Up-regulation of CB$_2$ receptors in reactive astrocytes in canine degenerative myelopathy, a disease model of amyotrophic lateral sclerosis

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Summary statement

CB$_2$ receptors become up-regulated in activated astrocytes recruited at the damaged spinal cord in dogs with degenerative myelopathy, a canine model of amyotrophic lateral sclerosis.
Targeting the CB₂ receptor afforded neuroprotection in SOD1\textsuperscript{G93A} mutant mice, a model of amyotrophic lateral sclerosis (ALS). The neuroprotective effects of CB₂ receptors were facilitated by their up-regulation in the spinal cord in SOD1\textsuperscript{G93A} mutant mice. Herein, we have investigated whether a similar CB₂ receptor up-regulation, as well as parallel changes in other endocannabinoid elements, are evident in the spinal cord of dogs with degenerative myelopathy (DM), caused from mutations in the superoxide dismutase 1 gene (SOD1). We used well-characterized post-mortem spinal cords from unaffected and DM-affected dogs. Tissues were used first to confirm the loss of motor neurons using Nissl staining, which was accompanied by glial reactivity (elevated GFAP and Iba-1 immunoreactivity). Next, we investigated possible differences in the expression of endocannabinoid genes measured by qPCR between DM-affected and control dogs. We found no changes in the CB₁ receptor (also found with CB₁ receptor immunostaining) as well as in NAPE-PLD, DAGL, FAAH and MAGL enzymes. In contrast, CB₂ receptor levels were significantly elevated in DM-affected dogs determined by qPCR and Western-blotting, results reconfirmed in the grey matter using CB₂ receptor immunostaining. Using double-labelling immunofluorescence, CB₂ receptor immunolabelling co-localized with GFAP but not Iba-1, indicating up-regulation of CB₂ receptors on astrocytes in DM-affected dogs. In summary, our results demonstrated a marked up-regulation of CB₂ receptors occurring in the spinal cord in canine DM, which was concentrated in activated astrocytes. Such receptors may be used as a potential target to enhance the neuroprotective effects exerted by these glial cells.
Introduction

Amyotrophic lateral sclerosis (ALS) is progressive degeneration and loss of upper and lower motor neurons in the brain and spinal cord, causing muscle weakness and paralysis (Hardiman et al., 2011). In 1993, genetic studies identified the first mutations in the copper-zinc superoxide dismutase gene (SOD1), which encodes for a key antioxidant enzyme, SOD1 (Rosen et al., 1993). Mutations in SOD1 account for 20% of genetic ALS and 2% of all ALS. More recently, similar studies have identified mutations in other genes, such as TARDBP (TAR-DNA binding protein) and FUS (fused in sarcoma), which encode proteins involved in pre-mRNA splicing, transport and/or stability (Buratti and Baralle, 2010; Lagier-Tourenne et al., 2010), and, in particular, the CCGGGG hexanucleotide expansion in the C9orf72 gene which appears to account for up to 40% of genetic cases (Cruts et al., 2013). Their pathogenic mechanisms, which differ, in part, from the toxicity associated with mutations in SOD1, led to a novel molecular classification of ALS subtypes (Al-Chalabi and Hardiman, 2013; Renton et al., 2014).

The ultimate goal in ALS is to develop novel therapeutics that will slow disease progression. Rilutek® has been the only FDA approved drug but limited in efficacy (Habib and Mitsumoto, 2011). Recently cannabinoids have been shown to have neuroprotective effects in transgenic rodent ALS models (Bilsland and Greensmith, 2008; de Lago et al., 2015, for review). Chronic treatment with the phytocannabinoid Δ9-tetrahydrocannabinol (Δ9-THC) delayed motor impairment and improved survival in the SOD-1G93A transgenic mouse (Raman et al., 2004). Other cannabinoid compounds, including the less psychotropic plant-derived cannabinoid cannabidiol (Weydt et al., 2005), the non-selective synthetic agonist WIN55,212-2 (Bilsland et al., 2006), and the selective cannabinoid receptor type-2 (CB2) agonist AM1241 (Kim et al., 2006; Shoemaker et al., 2007), produced similar effects. Genetic or pharmacological inhibition of fatty acid amide hydrolase (FAAH), one of the key enzymes in endocannabinoid degradation, was also beneficial in SOD-1G93A transgenic mice (Bilsland et al., 2006). The efficacy shown by compounds that target the CB2 receptor (Kim et al., 2006; Shoemaker et al., 2007) appears to be facilitated by the fact that this receptor was found to be up-regulated in reactive glia in post-mortem spinal cord tissue from ALS patients (Yiangou et al., 2006). Such elevation of...
CB$_2$ receptors has been also described in SOD$_1^{G93A}$ transgenic mice (Shoemaker et al., 2007; Moreno-Martet et al., 2014), and we recently found that the response occurred predominantly in activated astrocytes recruited at lesion sites in the spinal cord (Espejo-Porras et al., unpublished results). We have also described a similar increase in CB$_2$ receptors on reactive microglia in TDP-43 transgenic mice (Espejo-Porras et al., 2015). Based on these studies, the CB$_2$ receptor may be a novel target in altering disease progression in ALS, given its effective control of glial influences exerted on neurons, as has been investigated in other disorders (Fernández-Ruiz et al., 2007, 2015; Iannotti et al., 2016 for review).

A challenge of preclinical studies of novel neuroprotective agents in ALS is poor translation of therapeutic success in small animal (e.g. rodents, zebrafish, flies, nematodes) to human ALS patients. In most of cases, they were based on the over-expression of specific human gene mutations. In this context, we have recently paid attention to canine degenerative myelopathy (DM), a multisystem central and peripheral axonopathy described in dogs in 1973 (Averill, 1973), with an overall prevalence of 0.19% (Coates and Wininger, 2010 for review), which shares pathogenic mechanisms with some forms of human ALS, including mutations in SOD1 as one of the major causes of the disease (Awano et al., 2009). With some differences depending on the type of breeds, DM is characterized by degeneration in the white matter of the spinal cord and the peripheral nerves, then affecting both upper and lower motor neurons (Coates and Wininger, 2010 for review). The disease appears at 8-14 years of age with an equivalent affectation in both genders and necessitating euthanasia (Coates and Wininger, 2010 for review). This canine pathology represents a unique opportunity to investigate ALS in a context much more close to the human pathology, using animal species which, in the phylogeny, are closest to humans, and in which the disease occurs spontaneously. Our objective in the present study has been to investigate the changes that the development of DM produces in endocannabinoid elements in those CNS sites (spinal cord) most affected in this disease. It is important to remark that such elements may derive in potential targets for a pharmacological therapy with cannabinoid-based therapies (e.g. Sativex) aimed at delaying/arresting the progression of the disease in these dogs, and furtherly in humans. The study has been carried out with post-mortem tissues (spinal cords) from dogs affected by DM kindly provided by Dr. Joan R.
Coates (University of Missouri, Columbia, MO, USA), adequately classified in different disease stages (Coates and Wininger, 2010). All DM tissues included the necessary clinical, genetic and neuropathological information, and they were accompanied by adequate matched control tissues. Both DM-affected and control tissues were used for analysis of endocannabinoid receptors and enzymes using biochemical (qPCR, Western blot) and, in some cases, histological (immunohistochemistry) procedures, including the use of double immunofluorescence staining to identify the cellular substrates in which the changes in endocannabinoid elements (CB2 receptors) take place.

Results

Validation of the expected histopathological deterioration in DM-affected dogs

The data provided by the biobank confirmed that all tissues obtained from DM-affected dogs had a clinical diagnosis of DM in all cases supported by the genetic analysis which confirmed the presence of the SOD1 mutation. They corresponded to two different breeds, which are within the most affected by this disease (Coates and Wininger, 2010), and animals were all euthanized in an age interval of 9-13.6 years (11.8 ± 0.6), with a grade of the disease of 1-3 (2.2 ± 0.3). DM-affected dogs included 6 spayed females and 2 castrated males (see details in Table 1). The control tissues were selected from normal dogs or with no clinical diagnosis of DM. All control dogs were homozygous wild-type and age-matched (8-13.6 years; 10.0 ± 0.8). They included 6 females, 1 of them spayed, and 1 castrated male (see details in Table 1).

We found a significant reduction in the number of Nissl-stained cell bodies corresponding to lower motor neurons located in the ventral horn of DM-affected spinal cords (Figures 1A and 1B). The neuronal loss was accompanied by an intense glial reactivity in the affected areas, in particular we detected a 3-fold increase in GFAP immunolabelling in the spinal grey matter (Figures 1C and 1D). We also found microgliosis in both white and grey matter of the spinal cord, detected with DAB immunostaining for the microglial marker Iba-1 (Figure 2A), which we quantitated in a 2.5-fold elevation in the grey matter using Iba-1 immunofluorescence (Figures 2B and 2C).
Changes in the endocannabinoid receptors and enzymes in DM-affected dogs

Next, we investigated possible differences between DM-affected dogs and control animals in the expression of endocannabinoid genes measured by qPCR. Although certain trends towards an elevation may be apparently appreciated, there were no significant changes in the CB\textsubscript{1} receptor, as well as in FAAH, monoacylglycerol lipase (MAGL), N-arachidonoyl-phosphatidylethanolamine phospholipase D (NAPE-PLD), diacylglycerol lipase (DAGL) enzymes between the two groups (Figure 3A). We attempted to determine whether the trends detected for these five parameters may correspond to a greater affectation in DM dogs with advanced disease, but we did not find any statistically significant correlation (data not shown). They were not related to gender-dependent differences (data not shown). The absence of changes in CB\textsubscript{1} receptor gene expression was observed at the protein level using DAB immunostaining in the grey matter (Figures 3B and 3C). This happened despite the reduction in the number of motor neurons described before with Nissl staining.

Next, we investigated the CB\textsubscript{2} receptor, an endocannabinoid element that is frequently altered in conditions of neurodegeneration (Fernández-Ruiz et al., 2007, 2015; Iannotti et al., 2016), including ALS (Yiangou et al., 2006; Shoemaker et al., 2007; Moreno-Martet et al., 2014; Espejo-Porras et al., 2015). First, we detected an increase of more than 2-fold in CB\textsubscript{2} receptor expression, measured by qPCR, in DM-affected dogs (Figure 4A). We also investigated whether this increase occurred predominantly in the tissues obtained from DM-affected dogs at the intermediate and advanced stages, but we did not find any significant correlation between both variables (data not shown). This increase in gene expression was confirmed at the protein level using Western blotting first (2-fold increase; Figure 4B), as well as using DAB immunostaining which showed that the elevation of CB\textsubscript{2} receptors occurred predominantly in the grey matter (Figures 5A and 5B).

Double-labelling analyses to identify the CB\textsubscript{2} receptor-positive cellular substrates

The examination of the morphology of those cells positive for the CB\textsubscript{2} receptor in DAB immunostaining (Figure 5A) suggested they should be glial cells. We wanted to confirm this fact by using double-labelling immunofluorescence analysis. We found
that CB$_2$ receptor immunolabelling colocalized with GFAP immunofluorescence (Figure 6), thus indicating that the up-regulation of CB$_2$ receptor in the spinal cord of DM-affected dogs occurred in reactive astrocytes. Similar double-labelling immunofluorescence with Iba-1 did not detect any colocalization with the CB$_2$ receptor immunostaining, indicating that the receptor is not located in the microglial cells in the spinal cord of DM-affected dogs (Figure 7).

**Discussion**

Our study addressed changes in specific endocannabinoid elements in canine DM, a disease of older dogs with similarities to ALS (Coates and Winninger, 2010 for review). The endocannabinoid system has been previously investigated in different regions of the canine brain (Pirone et al., 2016), but this is the first time that these elements are investigated in the context of an important neurodegenerative disorder occurring in dogs. The benefits that such investigation may have would impact in a better development of cannabinoid-based therapies for human ALS, but also these studies may serve as a first step in the process to have also a cannabinoid-based pharmacotherapy useful in the veterinary medicine. Our study has investigated the six endocannabinoid elements commonly recognized to develop pharmacological therapies, and has identified the CB$_2$ receptor as promising potential target. It is also important to mention that our study demonstrates no losses of CB$_1$ receptors, which are typically located in neurons, despite the losses of motor neurons occurring in the disease. This supports that, contrarily to other neurodegenerative conditions in humans with profound losses of neuronal CB$_1$ receptors, e.g. Huntington’s disease (Fernández-Ruiz et al., 2015), this receptor may also serve as a potential target in canine DM (shown here) and also in human ALS (de Lago et al., 2015).

Regarding the CB$_2$ receptor, what we found here has been similarly observed in transgenic ALS rodent models (Shoemaker et al., 2007; Moreno-Martet et al., 2014; Espejo-Porras et al., 2015) and ALS patients (Yiangou et al., 2016), that the receptor becomes strongly up-regulated in activated glia in response to neuronal damage. The response is not exclusive of ALS but also observed in other acute or chronic neurodegenerative disorders (e.g. ischemia, Alzheimer’s disease, Parkinson’s disease, Huntington’s chorea; reviewed in Fernández-Ruiz et al., 2007, 2015; Iannotti
et al., 2016). These findings support that the elevation of CB$_2$ receptors in activated glial elements is an endogenous response of the endocannabinoid signaling aimed at protecting neurons against cytotoxic insults, as well as at restoring neuronal homeostasis and integrity (Pacher and Mechoulam, 2011; de Lago et al., 2015; Fernández-Ruiz et al., 2015).

Result of our study further demonstrated that the elevation of CB$_2$ receptors occurred in activated astrocytes rather than in microglial cells. This finding has been previously described in the spinal cords of transgenic SOD1 mice (Espejo-Porras et al., unpublished results). In other transgenic models of ALS, e.g. TDP-43 transgenic mice, the up-regulatory response of these receptors occurred predominantly in reactive microglial cells (Espejo-Porras et al., 2015) and in tissues of human ALS patients (Yiangou et al., 2006). In multiple sclerosis and Huntington’s chorea, the overexpression of CB$_2$ receptors occurred in both activated astrocytes and reactive microglia (Benito et al., 2007; Sagredo et al., 2009). The CB$_2$ receptor elevations in activated glial elements may be related to the control of the influence of these cells on neuronal homeostasis, for example, by enhancing the metabolic support and the glutamate reuptake activity exerted by astrocytes (Fernández-Ruiz et al., 2015 for review), by facilitating the transfer of microglial cells from the M1 to the M2 phenotypes (Mecha et al., 2016 for review), or by attenuating the generation of proinflammatory cytokines, chemokines, nitric oxide and reactive oxygen species by either astrocytes or microglial cells when they become activated (Fernández-Ruiz et al., for review). These potentialities situate this receptor in a promising position to serve for the development of novel therapies. In the case of our present study, and given their preferential location in activated astrocytes, we will need to conduct additional research aimed at investigating the consequences of the selective CB$_2$ receptor activation in these glial cells during the progression of this canine disease.

In conclusion, our results demonstrated a marked up-regulation of CB$_2$ receptors occurring in the spinal cord of dogs affected by DM. Such up-regulation occurred in absence of changes in other endocannabinoid elements and was concentrated in activated astrocytes, then becoming a potential target to enhance the protective effects exerted by these glial cells to improve neuronal homeostasis and integrity.
Materials and Methods

Management of the postmortem tissues

All experiments were conducted on post-mortem spinal cord tissues collected from DM affected and unaffected dogs. All tissues (formalin-fixed tissues for routine histopathology and frozen tissues for qPCR and Western blotting) were provided by Dr. Joan R. Coates (Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO, USA). Protocols for tissue collection were approved by the University of Missouri Animal Care and Use Committee.

Tissues provided included those of DM affected dogs and age-matched controls and accompanied by adequate clinical and genetic testing information (see details in Table 1). DM diagnoses were confirmed histopathologically by assessing the mid- to lower thoracic spinal cord segment for evidence of myelinated axon loss and pronounced astrogliosis in the dorsal portion of the lateral funiculus (Awerill, 1973; March et al., 2009). Dogs that had exhibited clinical signs of DM but did not show the typical histopathology were presumed to have another cause for the myelopathy and excluded from the study. The spinal cord segments were examined for the presence of SOD1 immunoreactive aggregates within ventral horn motor neurons (Awano et al., 2009). Dogs that had not exhibited any clinical signs of DM prior to euthanasia and whose thoracic spinal cords were histologically normal were used as controls.

Tissues from DM-affected dogs were sorted by different stages of disease progression characterized at origin according to the following clinical and histopathological characteristics: (i) Stage 1 (upper motor neuron paraparesis): progressive general proprioceptive ataxia and asymmetric spastic paraparesis, but intact spinal reflexes; (ii) Stage 2 (non-ambulatory paraparesis to paraplegia): mild to moderate loss of muscle mass, reduced to absent spinal reflexes in pelvic limbs, and possible urinary and fecal incontinence; (iii) Stage 3 (lower motor neuron paraplegia to thoracic limb paresis): signs of thoracic limb paresis, flaccid paraplegia, severe loss of muscle mass in pelvic limbs, and urinary and fecal incontinence; and (iv) Stage 4 (lower motor neuron tetraplegia and brainstem signs): flaccid tetraplegia,
difficulty with swallowing and tongue movements, reduced to absent cutaneous trunci reflex, generalized and severe loss of muscle mass, and urinary and fecal incontinence (see details in Coates and Wininger, 2010). All DM tissues were accompanied by the necessary signalment, genotype and clinical diagnosis (see details in Table 1). Tissue studies confirm the loss of motor neurons using Nissl staining and accompanied by analysis of glial reactivity using GFAP and Iba-1 immunostaining. Next, we investigated the status of endocannabinoid receptors and enzymes using biochemical (qPCR, Western blot) and, in some cases, immunostaining procedures, included double immunofluorescence staining to identify the cellular substrates in endocannabinoid elements (CB₂ receptors) take place. For all measures, tissues used corresponded to 7-8 different animals per experimental group.

Real time qRT-PCR analysis

Total RNA was extracted from spinal cord samples (from T7 to T10) using TRI Reagent® (Sigma Chem., Madrid, Spain). The total amount of RNA extracted was quantitated by spectrometry at 260 nm and its purity was evaluated by the ratio between the absorbance values at 260 and 280 nm, whereas its integrity was confirmed in agarose gels. To prevent genomic DNA contamination, DNA was removed and single-stranded complementary DNA was synthesized from 0.6 μg of total RNA using a commercial kit (Rneasy Mini Quantitect Reverse Transcription, Qiagen, Izasa, Madrid, Spain). The reaction mixture was kept frozen at -80°C until enzymatic amplification. Quantitative real-time PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, U.S.A.) to quantify mRNA levels for CB₁ receptor (ref. Cf02716352_u1), CB₂ receptor (ref. Cf02696139_s1), DAGL (Cf02705627_m1), FAAH (ref. Cf02648944_m1) and MAGL (ref. Cf02662432_m1). For NAPE-PLD, we used one custom designed assay (Custom Plus Taqman® RNA Assay Design, Applied Biosystems, Foster City, CA, USA). In all cases, we used GAPDH expression (ref. Cf04419463_gH) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the threshold cycle (Ct) was calculated by the instrument’s software (7300 Fast
System, Applied Biosystems, Foster City, CA, USA). Values were normalized as percentages over the control group.

**Western blot analysis**

Purified protein fractions were isolated using ice-cold RIPA buffer. Then, 20 μg of protein were boiled for 5 min in Laemmlı SDS loading buffer (10% glycerol, 5% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue and 125 mM TRIS-HCl, at pH 6.8) and loaded onto a 12% acrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA), and then transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA) using mini Trans-Blot Electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with the mouse anti-CB2 receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by a second incubation during 2 hours at room temperature with an ECL™ Horseradish Peroxidase-linked whole secondary antibody (GE Healthcare UK Limited, Buckinghamshire, UK) at a 1:5000 dilution. Reactive bands were detected by chemiluminescence with the Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare UK Limited, Buckinghamshire, UK). Images were analyzed on a ChemiDoc station with Quantity one software (Bio-Rad Laboratories, Madrid, Spain). Data were calculated as the ratio between the optical densities of the specific protein band and the housekeeping protein GAPDH, and they were normalized as percentages over the control group.

**Histological procedures**

*Tissue slicing.* Fixed spinal cords were sliced with a cryostat at the thoracic level, always between T7-T10, which correspond to the spinal level in which the axonal degeneration was most severe (Coates and Wininger, 2010). Coronal sections (20 μm thick) were collected on gelatin-coated slides. Sections were used for procedures of Nissl-staining, immunohistochemistry and immunofluorescence.

*Nissl staining.* Slices were used for Nissl staining using cresyl violet, as previously described (Alvarez et al., 2008). A Leica DMRB microscope (Leica, Wetzlar, Germany) and a DFC300FX camera (Leica) were used for the observation and
photography of the slides, respectively. For counting the number of Nissl-stained large motor neurons in the anterior horn, high resolution photomicrographs were taken with the 20x objective under the same conditions of light, brightness and contrast. Four images coming from at least 3 sections per animal were analyzed. The final value is the mean for all animals included in each experimental group.

**Immunohistochemistry.** Slices were preincubated for 20 min in 0.1M PBS with 0.1% Triton X-100, pH 7.4, and subjected to endogenous peroxidase blockade by 1 hour incubation at room temperature in peroxidase blocking solution (Dako Cytomation, Glostrup, Denmark). Then, they were incubated in 0.1M PBS with 0.01% Triton X-100, pH 7.4, with one of the following primary antibodies: (i) polyclonal anti-rabbit Iba-1 antibody (Wako Chemicals, Richmond, VI, USA) used at 1/500; (ii) polyclonal anti-rabbit CB1 receptor (Thermo Scientific, MA, USA) used at 1/400; and (iii) polyclonal anti-goat CB2 receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) used at 1/100. Incubation was prolonged overnight at 4°C, then sections were washed in 0.1M PBS and incubated for 2 hours at room temperature with the appropriate biotin-conjugated anti-goat or anti-rabbit (1:200; Vector Laboratories, Burlingame, CA, USA) secondary antibodies. Vectastain® Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and a DAB substrate–chromogen system (Dako Cytomation, Glostrup, Denmark) were used to obtain a visible reaction product. Negative control sections were obtained using the same protocol with omission of the primary antibody. All sections for each immunohistochemical procedure were processed at the same time and under the same conditions. A Leica DMRB microscope (Leica, Wetzlar, Germany) and a DFC300FX camera (Leica) were used for slide observation and photography.

**Immunofluorescence.** Quantification of GFAP and Iba-1 immunoreactivity was also carried out using immunofluorescence, and this procedure was also used for double-labelling studies. Slices were preincubated for 1 hour with Tris-buffered saline with 1% Triton X-100 (pH 7.5). Then, sections were sequentially incubated overnight at 4°C with a polyclonal anti-Iba-1 (1/500; Wako Chemicals, Richmond, VI, USA) or polyclonal anti-GFAP (1/200; Dako Cytomation, Glostrup, Denmark), followed by washing in Tris-buffered saline and a new incubation (at 37°C for 2 hours) with an Alexa 488 anti-rabbit antibody conjugate made in donkey (1/200; Invitrogen,
Carlsbad, CA, USA), rendering green fluorescence for anti-Iba-1 or anti-GFAP. The immunofluorescence was quantified using a SP5 Leica confocal microscope and the ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA). For double-labelling studies, sections were then washed again and incubated overnight at 4°C with a polyclonal anti-CB2 receptor (1/100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). This was followed by washing in Tris-buffered saline and a further incubation (at room temperature for 2 hours) with a biotin-conjugated anti-goat (1:200; Vector Laboratories, Burlingame, CA, USA) secondary antibody, followed by a new washing and an incubation (at 37°C for 2 hours) with red streptavidin (Vector Laboratories, Burlingame, CA, USA) rendering red fluorescence for anti-CB2 receptor. Sections were counter-stained with nuclear stain TOPRO-3-iodide (Molecular Probes, Eugene, OR, USA) to visualize cell nuclei. To quench endogenous autofluorescence, tissue sections were also treated with 0.5% Sudan Black (Merck, Darmstadt, Germany) in 70% ethanol for 1 min and differentiated with 70% ethanol (Schnell et al., 1999). A Leica TCS SP5 microscope was used for slide observation and photography. Differential visualization of the fluorophores was accomplished through the use of specific filter combinations. Samples were scanned sequentially to avoid any potential for bleed through of fluorophores.

Statistics

Data were assessed by unpaired Student’s t-test or one-way ANOVA followed by the Student-Newman-Keuls test, as required.
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Authors’ contribution:

- **Study design:** Eva de Lago, Carmen Pérez-Díaz and Javier Fernández-Ruiz
- **Sample collection and clinical/genetic characterization:** Joan R. Coates
- **Sample analysis:** María Fernández-Trapero, Francisco Espejo-Porras and Carmen Rodríguez-Cueto
- **Data interpretation (including statistical assessment):** Eva de Lago and Javier Fernández-Ruiz
- **Manuscript draft:** Javier Fernández-Ruiz (revised, corrected and approved by all authors)

Disclosure of potential conflicts of interest

Authors declare that they have no conflicts of interest in relation with this submission.

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References


## Table 1
Clinical, genetic and histopathological characteristics of DM-affected and control dogs whose spinal tissues have been used in this study.

<table>
<thead>
<tr>
<th>UCM animal code</th>
<th>Genotype</th>
<th>Age at death (years)</th>
<th>Gender</th>
<th>Breed</th>
<th>Diagnosis</th>
<th>Disease grade</th>
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**Figure 1.** Representative microphotographs and quantification for Nissl staining (panels A and B, respectively) and GFAP immunofluorescence (panels C and D, respectively) in the spinal cord sections (grey matter in the ventral horn at T7-T10) of DM-affected and age-matched control dogs. Values are expressed as means ± SEM for 6-7 animals per group. Data were analyzed using the unpaired Student’s t-test (*p<0.05, **p<0.01 compared to control animals). Bar scale = 300 µm (Nissl staining) and 200 µm (GFAP immunofluorescence).
Figure 2. Representative microphotographs for Iba-1 immunostaining using DAB (panel A), as well as for Iba-1 immunofluorescence (panel B) and its quantification (panel C), in the spinal cord sections (grey matter in the ventral horn and white matter in the dorsal area, both at T7-T10) of DM-affected and age-matched control dogs. Values are expressed as means ± SEM for 5-7 animals per group. Data were analyzed using the unpaired Student’s t-test (**p<0.01 compared to control animals). Bar scale = 300 µm for DAB immunostaining and 200 µm for immunofluorescence.
Figure 3. Gene expression for the CB₁ receptor and the NAPE-PLD, DAGL, FAAH and MAGL enzymes measured by qPCR (panel A), and representative microphotographs for CB₁ receptor immunostaining using DAB (panel C) and its quantification in the grey matter in the ventral horn (panel B), in the spinal cord samples (for qPCR) or T7-T10 sections (for immunostaining) of DM-affected and age-matched control dogs. Values are expressed as means ± SEM for 7-8 animals per group. Data were analyzed using the unpaired Student’s t-test. Bar scale = 150 µm.
Figure 4. Gene expression for the CB₂ receptor measured by qPCR (panel A), as well as Western blot analysis for this receptor (panel B) in spinal cord samples of DM-affected and age-matched control dogs. Values correspond to % over control animals and are expressed as means ± SEM for 7 animals per group. Data were analyzed using the unpaired Student’s t-test (*p<0.05 compared to control animals).
Figure 5. Representative microphotographs for CB$_2$ receptor immunostaining using DAB (panel A) and its quantification (panel B) in the grey matter of the ventral horn in T7-T10 spinal cord sections of DM-affected and age-matched control dogs. Values are expressed as means ± SEM for 5-6 animals per group. Data were analyzed using the unpaired Student’s t-test (*p<0.05 compared to control animals). Bar scale = 150 µm and 50 µm for the detail. Arrows indicate the CB$_2$ receptor-positive cells.
Figure 6. Representative microphotographs corresponding to a double immunofluorescence analysis for the CB$_2$ receptor and GFAP, using TOPRO-3 for labelling cell nuclei, in the grey matter of the ventral horn in T7-T10 spinal cord sections of DM-affected and age-matched control dogs ($n=3$/group). Bar scale = 50 µm. Arrows indicate cells labelled with the antibodies for the two markers.
**Figure 7.** Representative microphotographs corresponding to a double immunofluorescence analysis for the CB$_2$ receptor and Iba-1, using TOPRO-3 for labelling cell nuclei, in the grey matter of the ventral horn in T7-T10 spinal cord sections of DM-affected and age-matched control dogs (n=3/group). Bar scale = 50 µm.