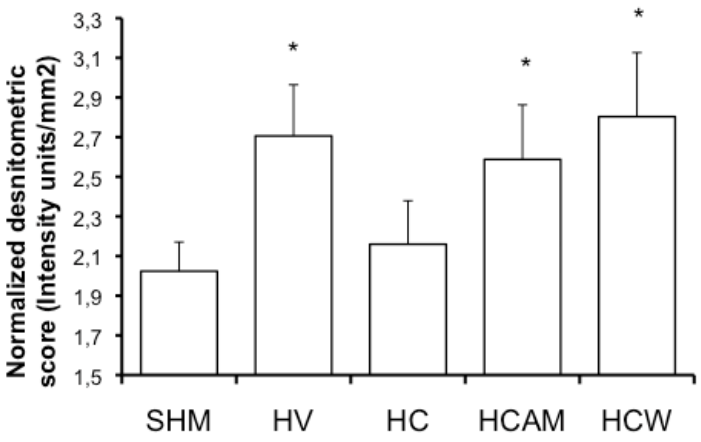



Cr
NAA
Lac
Cho
GSH
Glu

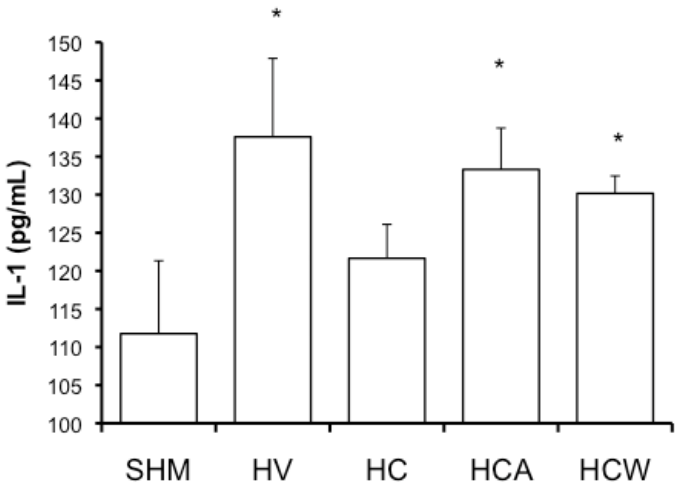

Lac/NAA
NAA/Cho
Glu/NAA
GSH/Cr
Ratio
SHM
HV
HC
HCA
HCW

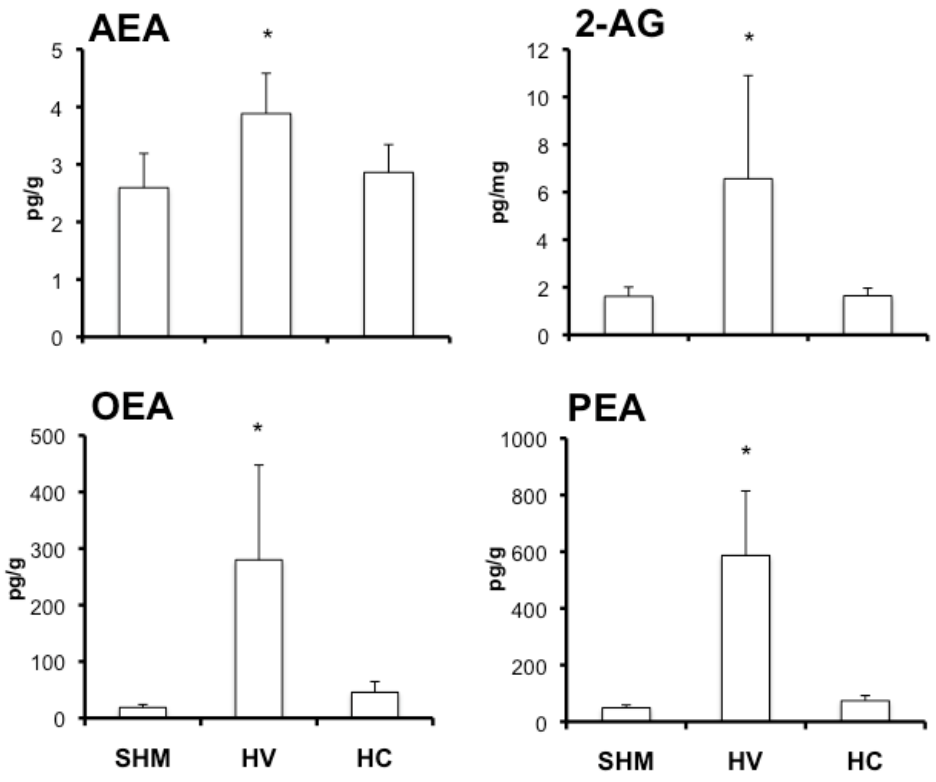
A)


SHM
HV
HC
HCA
HCW


Normalized desnitometric score (Intensity units/mm2)
3,3
3,1
2,9
2,7
2,5
2,3
2,1
1,9
1,7
1,5
*
*
*
SHM
HV
HC
HCAM
HCW

B)


IL-1 (pg/mL)
150
145
140
135
130
125
120
115
110
105
100
*
*
*
SHM
HV
HC
HCA
HCW


AEA
pg/g
0
1
2
3
4
5
*
2-AG
pg/mg
0
2
4
6
8
10
12
*
OEA
pg/g
0
100
200
300
400
500
*
SHM
HV
HC
PEA
pg/g
0
200
400
600
800
1000
*
SHM
HV
HC