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Cannabidiol administration after hypoxia–ischemia to newborn rats reduces long-term brain injury and restores neurobehavioral function

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ABSTRACT

Cannabidiol (CBD) demonstrated short-term neuroprotective effects in the immature brain following hypoxia–ischemia (HI). We examined whether CBD neuroprotection is sustained over a prolonged period. Newborn Wistar rats underwent HI injury (10% oxygen for 120 min after left carotid artery electrocoagulation) and then received vehicle (HV, $n = 22$) or 1 mg/kg CBD (HC, $n = 23$). Sham animals were similarly treated (SV, $n = 16$ and SC, $n = 16$). The extent of brain damage was determined by magnetic resonance imaging, histological evaluation (neuropathological score, 0–5), magnetic resonance spectroscopy and Western blotting. Several neurobehavioral tests (RotaRod, cylinder rear test[CRT],and novel object recognition[NOR]) were carried out 30 days after HI (P37). CBD modulated brain excitotoxicity, oxidative stress and inflammation seven days after HI. We observed that HI led to long-lasting functional impairment, as observed in all neurobehavioral tests at P37, whereas the results of HC animals were similar to those of sham animals (all $p < 0.05$ vs. HV). CBD reduced brain infarct volume by 17% ($p < 0.05$) and lessened the extent of histological damage. No differences were observed between the SV and SC groups in any of the experiments. In conclusion, CBD administration after HI injury to newborn rats led to long-lasting neuroprotection, with the overall effect of promoting greater functional rather than histological recovery. These effects of CBD were not associated with any side effects. These results emphasize the interest in CBD as a neuroprotective agent for neonatal HI.

1. Introduction

The phytocannabinoid cannabidiol (CBD) is the major non-psychoactive constituent of *Cannabis sativa*; the lack of psychoactive effects is attributable to its non-significant binding to CB_1 receptors (Pertwee, 2004; Mechoulam et al., 2007). However, CBD has been demonstrated to have a broad spectrum of therapeutic properties, including neuroprotective effects in numerous pathological conditions (Pertwee, 2004; Mechoulam et al., 2007; Hayakawa et al., 2010). These neuroprotective effects are thought to be due to the potent anti-inflammatory and anti-oxidant properties of CBD, although other actions of CBD that might also account for the CBD-induced neuroprotection include inhibition of calcium transport across membranes, inhibition of anandamide uptake and enzymatic hydrolysis, activation of nuclear receptors of the PPAR family, inhibition of NF-κB activation, iNOS expression, adenosine uptake, and activation of 5HT-1A receptor (see details in Marsicano et al., 2002; Pertwee, 2004; Sacerdote et al., 2005; Carrier et al., 2006; Mechoulam et al., 2007; Ryan et al., 2009; Castillo et al., 2010; Jones et al., 2010). The neuroprotective properties of CBD have been studied in numerous chronic neurodegenerative disorders and in acute episodes of brain damage. For example, CBD reduces brain damage after ischemic injury in adult animals (Hayakawa et al., 2010).

The study of neuroprotective therapies for reducing brain damage due to hypoxia–ischemia (HI) in newborns is of utmost importance. One to two per one thousand live births develop mild

Abbreviations: CBD, cannabidiol; CRT, cylinder rearing test; Epo, erythropoietin; HI, hypoxia–ischemia; NOR, novel object recognition.

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to severe perinatal HI encephalopathy, which usually leads to death or permanent sequelae, imposing considerable lifelong socioeconomic costs on families and society (Cilio and Ferrierro, 2010). Hypothermia, the only treatment currently available, reduces mortality or severe sequelae, but only in mild cases. Thus, complementary therapies that can be combined with hypothermia to enhance its neuroprotective properties and/or extend its therapeutic time window are needed (Cilio and Ferriero, 2010). We recently reported that administration of CBD to newborn piglets after HI has a protective effect on neurons and astrocytes, preserves brain activity, prevents seizures and improves neurobehavioral performance when given shortly after HI (Alvarez et al., 2008; Lafuente et al., 2011). We also examined the effect of CBD in an in vitro model of HI damage to newborn brains by exposing forebrain slices from newborn mice to oxygen–glucose deprivation. Our results demonstrated that CBD-mediated prevention of necrotic and apoptotic cell death is related to the modulation of excitotoxicity, inflammation and toxic NO production. We also observed that CB2 and adenosine receptors are involved in these effects (Castillo et al., 2010). All of these data strongly support CBD as a novel and promising therapy for asphyxiated newborns. However, the beneficial effects of CBD in neonatal HI were observed shortly after the ischemic insult and, with the purpose to translate these observations to a clinical setting, it will be necessary to demonstrate that CBD provides sustainable and long-term neuroprotective effects without severe side effects (Cilio and Ferriero, 2010).

The aim of the present work was to determine whether CBD administration after HI injury to newborn animals leads to long-term neuroprotective effects, which are demonstrable not only by histological and anatomical observations but also by improved functional outcomes. We employed a widely used rat model to study the long-term effects following newborn HI brain injury due to its ease of use and the ability to assess the results in the context of previous studies (Vexler et al., 2006).

2. Methods

2.1. Induction of HI brain damage

The experimental protocol met European and Spanish regulations for the 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. We employed the minimum number of animals necessary to achieve statistical significance. The protocol was based on a model extensively described elsewhere (Fernández-López et al., 2007). Briefly, the left common carotid artery was exposed and electrocoagulated in 7-to10-day-old (P7-P10) Wistar rats (HI rats) under anesthesia with sevoflurane (5% induction, 1% maintenance). After a 3-h recovery period with their dam, rat pups were placed in groups of three into 500-mL jars maintained at 37 °C by a warm water bath and then exposed to hypoxia (10% O_2) for 120 min. Six animals from the same litter were selected for HI. The control group (sham rats) consisted of the remaining pups from the litter that underwent a similar surgical procedure but without the carotid electrocoagulation and subsequent hypoxia. Ten minutes after the end of HI, animals from the HI and sham groups received s.c. injections of pure CBD or vehicle. Pups were randomly assigned to either the sham or HI group prior to surgery, and rats from each group were randomly assigned to receive either CBD or vehicle. The CBD, which was a generous gift from GW Pharma (Leeds, UK), was prepared in a 5 mg/mL formulation of ethanol:solutol:saline at a ratio of 2:1:17. In a pilot experiment, we tested three different schedules for CBD administration: 1 mg/kg single dose, 1 mg/kg every 24 h for 72 h, or 1 mg/kg every 8 h for 72 h. In all cases, the volume of injection of CBD or vehicle was adjusted to 0.1 mL. Because we did not observe any differences due to the administration schedule, the simpler single dose was selected. Following the injection, the pups were returned to their dam. Seven (P14) or 30 (P37) days later, they were used for different analyses. Rats were weaned at P30.

2.2. Functional studies

At P37, three different functional tests were performed by an examiner blinded to the experimental group. All tests were performed for five consecutive days and in the same order as presented below for all rats. First, two sensorimotor tests were conducted. The first assessed motor and balance coordination (RotaRod), and the second examined spontaneous forelimb use and motor deficit (cylinder rearing test, CRT). Finally, a cognitive test was conducted to explore memory deficits (novel object recognition, NOR). All trials were video recorded and scored by three different examiners who were blinded to the experimental groups. The mean score from the three evaluations was calculated and used for statistical analysis. After each test, the apparatus was thoroughly cleaned with 70% alcohol to avoid olfactory stimuli.

2.2.1. RotaRod

This test examines motor and coordination deficits (Balduini et al., 2000; Lubics et al., 2005). Animals were tested on a RotaRod treadmill (LE 8500, Panlab SL, Barcelona, Spain) with a diameter of 7 cm elevated 50 cm above the bottom of the apparatus and attached to a motor to control speed. Briefly, rats were trained for two days. On the first training day, rats were placed on the RotaRod at a constant speed of 4 rpm (revolutions per minute) for 1 min. On the second day, animals were trained with accelerated speed from 4 to 40 rpm for a maximum of 10 min. Finally, the rats were subjected to two trials. In each trial, animals were scored for their latency to fall (in seconds). Animals rested a minimum of 1 h between trials to avoid fatigue. The average latency to fall for the two trials was used as the measured parameter.

2.2.2. Cylinder rearing test (CRT)

This test examines lateral bias of sensorimotor deficits (Grow et al., 2003). Each rat was placed in a transparent methacrylate cylinder 20 cm in diameter and 30 cm in height. The initial forepaw (left, right, or both) preference was scored based on the initial weight-bearing contact of the forepaw with the wall during each full rearing event over a 2-min trial. The relative proportion of left (ipsilateral) forepaw contacts was calculated as: (left-right)/(left + right + both)*100. For each animal, a minimum of 4 wall contacts was required for trial analysis.

2.2.3. Novel object recognition (NOR)

This test explores non-spatial working memory (Dere et al., 2007; Broadbent et al., 2010). Before training the animals with objects, they were allowed to acclimate to the testing environment. The day before testing, each rat was placed in a methacrylate box (40 × 40 × 35 cm) for 5 min. On the testing day, each rat completed two 5-min sessions with an intertrial interval of 1 h. In the first session, the rat was allowed to explore the box, which contained two identical objects. For the second session, each rat was returned to the box where one of the original objects was replaced by a new one. Exploration was scored when the rat sniffed at the novel object within a distance of 1 cm or touched the novel object with its nose. The time spent on the exploration of the familiar (Tf) and the novel object (Tn) was recorded separately, and a discrimination index, (Tn − Tf)/(Tn + Tf), was calculated.

2.3. Measurement of the extent of brain injury: magnetic resonance imaging (MRI)

At P14 or P37 (after the last functional test), rats were sacrificed by a lethal i.p. injection of diazepam + ketamine and transcardially perfused, and the brains were removed and placed in 10% formalin. The MRI scan of the brains was carried out in the MRI Unit of the Instituto Pluridisciplinar, (Universidad Complutense, Madrid, Spain) on a BIOSPEC BMT 47/40 (Bruker-Medical, Ettlingen, Germany) operating at 4.7 T, equipped with an actively shielded gradient insert with an 11.2-cm bore, a maximal gradient strength of 200 mT/m, an 80-μs rise time, and a homemade 4-cm surface coil. T2WI were acquired with multislice rapid acquisition (TR = 3.4 s, RARE factor = 8, interecho interval = 30 ms, TEeff = 120 ms; matrix size = 256 × 256 (pixel dimensions 117 × 117 μm), field of view (FOV) = 3 cm^2). The slice package consisted of 26 consecutive 0.5-mm-thick slices in the axial plane with an interslice gap of 0.1 mm to image the entire brain. Brains were placed in Fluorinert FC-40 (3 M, Minnesota, USA) for the MRI scan and then replaced in PFH.

Volumetric analyses of the MRI slices were performed using ImageJ 1.43u software (U.S. National Institutes of Health). In each slice, the area of brain parenchyma was manually outlined, and the size of the selected area was calculated by the software program. The area calculated for each slice was then combined to determine the entire volume. The analysis of brains from sham rats revealed that the mean left hemisphere volume (LHV) was 0.97 times that of the mean right hemisphere volume (RHV). In HI rats, the volume of lesion was calculated by subtracting the volume of intact brain parenchyma in the left hemisphere from the theoretical LHV, calculated as RHV × 0.97. The boundary of each lesion was identified by a well-defined hyperintense and/or infarcted area. The lesion volume was expressed as a percentage of the theoretical whole brain volume, calculated as RHV + theoretical LHV (=RHV × 0.97). Thus, the final formula volume of lesion (%) = 49.23 − 100 × intact left volume/(RHV*1.97).

2.4. Histological evaluation

After the MRI scans, the brains were embedded in paraffin, and coronal sections (4 μm) were cut and mounted on glass slides for Nissl staining. Three consecutive sections corresponding to the plate 35 of the rat brain atlas (Paxinos and Watson, 1997) were selected for analysis by an examiner blinded to the experimental group of the animal. The degree of brain damage in the CA1 area of the ipsilateral hippocampus was scored as follows: 0 = normal, 1 = few neurons damaged (1–5%), 2 = several neurons damaged (6–25%), 3 = moderate number of neurons damaged

(26–50%), 4 = more than half of neurons damaged (51–75%), 5 = majority of neurons damaged (>75%), or absent hippocampus. The mean of 3 sections from each animal was determined.

2.5. Proton magnetic resonance spectroscopy (H^+-MRS)

For this purpose, a subset of rats was killed by lethal injection of diazepam + ketamine i.p, prior to decapitation, and their brains were immediately frozen in isopentane and stored at −80 °C. H^+-MRS was performed at 500.13 MHz using a Bruker AMX500 spectrometer 11.7 T operating at 4 °C on frozen samples from ipsilateral and contralateral parietal cortex (3 mg) placed within a 50-μl zirconium oxide rotor with a cylindrical insert and spun at 4000 Hz. Standard solvent-suppressed spectra were acquired as 16 k data points and averaged over 256 acquisitions. The total acquisition time was ~14 min using a sequence based on the first increment of the NOESY pulse sequence to affect suppression of the water resonance and limit the effect of Bo and B1 inhomogeneities in the spectra (relaxation delay-90°-t1-90°-tm-90°-acquire free induction decay (FID)), in which a secondary radio frequency irradiation field was applied at the water resonance frequency during the relaxation delay of 2 s and during the mixing period (tm = 150 ms), 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 0 ppm.

Using the 3.1.7.0 version of SpinWorks software (University of Manitoba, Canada), curve fitting was performed and several ratios were calculated, including N-acetylaspartate/choline (NAA/Cho), which inversely correlates with the severity of neuronal damage (Penrice et al., 1997; Li et al., 2010); lactate/creatine (Lac/Cr), which is proportional to changes in phosphorylation potential (Penrice et al., 1997); glutamate/N-acetylaspartate (Glu/NAA), which is proportional to increased excitotoxicity (Groenendaal et al., 2001); and reduced glutathione/creatine (GSH/Cr), which correlates with oxidative stress (Satoh and Yoshioka, 2006).

2.6. Western blotting

Frozen brain tissue was homogenized in tissue protein extraction reagent (T-PER; 1 g of tissue/10 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. Then, 20 μg of protein from each extract was reduced, denatured, and separated on 10% sodium dodecyl-sulfate–polyacrylamide gels (50 mA). Next, the proteins were transferred to polyvinylidene fluoride membranes (GE Healthcare; Buckinghamshire, UK). The membranes were blocked by overnight incubation in PBS-Tween (PBST) containing 5% nonfat dried milk at 4 °C. The resultant blots were incubated with mouse monoclonal anti-TNF-alpha antibody overnight at 4 °C (1:1500; Serotec; Oxford, UK) or rabbit polyclonal anti-β-actin (1:3000; Abcam, Cambridge, UK) for 1 h at room temperature in PBST containing 5% nonfat dried milk. Finally, blots were incubated with anti-rabbit or anti-mouse HRP-labeled secondary antibodies (1:4000; GE Healthcare; Buckinghamshire, UK) for 1 h at RT. The peroxidase reaction was developed with an ECL Kit (GE Healthcare; Buckinghamshire, UK). Films were scanned and analyzed with ImageJ. β-actin was used to normalize the protein lane charge of the blot.

2.7. Determination of the CBD concentration in brain tissue

To determine the concentration of CBD in brain tissue, healthy P7 pups received s.c. injections (0.1 mL) of a solution containing 1 mg/kg CBD. Then, pups were sacrificed 0, 3, 6, 12, 24 or 36 h after injection ($n = 6$ each), and their brains were immediately removed, frozen in isopentane, and 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/MSat Quotient Bioresearch Ltd. (Fordham, UK).

2.8. Statistical analysis

The results are expressed as the mean ± standard error. Statistical comparisons of injury volumes or functional test results were made with ANOVA tests followed by Scheffé's test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant. All analyses were performed using the 15.0.0 version of SPSS software (SPSS Inc., Corpus Christi, TX, USA).

3. Results

Eleven out of 86 pups died during the surgical procedure or the hypoxic episode. The remaining pups (38 males and 37 females) were assigned to the different experimental groups: Sham + vehicle (SV, $n = 16$), Sham + CBD (SC, $n = 16$), HI + vehicle (HV, $n = 22$) or HI + CBD (HC, $n = 23$). One of the SV, none of the SC, 1 of the HV, and1 of the HC pups died in the first few days after surgery ($p > 0.05$).

3.1. Functional tests

3.1.1. RotaRod

The HI injury led to poorer performance on this motor coordination test; the HV animals showed a 21% decrease in time spent on the rod compared to the SV animals ($p < 0.05$ vs. sham; Fig. 1). CBD administration after HI improved RotaRod performance, and the HC animals' latency to fall was similar to that of SV rats and significantly better than that of the HV animals ($p < 0.05$ vs. HV; Fig. 1). There were no differences between the SV and SC groups.

3.1.2. CRT

Animals in the Sham groups did not show any paw preference during rearing in the CRT (Fig. 1). The HI injury led to a motor deficit in the contralateral (right) forepaw, which was indicated by a significant preference in use of the ipsilateral (left) forepaw ($p < 0.05$ vs. sham; Fig. 1). CBD administration improved the performance of the HC rats in the CRT; there was no paw preference exhibited by the HC animals ($p < 0.05$ vs. HV; Fig. 1). There were no differences between the SV and SC animals with regard to CRT performance.

3.1.3. NOR

The HI injury resulted in working memory impairment, as the preference for novel objects was reduced by 30% in HV rats ($p < 0.05$ vs. sham; Fig. 1). HC rat test performance was similar to that of sham animals ($p < 0.05$ vs. HV; Fig. 1), suggesting that CBD treatment after HI prevented memory impairment. There were no differences between the animals in the SV and SC groups in the NOR test.

3.2. Measurement of the extent of brain injury by MRI

At P14, the injured area appeared on MRI as a hyperintense area that was equivalent to 50% of the hemisphere volume; there were no differences between HV and HC at that time (Fig. 2). At P37, the injured area appeared mostly as an infarcted area surrounded by a thin hyperintense area; together, the injured area in the left hemisphere after HI injury was equivalent to 40% of the hemisphere volume in the HV animals (Fig. 2), which represents a non-significant reduction of the injured area from P14. Treatment with CBD reduced the brain injury volume. Thus, in the HC animals, the area of injury at P37 was reduced by 30% compared with HC at P14 and by 17% compared with HV at P37 (both $p < 0.05$; Fig. 2). There were no differences in brain volume between animals in the SV and SC groups (1332.13 ± 38.1 vs. 1329.3 ± 27 mm^3 for SV and SC, respectively; $p > 0.05$).

3.3. Histological evaluation

HI leads to an ipsilateral area of infarction with damage to the surrounding tissues, including the temporoparietal cortex and the hippocampus. Thus, most neurons in the brains of HV animals appeared necrotic (Fig. 3). The histological evaluation demonstrated that treatment with CBD reduced the extent of brain damage (Fig. 3). Overall, there was a mean neuropathological score reduction of 1 point in the HC animals when compared with the brains of HV animals ($p < 0.05$ vs. HV). There were no differences between the SV and SC animals.

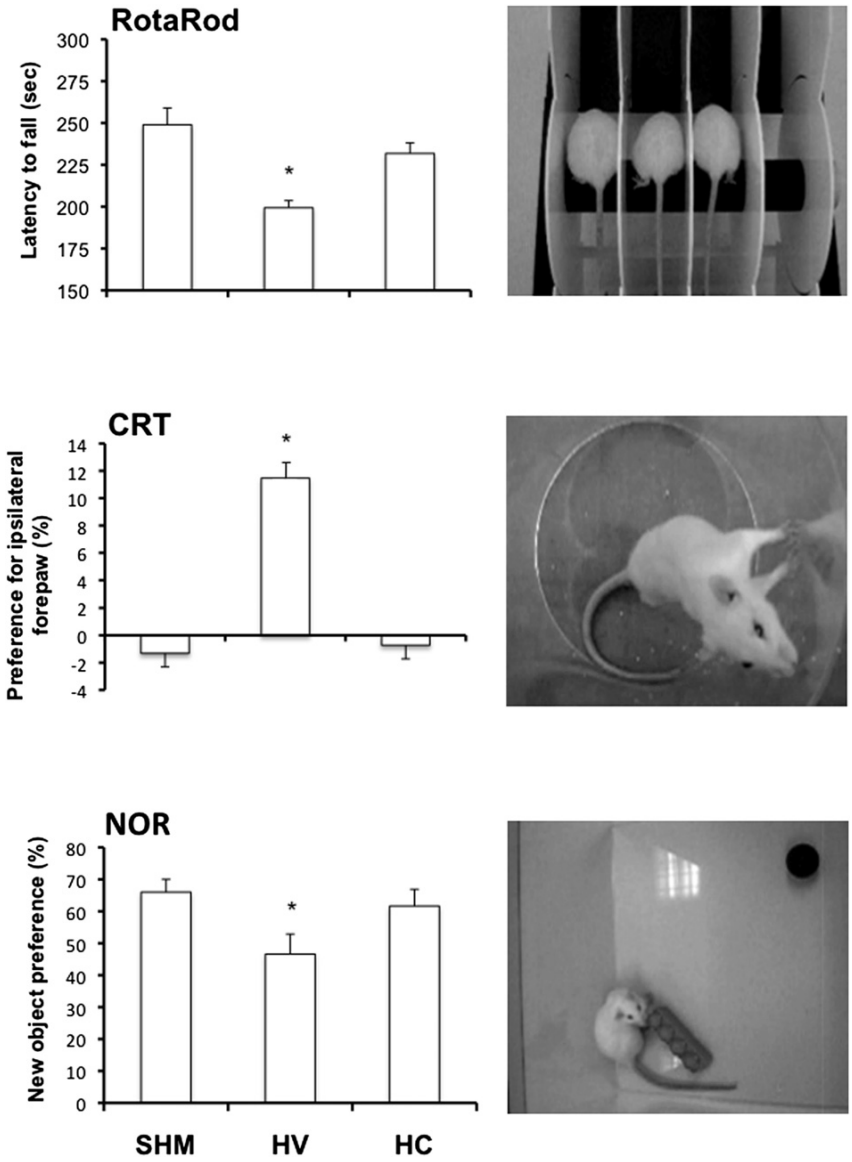

Fig. 1. Neurobehavioral tests conducted at P37 in Wistar rats that received an injection of vehicle or 1 mg/kg cannabidiol s.c. after hypoxic-ischemic injury (HV and HC, respectively) or underwent a sham operation (SHM) on P7. Bars represent the mean ± SEM of results from 15 to 23 animals. Photographs on the right are of actual experiments. CRT: cylinder rear test. NOR: novel object recognition. See Methods (2.2. Functional studies) for more details. (*) $p < 0.05$ vs. SHM.

3.4. H^+-MRS studies

HI insult led to decreased NAA/Cho in the ipsilateral cortex of HV animals at P14, which reflected the loss of neural cells (Fig. 4). We also observed an increase of Lac/Cr, which was suggestive of persistent metabolic derangement (Fig. 4). This was associated with an increase of Glu/NAA and a decrease of GSH/Cr, implying that brain damage in HV was associated with increased excitotoxicity and oxidative stress (Fig. 4). All of these parameters were milder but still significantly impaired in the contralateral cortex of HV animals (Figs. 4 and 5).

CBD administration prevented the NAA/Cho decrease and reduced the Lac/Cr increase in the ipsilateral cortex at P14 (Fig. 4). In addition, CBD reduced the Glu/NAA increase and blunted the increase of GSH/Cr (Fig. 4). There were no differences between the Sham and HC groups with regard to the contralateral cortex (Fig. 4).

At P37, a decrease of NAA/Cho was still observable in the ipsilateral cortex of HV animals (Fig. 4). No other differences between groups were noticeable at that time point.

There were no differences between the SV and SC groups in the H^+-MRS studies.

3.5. TNFα concentration

HI led to an increase in the TNFα concentration at P14 as shown by Western blot analysis (Fig. 5). This increase was blunted by CBD treatment (Fig. 4). There were no differences in TNFα concentration among the groups at P37 (data not shown). There were no differences between the SV and SC animals.

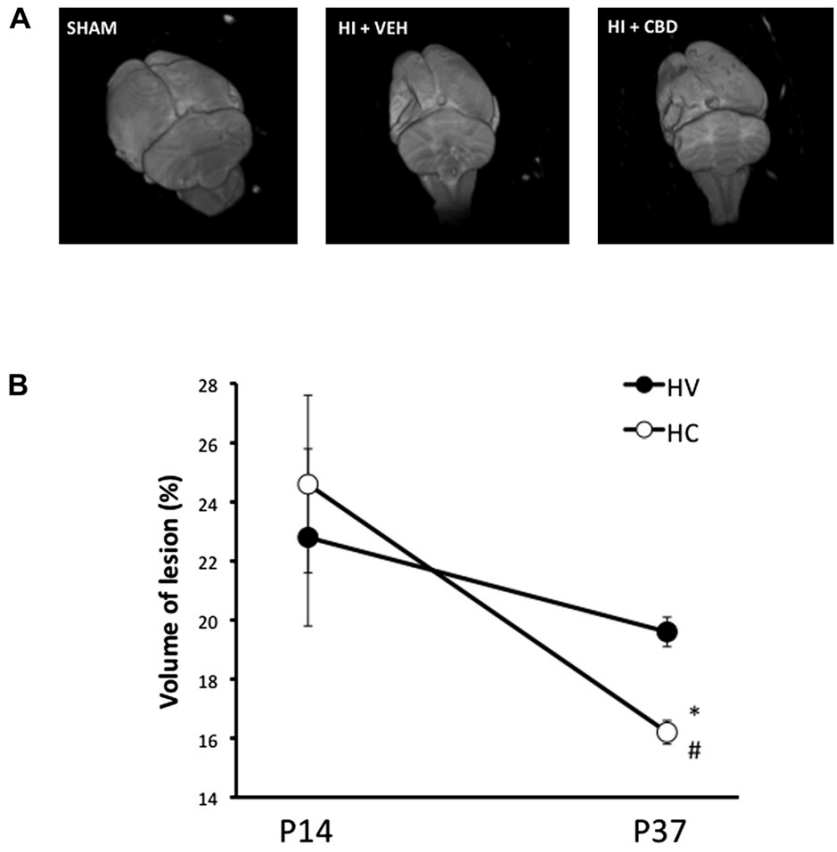

Fig. 2. *Top*: Representative 3D reconstruction of magnetic resonance (MR) images obtained at P37 of Wistar rats that received an injection of vehicle or 1 mg/kg cannabidiol s.c. after a hypoxic–ischemic injury on P7 (HV and HC, respectively). *Bottom*: Results of the residual lesion volume as calculated from the MR images. See Methods (2.3. Measurement of the extent of brain injury) for more details. Bars represent the mean ± SEM of results from 15 to 23 animals. (*) $p < 0.05$ vs. sham.

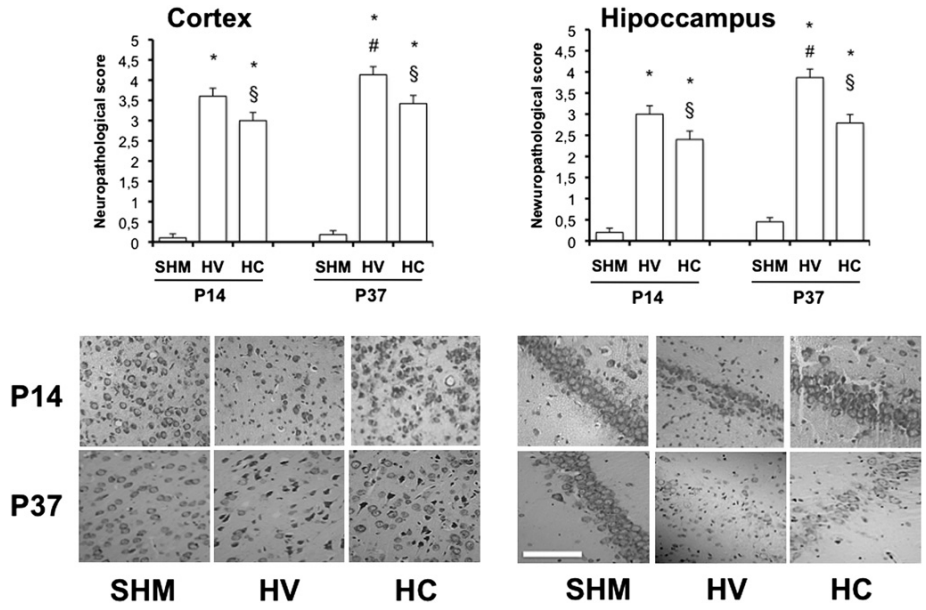

Fig. 3. Representative micrographs of Nissl staining of brain slices at P14 or P37 from Wistar rats that received an injection of vehicle or 1 mg/kg cannabidiol s.c. after a hypoxic–ischemic injury (HV and HC, respectively) or underwent a sham operation (SHM) on P7. Original magnification: ×200. Scale: 100 μm. Bars in the top graph represent the mean ± SEM of neuropathological score results. See Methods (2.4. Histological evaluation) for more details. (*) $p < 0.05$ vs. SHM. (§) $p < 0.05$ vs. HV. (#) $p < 0.05$ vs. P14.

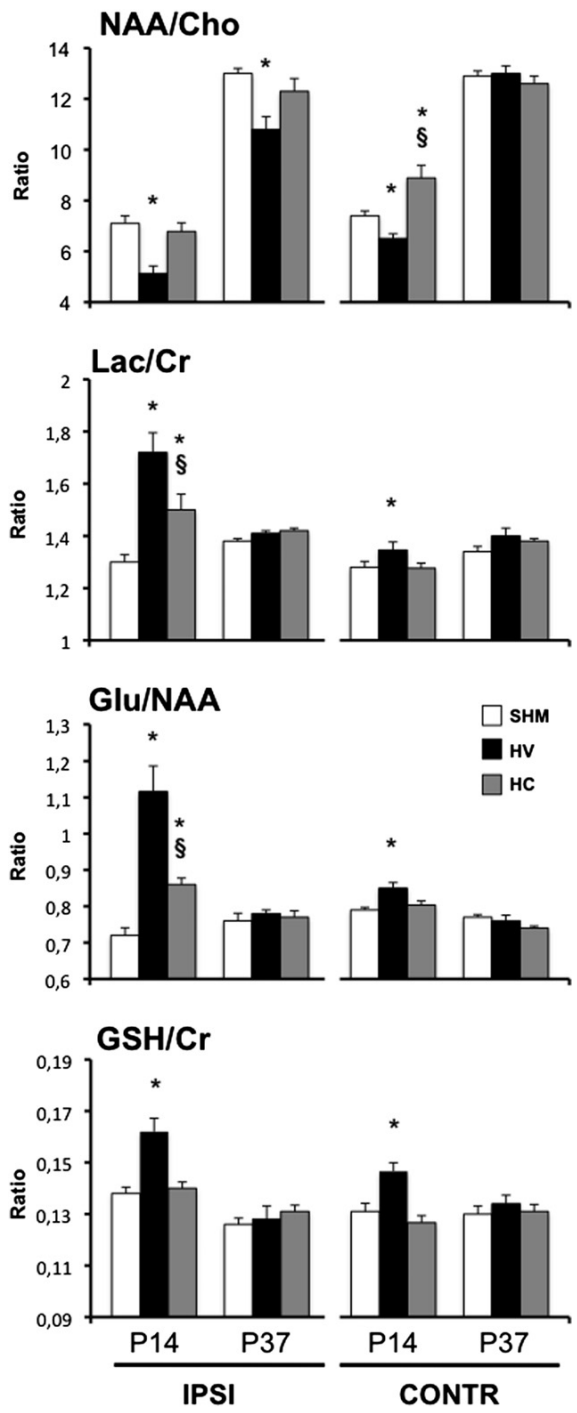

Fig. 4. Results from H^+-MRS studies carried out on brain samples obtained at P14 or P37 from Wistar rats that received an injection of vehicle or 1 mg/kg cannabidiol s.c. after a hypoxic–ischemic injury (HV and HC, respectively) or underwent a sham operation (SHM) on P7. Bars in the graph represent the mean ± SEM of results from 10 to 15 samples. See Methods (2.5. Proton Magnetic resonance spectroscopy (H^+-MRS)) for more details. IPSI: ipsilateral brain hemisphere. CONTR: contralateral brain hemisphere. (*) $p < 0.05$ vs. SHM. (§) $p < 0.05$ vs. HV.

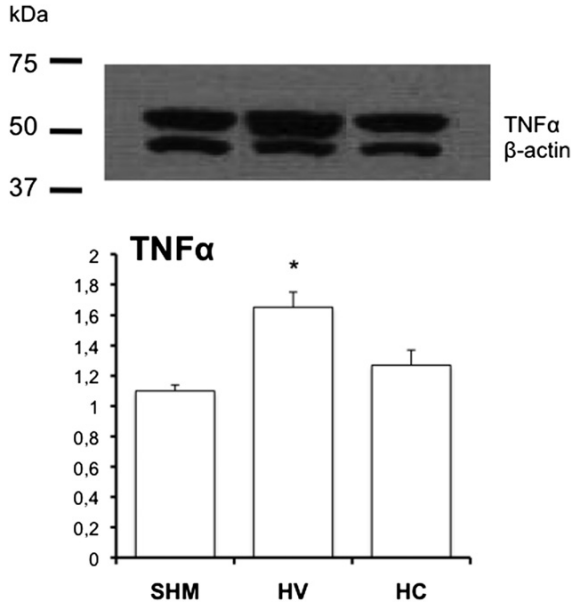

Fig. 5. Results from Western blotting studies carried out on brain samples obtained at P14 from Wistar rats that received an injection of vehicle or 1 mg/kg cannabidiol s.c. after a hypoxic–ischemic injury (HV and HC, respectively) or underwent a sham operation (SHM) on P7. Bars represent the mean ± SEM of results from 5 samples. See Methods (2.6. Western blotting) for more details. (*) $p < 0.05$ vs. SHM. (§) $p < 0.05$ vs. HV.

3.6. Determination of the CBD concentration of CBD in brain tissue

The CBD concentrations in brain tissue 0, 3, 6, 12, 24 and 36 h after injection were 0, 28.7 ± 3.2, 13.9 ± 2.6, 10.4 ± 1.2, 5.1 ± 0.2 and 0.6 ± 0.6 ng/g, respectively.

4. Discussion

In the present study, we found that the administration of CBD to newborn rats after HI injury led to a sustained neuroprotective effect that persisted at 30 days after HI. At this age, we observed that CBD-treated animals had smaller brain lesions and improved neurobehavioral performance when compared with non-treated animals of similar age. In addition, we observed that CBD administration to newborn animals was not associated with any noticeable side effects, as brain volume and neurobehavioral performance were similar in CBD- and vehicle-treated sham animals.

When exploring neuroprotective effects with a novel therapy, it is important to confirm that the benefits are reflected in neurological status. To this end, we employed two sensorimotor tests to assess possible impairments in motor coordination and performance (RotaRod and CRT) and one test assessing non-spatial memory deficits (NOR). We administered the tests 4 weeks after HI (P35-P38),which is consistent with previous studies (Balduini et al., 2000; Wagner et al., 2002; Grow et al., 2003; Lubics et al., 2005; Caceres et al., 2010; Lee et al., 2010). At that time, rats had already been weaned and could be considered as young adult animals capable of mature responses in the three tests. This is important because younger animals have poorer responses. The RotaRod treadmill is a classic sensorimotor test widely used for evaluating motor coordination impairments following ischemic brain injuries. A more severely impaired animal will have a shorter latency to fall from the accelerating treadmill than a less impaired animal (Balduini et al., 2000; Lubics et al., 2005; Spandou et al., 2005). The CRT is a standard test for studies on motor

impairment after ischemic brain injuries in adult rats. It is becoming increasingly popular because of its simplicity, reliability and reproducibility (Chang et al., 2005), and it has been adapted for studies after HI in newborn rodents (Grow et al., 2003). The CRT quantifies the lateral bias of sensorimotor deficits, which is of particular interest in models that result in unilateral brain damage (Grow et al., 2003; Lee et al., 2010; Fan et al., 2011). Administration of CBD after neonatal HI returned the performance in both sensorimotor tests to normal levels at P37. The result is important because although other well-recognized neuroprotective strategies, such as hypothermia, improve the performance in these sensorimotor tests several weeks after HI, the behaviors do not return to control levels (Lee et al., 2010). Similarly, erythropoietin (Epo) treatment restores CRT performance to control levels in mice (Fan et al., 2011) but only partially restores RotaRod performance in rats (Spandou et al., 2005).

The NOR test explores deficits in non-spatial working memory and is based on rodents' innate preference to examine novel rather than familiar objects (Dere et al., 2007; Broadbent et al., 2010). The NOR test is less stressful to the animal than tasks based on negative reinforcement of behavior, and it is independent of food palatability or intake. Thus, the NOR test is especially well suited to examine the effects of pharmacological interventions on memory and is particularly appropriate for treatments with unknown effects on food behavior and reward processes (Dere et al., 2007). We observed that HI led to memory deficits, which were indicated by a 30% reduction in preference for new objects in the HV group. Previous experiments that induced brain damage in immature rats by hypoxic injury (Simola et al., 2008) or ionic radiation (Caceres et al., 2010) produced similar results. This memory deficit may be explained by the extensive damage observed in the hippocampus and cerebral cortex of these animals. The hippocampus is the main brain region responsible for recognition memory, but this behavior is also dependent upon the cerebral cortex, in particular the perirhinal and prefrontal areas (Dere et al., 2007; Broadbent et al., 2010). CBD treatment prevented the memory deficit caused by the HI insult and reduced hippocampal and cortical damage. Few studies have explored the effect of neuroprotective strategies on memory deficits in neonatal HI. Hypothermia (Wagner et al., 2002) and Epo treatment (Kumral et al., 2004) have been demonstrated to reduce but not prevent spatial memory deficits after HI in newborn rats.

HI injury to newborn rats resulted in long-lasting brain damage. The ipsilateral brain hemisphere volume was reduced by 40%, which was similar to previous studies (Wagner et al., 2002; Lubics et al., 2005; Lee et al., 2010). The tissue surrounding the cortical infarcted area appeared damaged, which is typical in this experimental model (Bona et al., 1998; Wagner et al., 2002; Spandou et al., 2005; Hobbs et al., 2008). At P14, the volume of brain damage assessed by MRI was similar in HV and HC, whereas the volume of damage was reduced in HC by 17% compared with HV at P37. This discrepancy is likely because hyperintense areas in T2WI shortly after HI correspond to areas of brain edema, which includes necrotic tissue and injured but recoverable tissue, the so-called "penumbral" area (Fernandez-Lopez et al., 2007). CBD exerted a protective effect on brain tissue surrounding the cortical infarcted area at P14, as shown by the results from histological, H^+-MRS and Western blot studies. These findings suggest that CBD administration would recover the "penumbral" area, preventing it from becoming necrotic, thus reducing brain damage. In HV, however, most of the injured area evolved to necrosis, so that the damage observed in the MRI study at P37 was essentially the same as the area of injury observed at P14. In agreement with this result, the neuropathological score worsened in HV both in the cortex and the hippocampus from P14 to P37 but not in HC. The final reduction of brain damage volume by CBD was significant, but it was less impressive than that previously reported for hypothermia; MRI studies have demonstrated that hypothermia reduces brain lesion volume by 23–27% (Wagner et al., 2002; Lee et al., 2010). Similarly, CBD administration reduced histological brain damage, and the neuropathological scores were 23% and 30% lower in the HC hippocampus and cerebral cortex compared to HV, respectively. Like the lesion volume results, this neuropathological improvement was less striking for CBD than has been reported for hypothermia, which is associated with pathological score reductions of 38–50% (Bona et al., 1998; Hobbs et al., 2008). Rather, the results for CBD are similar to those reported for xenon (Hobbs et al., 2008). Overall, these findings indicate that CBD was not superior to other neuroprotective treatments in reducing histological brain damage. However, CBD was much more effective in promoting functional recovery as we observed that the neurobehavioral measures returned to normal levels.

The H^+-MRS studies corroborate the protective effect of CBD. At P14,CBD blunted the decrease of the NAA/Cho ratio that reflects neural cell loss (Penrice et al., 1997; Li et al., 2010). This effect was associated with a reduction of the observed increase of Lac/Cr in HV rats, a measure that reflects the sustained metabolic derangement observed in injured brain tissue (Penrice et al., 1997). Interestingly, both NAA/Cho and Lac/Cr are considered good predictors of newborn brain damage severity after HI insults (Li et al., 2010). The protective effect of CBD was associated with a reduction of the increase of Glu/NAA, which is indicative of excitotoxicity (Groenendaal et al., 2001) and the prevention of increased GSH/Cr, which is due to the increase of oxidative stress (Satoh and Yoshioka, 2006). These results agree with the reported reduction of glutamate release by CBD in newborn mice forebrain slices exposed to oxygen–glucose deprivation (Castillo et al., 2010), as well as with the strong antioxidant effect attributed to CBD (Pertwee, 2004; Mechoulam et al., 2007; Ryan et al., 2009). In addition, the Western blot analysis revealed that CBD ameliorated the HI-induced increase of TNFα concentration observable at P14, which is in agreement with the anti-inflammatory effect demonstrated by CBD in newborn mice forebrain slices exposed to oxygen–glucose deprivation (Castillo et al., 2010).

The study on CBD concentration in brain tissue revealed a maximum concentration of approximately 30 ng/g. This is equivalent to 100 nM, a concentration at which CBD has been demonstrated to exert anticonvulsant effects (Jones et al., 2010) as well as to modulate excitotoxicity (Ryan et al., 2009) and inflammation (Sacerdote et al., 2005). It would be interesting to determine how a single dose of CBD injected close to the end of HI insult promotes functional and histological recovery that is evident 30 days later. We tested other administration schedules with repeated CBD doses and did not obtain further benefits. We observed that the maximum concentration of CBD in the brain (approximately 30 ng/g) was achieved 3 h after the injection of 1 mg/kg CBD, and it was still detectable in the brain 24 h after the injection. This result contrasted with work by Deiana et al. (2011), who reported that after administering CBD (120 mg/kg) to adult rats, the drug was completely cleared from the brain 24 h after injection. This was despite the fact that the maximum concentration of CBD, observed 4 h after CBD injection, was proportionally higher than in our experiments (Deiana et al., 2011). These data suggest that CBD remained in the immature brain for longer and was present during the period when most of the pathophysiological processes leading to HI brain damage took place (Cilio and Ferriero, 2010). An additional point of interest is that in the cited work (Deiana et al., 2011), the administration of 30% solutol as a vehicle was associated with undesirable side effects. In our work, the administration of 6% solutol did not result in undesirable side effects in sham or HI rats.

Finally, it is worth noting that CBD metabolites are active substances with poorly understood effects (Jiang et al., 2011).

However, it is unlikely that those metabolites could have relevant effects on CBD neuroprotection in newborns because the major CYP isoforms responsible for CBD metabolism, CYP3A4 and CYP2C19 (Jiang et al., 2011), have very little activity in neonates (Yokoi, 2009).

5. Conclusion

In conclusion, CBD administration after HI injury to newborn rats leads to long-lasting neuroprotective effects. In contrast to results reported with other neuroprotective treatments, CBD was more effective in recovering functional parameters than restoring histological markers. The neuroprotective action of CBD was associated with the modulation of excitotoxicity, oxidative stress and inflammation. CBD was not associated with side effects. These results indicate that CBD has potential as a neuroprotective treatment and a useful partner for other neuroprotective strategies, such as hypothermia.

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