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RESEARCH PAPER

Changes in CB₁ and CB₂ receptors in the post-mortem cerebellum of humans affected by spinocerebellar ataxias

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BACKGROUND AND PURPOSE

Spinocerebellar ataxias (SCAs) are a family of chronic progressive neurodegenerative diseases, clinically and genetically heterogeneous, characterized by loss of balance and motor coordination due to degeneration of the cerebellum and its afferent and efferent connections. Unlike other motor disorders, the possible role of changes in the endocannabinoid system in the pathogenesis of SCAs has not been investigated.

EXPERIMENTAL APPROACH

The status of cannabinoid receptor type 1 (CB₁) and cannabinoid receptor type 2 (CB₂) receptors in the post-mortem cerebellum of SCA patients and controls was investigated using immunohistochemical procedures.

KEY RESULTS

Immunoreactivity for the CB₁ receptor, and also for the CB₂ receptor, was found in the granular layer, Purkinje cells, neurons of the dentate nucleus and areas of white matter in the cerebellum of SCA patients at levels notably higher than controls. Double-labelling procedures demonstrated co-localization of CB₁ and, in particular, CB₂ receptors with calbindin, supporting the presence of these receptors in Purkinje neurons. Both receptors also co-localized with Iba-1 and glial fibrillary acidic protein in the granular layer and white matter areas, indicating that they are present in microglia and astrocytes respectively.

CONCLUSIONS AND IMPLICATIONS

Our results demonstrate that CB₁ and CB₂ receptor levels are significantly altered in the cerebellum of SCA patients. Their identification in Purkinje neurons, which are the main cells affected in SCAs, as well as the changes they experienced, suggest that alterations in endocannabinoid receptors may be related to the pathogenesis of SCAs. Therefore, the endocannabinoid system could provide potential therapeutic targets for the treatment of SCAs and its progression.

LINKED ARTICLES

This article is part of a themed section on Cannabinoids 2013. To view the other articles in this section visit <http://dx.doi.org/10.1111/bph.2014.171.issue-6>

Abbreviations

CB₁, cannabinoid receptor type 1; CB₂, cannabinoid receptor type 2; DAB, 3,3'-Diaminobenzidine; GFAP, glial fibrillary acidic protein; KPBS, potassium phosphate-buffered saline; SCA, spinocerebellar ataxia; NBB, The Netherlands Brain Bank; TBS, Tris-buffered saline

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Introduction

During the last decade, robust preclinical evidence has accumulated demonstrating the neuroprotective potential of compounds that affect the activity of the endocannabinoid system, including both plant-derived cannabinoids and certain signalling lipids (reviewed in Fernández-Ruiz *et al.*, 2010; 2011). This potential has been demonstrated for both acute and chronic brain damage and, in some cases (e.g. Huntington's disease), it is already being investigated at the clinical level (de Yébenes, 2010) based on preclinical results obtained with specific cannabinoid-based drugs (Sagredo *et al.*, 2011; Valdeolivas *et al.*, 2012). The evidence that cannabinoids are neuroprotective compounds is mostly derived from the pharmacological correction of alterations in the endocannabinoid communicating system observed in neurodegenerative disorders (reviewed in Fernández-Ruiz *et al.*, 2007; 2010), alterations that several authors have related to the pathogenesis of these disorders (reviewed in Centonze *et al.*, 2007; Fernández-Ruiz *et al.*, 2010). These alterations are usually deficits, but increases in specific elements of this communicating system (i.e. ligands, receptors, enzymes) have also been found even in early and presymptomatic phases of these disorders, and have been characterized in experimental models and, to a lesser extent, in human subjects (reviewed in Centonze *et al.*, 2007; Fernández-Ruiz *et al.*, 2010). These changes could play a pivotal role in the pathogenesis of these conditions, presumably by aggravating the neuronal injury; although, in some cases, these alterations have been interpreted as being part of an endogenous protectant response against brain damage (reviewed in Fernández-Ruiz *et al.*, 2010; Pacher and Mechoulam, 2011). For example, several studies have described an increased accumulation of arachidonoyl-ethanolamide, 2-arachidonoyl-glycerol or both in specific brain structures in experimental models of different neurodegenerative disorders including multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, ischaemia and brain trauma (Baker *et al.*, 2001; Panikashvili *et al.*, 2001; Witting *et al.*, 2004). Data from patients are also available (Pisani *et al.*, 2005). Experimental brain injury has also been found to be associated with an up-regulation of cannabinoid receptors, including cannabinoid receptor type 1 (CB₁) receptors (Jin *et al.*, 2000; Hansen *et al.*, 2001), although the most pertinent data were obtained with the cannabinoid receptor type 2 (CB₂) receptor. The CB₂ receptor is mostly absent in the brain of healthy individuals but has been reported to be significantly up-regulated in many degenerative pathologies. These results were obtained mainly in animal models of disease but also from a few studies in human samples (reviewed in Fernández-Ruiz *et al.*, 2007; Benito *et al.*, 2008). This response occurs preferentially in activated astrocytes and, in particular, reactive microglial cells (note that resident microglial cells do not express CB₂ receptors), which indicates that CB₂ receptors may play a role in regulating the trophic and/or the cytotoxic influences of these cells on neurons (Fernández-Ruiz *et al.*, 2007; Benito *et al.*, 2008). The induction/up-regulation of CB₂ receptors has also been observed in structures undergoing neuronal damage in patients and animal models of stroke (Ashton *et al.*, 2007), Alzheimer's disease (Benito *et al.*, 2003), Huntington's disease (Palazuelos *et al.*, 2009; Sagredo *et al.*,

2009), Parkinson's disease (Price *et al.*, 2009; García *et al.*, 2011), multiple sclerosis (Maresz *et al.*, 2005; Benito *et al.*, 2007), amyotrophic lateral sclerosis (Yiangou *et al.*, 2006), simian immunodeficiency virus encephalitis (Benito *et al.*, 2005), Down's syndrome (Núñez *et al.*, 2008) and neuropathic pain (Zhang *et al.*, 2003), but not in cases of spinocerebellar ataxias (SCAs), the pathology under investigation in the present study. Importantly, preclinical evaluations of different selective agonists of this receptor have generally demonstrated that these drugs delay the progression of brain damage in most of these disorders, thus indicating their potential to act as neuroprotectants (see below and some reviews in Fernández-Ruiz *et al.*, 2007; Benito *et al.*, 2008).

Autosomal-dominant SCAs are a group of inherited neurodegenerative disorders. The most prevalent cases belong to the family of polyglutaminopathies, which also includes Huntington's disease, and are primarily caused by excessive CAG repeats leading to the expansion of a polyglutamine tract in different recipient proteins (i.e. huntingtin in Huntington's disease, frequently ataxins but also other proteins in SCAs) (Klockgether, 2011). Despite its ubiquitous distribution, the mutant protein usually only affects specific structures within the CNS, the cerebellum being the key structure affected in SCAs, which explains the specificity of the neurological symptoms, that is motor incoordination and ataxia (Matilla-Dueñas *et al.*, 2012). The age of onset of the clinical symptoms is typically between 30 and 50 years of age, although early onset in childhood, as well as cases in which the pathology initiates after 60 years, have been reported for specific SCA subtypes, frequently related to longer or shorter polyglutamine expansions (Durr, 2010). Like other polyglutamine diseases, SCAs are characterized by protein misfolding, failed or incomplete proteolysis and the deposition and formation of intracellular protein aggregates, which represents the key neuropathological characteristic of these disorders (Orr, 2012). Although there is some controversy as regards the role of these aggregates in neurotoxicity, they appear to play a key role in eliciting transcription dysregulation, mitochondrial failure, excitotoxicity, alterations in calcium homeostasis, oxidative stress and local inflammation, which ultimately lead to cell death in specific subpopulations of cerebellar neurons (Fratkin and Vig, 2012). Hence cannabinoid agonists that have been demonstrated to be effective in reducing excitotoxicity (e.g. selective CB₁ agonists), oxidative injury (e.g. antioxidant cannabinoids) or inflammation (e.g. selective CB₂ agonists) in various chronic neurodegenerative disorders (reviewed in Fernández-Ruiz *et al.*, 2010) may also have a therapeutic effect in SCAs. However, it is also possible that cannabinoids could enhance autophagy, a cellular process responsible, among others, for eliminating the accumulation of toxic proteins, and this has been proposed as a possible therapeutic strategy for SCAs and other polyglutamine disorders, given that mutant proteins are autophagy substrates due to the failure of primary proteolytic processes (Williams *et al.*, 2006). Interestingly, certain cannabinoid agonists have been demonstrated to stimulate cell autophagy in human glioma cells (Salazar *et al.*, 2009), which may also have a therapeutic effect in polyglutamine disorders such as SCAs.

Whether cannabinoids can provide neuroprotection in SCAs as they do in preclinical models of other neurodegenerative disorders also needs to be investigated. However,

before this can be done, the type of changes that the development of cerebellar degeneration in the different SCAs produces in particular elements of the endocannabinoid signalling system, specifically alterations in endocannabinoid receptors, needs to be clarified. Hence, we used immunohistochemical procedures to identify and quantify the major endocannabinoid receptors, that is CB₁ and CB₂ receptors, in the post-mortem cerebellum of SCA patients and control subjects.

Methods

Subjects

We used post-mortem human cerebellum from control subjects and from patients diagnosed with an autosomal dominant cerebellar ataxia and these samples were obtained from The Netherlands Brain Bank (NBB), Institute for Neuroscience, Amsterdam, the Netherlands. All material was collected from donors for and from whom a written informed consent for a brain autopsy and the use of material and clinical information for research purposes had been obtained from the NBB. The samples received were fixed, paraffinized, sliced (8 µm) and mounted on glass slides. Table 1 summarizes the major characteristics (age, gender and post-mortem delay) of the individuals (patients and controls) included in our analyses. Unfortunately, the number of SCA types in the NBB, as well as in other biobanks that we contacted, is rather small, so our experimental group had to be formed using only one subject of the most representative types of SCAs. However, the neuropathological data provided by the biobank confirmed that all the SCA patients exhibited a marked atrophy in the cerebellum and the pons (small size, presence of gliosis, and evidence of neuronal loss), but with no important changes in the remaining brain areas, for example, the substantia nigra was generally normal, the locus coeruleus was visible and the ventricular system was not dilated. We also did a histopathological evaluation of all the tissues received so as to confirm the data provided by the biobank (see

Figures 1–3, and Results section). This evaluation was done before the tissues were used for the immunohistochemical analysis of CB₁ and CB₂ receptors. We also considered the pharmacological information (medication and intoxication history) of SCA patients and control subjects provided by the NBB. Most of SCA patients had been treated with benzodiazepines (e.g. clonazepam, temazepam, diazepam), GABA-acting substances (e.g. baclofen, GABApentine) and, in a few cases, tricyclic antidepressants (e.g. amitriptyline). They were also sporadically treated with antibiotics (e.g. amoxyciline), and all of them received morphine during the last days/hours before death. One patient (subject #7) was treated with nabilone 6 years before death but the treatment was not effective. Five of the patients with a genetic diagnosis of the most prevalent SCAs had to be discarded from the initial patient population ($n = 10$) because of a previous history of tobacco and/or alcohol addiction, or because they consumed cannabis; this was another reason why our sample size was small. Medication and intoxication history of control subjects was also available and used to select suitable control subjects. Some of the control subjects had been diagnosed with colon carcinoma and, therefore, also received morphine during the last days/hours before death.

Histochemical techniques

Immunohistochemistry. The protocol used was as described previously (Tsou *et al.*, 1998; Benito *et al.*, 2005) with slight modifications. Tissue sections were deparaffinized and washed extensively in 50 mM, pH 8, potassium phosphate-buffered saline (KPBS). To obtain more efficient immunostaining, samples were subjected to an antigen retrieval procedure (Shi *et al.*, 2001). Briefly, tissue sections were placed into a stainless steel pressure cooker containing Antigen Retrieval Solution pH 9 (Dako Cytomation, Glostrup, Denmark). After being heated under pressure for 2 min, samples were removed and washed extensively in KPBS. Then, endogenous peroxidase was blocked by 30 min incubation at room temperature in peroxidase blocking solution (Dako Cytomation). After several washes with KPBS, tissues were incubated with the primary antibody overnight at room

Table 1

Major characteristics of patients and control subjects whose post-mortem samples were used in this study (they were obtained from the Netherlands Brain Bank)

Subject #	Age	Gender	Diagnosis	Post-mortem delay
1	56	Male	Control	325 min
2	62	Male	Control	480 min
3	66	Male	Control	465 min
4	83	Female	Control	315 min
5	88	Female	Control	355 min
6	54	Male	SCA3	315 min
7	59	Male	SCA2	310 min
8	61	Male	SCA	325 min
9	82	Female	SCA7	305 min
10	86	Female	SCA6	395 min

Calbindin immunostaining

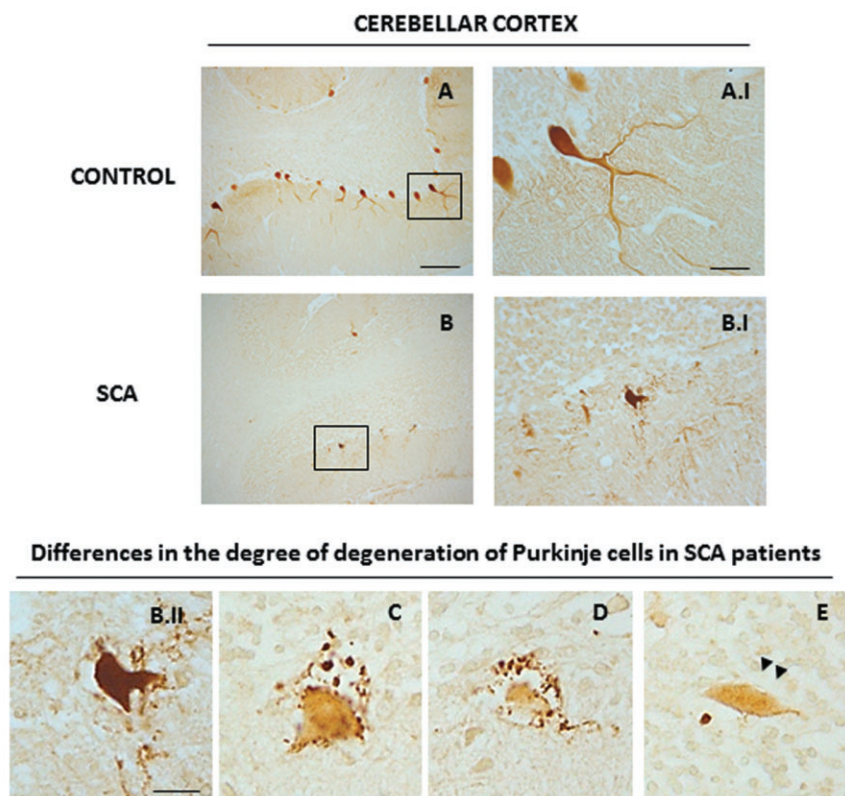


Figure 1

DAB immunostaining for calbindin, a marker of Purkinje cells, in the cerebellar cortex of SCA patients (B and B.I) and control subjects (A and A.I). Microphotographs shown in B.II, C, D and E correspond to details obtained in the cerebellar cortex of SCA patients proving the existence of Purkinje cells with different degrees of degeneration noted by loss in calbindin immunostaining (scale bars: A, B = 200 μ m; A.I, B.I = 50 μ m; B.II-E = 20 μ m). Arrowheads indicate the presence of axonal torpedoes in surviving Purkinje cells.

temperature. Antibodies (see those used and specific details in Table 2) were diluted in KPBS containing 1% BSA (Sigma Chem., Madrid, Spain) and 1% Triton X-100 (Sigma Chem.). After the incubation, sections were washed in KPBS, followed by incubation with biotinylated goat anti-rabbit antibody (1:200), biotinylated horse anti-mouse antibody (1:200) or biotinylated horse anti-goat antibody (1:200) for 2 h at room temperature. Avidin-biotin complex (Vector Elite; Vector Laboratories, Burlingame, CA, USA) and a 3,3'-Diaminobenzidine (DAB) substrate-chromogen system (Dako Cytomation) were used to obtain a visible reaction product. Negative controls were obtained for each sample by omitting primary antibody as well as by using a blocking peptide. Sections were dehydrated, sealed and coverslipped. A Leica DMRB microscope (Leica, Wetzlar, Germany) and DFC300FX camera (Leica) were used for the observations and photography of the slides respectively.

Quantification of CB₁ and CB₂ immunostaining was carried out on high-resolution digital microphotographs that were taken with the 20 $\times$ objective and under the same conditions of light and brightness/contrast. The analysis software

ImageJ (NIH, USA) was used to measure the mean density of labelling in a selected area, in the case of the granular layer and the white matter regions, or the mean density of labelling in individual cells in the case of the Purkinje cells (approximately 10 cells per sample in patients and 25 cells per sample in control subjects) and the neurons of the dentate nucleus (approximately 25 cells per sample in both groups). Five images were obtained for each sample. We carried out separate densitometrical analysis for each region. All data are expressed in arbitrary units.

Immunofluorescence. To identify specific cell populations, we performed co-localization studies with immunofluorescence combining the anti-CB₁ or the anti-CB₂ antibodies with antibodies for specific markers for Purkinje cells (i.e. calbindin), macrophages (i.e. Cd68), microglia (i.e. Iba-1) and astrocytes and Bergmann glia [i.e. glial fibrillary acidic protein (GFAP)]. After the antigen retrieval procedure, tissue sections were washed with pH 7.5 Tris-buffered saline (TBS) before overnight incubation at room temperature with the antibodies used for identification of the cell types followed by

Iba-1 immunostaining

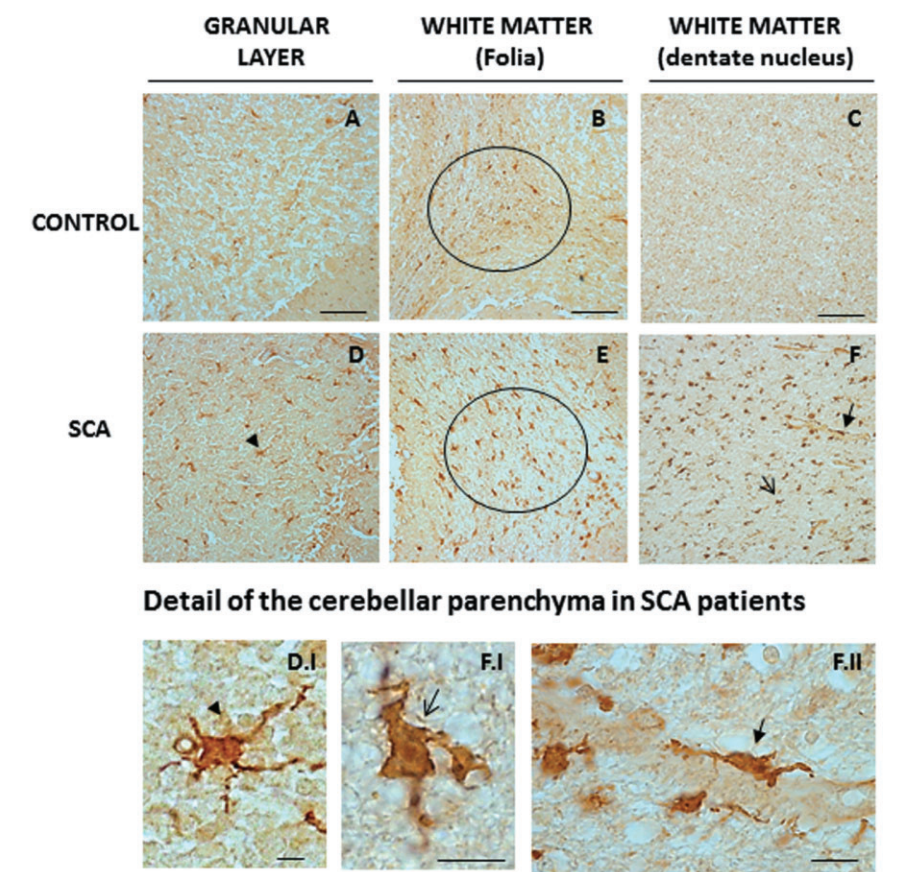


Figure 2

DAB immunostaining for Iba-1, a marker of microglial cells, in different areas of the cerebellum of SCA patients (D–F) and control subjects (A–C). Microphotographs shown in D.I, F.I and F.II correspond to details obtained in the cerebellar parenchyma of SCA patients proving the presence of Iba-1-positive cells probably corresponding to microglial cells (marked with arrows and arrowheads) (scale bars: A–F = 100 µm; D.I, F.I and F.II = 10 µm).

Table 2

List of antibodies used in immunohistochemical studies

Antibody	Dilution (IHC)	Dilution (IF)	Class	Manufacturer
CB ₁ receptor	1:300	1:100	Polyclonal	Affinity BioReagents, Rockford, IL, USA
CB ₂ receptor	1:100	1:50	Monoclonal	R&D systems, Minneapolis, MN, USA
Calbindin D-28 K	1:500	1:300	Monoclonal	Sigma Chem., Madrid, Spain
Calbindin D-28 K		1:250	Polyclonal	Millipore, Billerica, MA, USA
GFAP-Cy3		1:1500	Monoclonal	Sigma Chem., Madrid, Spain
GFAP	1:500		Monoclonal	AbD Serotec, Oxford, UK
Iba-1	1:1000	1:1000	Polyclonal	Wako, Osaka, Japan
Iba-1		1:75	Polyclonal	Abcam, Cambridge, UK
Cd68		1:50	Monoclonal	Dako Cytomation, Glostrup, Denmark

GFAP, glial fibrillary acidic protein; IF, immunofluorescence; IHC, immunohistochemistry.

GFAP immunostaining

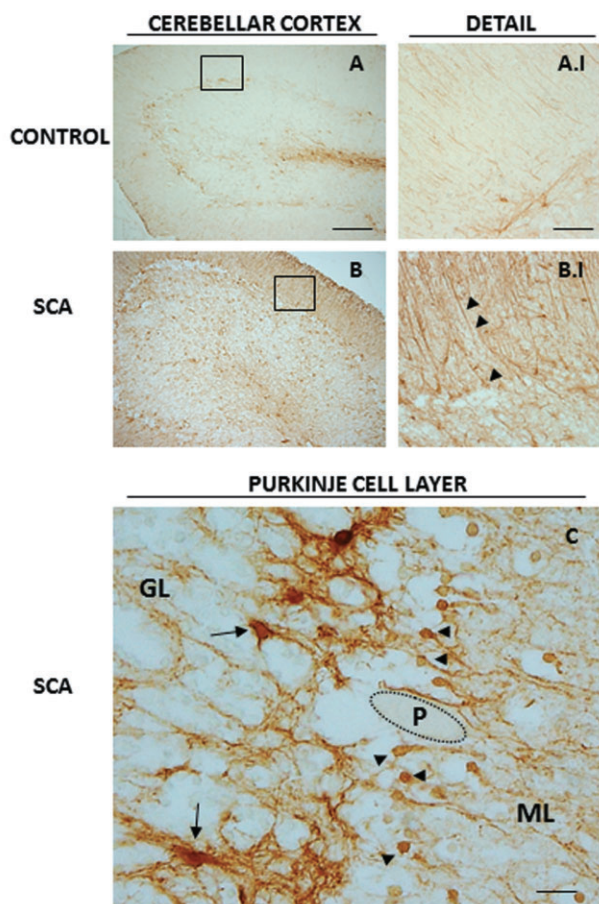


Figure 3

DAB immunostaining for GFAP, a marker of astrocytes, in the cerebellar cortex of SCA patients (B and B.I) and control subjects (A and A.I). The microphotograph shown in C corresponds to a detail of the Purkinje layer of SCA patients in which GFAP immunostaining was seen in two cell subpopulations that may correspond to protoplasmic astrocytes (marked with arrows) and Bergmann glia (marked with arrowheads). GL = granular layer; ML = molecular layer; P = Purkinje neurons (scale bars: A and B = 200 μ m; A.I and B.I = 50 μ m; C = 20 μ m).

incubation with an Alexa 546 secondary antibody conjugate (Invitrogen, Carlsbad, CA, USA) at 37°C for 2 h, rendering red fluorescence. In the case of the astrocyte marker GFAP, the tissue was incubated with GFAP-Cy3 antibody (Sigma Chem.) at 37°C for 2 h, rendering red fluorescence too. Afterwards, the primary antibodies for CB₁ or CB₂ were incubated overnight at room temperature after extensive washes in TBS, followed by incubation with Alexa 488 secondary antibody conjugate (Invitrogen), rendering green fluorescence. 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 treated with 1% Sudan Black (Merck, Darmstadt,

Germany) in 70% ethanol for 5 min and differentiated with 70% ethanol (Schnell *et al.*, 1999). Sections were mounted onto glass slides with aqueous solution (Vectashield; Vector Laboratories, Burlingame, CA, USA).

Confocal microscopy analysis. Samples were viewed on a Leica TCS SP5 microscope system using argon and helium-neon lasers. Differential visualization of the fluorophores Alexa 488 (excitation 496 nm and emission 519 nm), Alexa 546 (excitation 556 nm and emission 573 nm), Cy3 (excitation 550 nm and emission 565 nm) and Topro-3 (excitation 650 nm and emission 667 nm) was accomplished through the use of specific filter combinations. Samples were scanned sequentially to avoid any potential for bleed through of fluorophores. In some cases, images were processed with the Leica LAS AF software to obtain three-dimensional orthogonal projections.

Statistics

Data were assessed by Student's *t*-test using the GraphPad software (version 5.0).

The receptor nomenclature conforms to BJP's Concise guide to PHARMACOLOGY (Alexander *et al.*, 2013).

Results

Evidence of neuronal damage and glial activation in the cerebellum of SCA patients compared to control subjects

Before using the post-mortem samples for the analysis of endocannabinoid receptors, we analysed several parameters to confirm the presence of histopathological signs of dysfunction/degeneration in the patients affected by different types of SCAs. Our analyses have always concentrated in the cerebellum which is the main structure affected in this disease. As expected, the cerebellum of SCA patients had a notable loss of Purkinje cells (Figure 1B and B.I) compared to control subjects (Figure 1A and A.I), shown using immunolabelling with calbindin, a marker of these neurons. In addition, the immunostaining with calbindin also indicated that the surviving Purkinje cells in SCA patients showed a rare morphology, for example, they have a shrunken cell body and a notable reduction in the dendritic tree (Figure 1B.I). The surviving Purkinje cells present axonal swellings also termed axonal torpedoes (see arrowheads in Figure 1E). These structural alterations of Purkinje cells are evident to different extents in the cerebellum of each SCA patient, some have cells with strong calbindin immunostaining and others cells with a complete loss of immunostaining for this marker (Figure 1B.II, C and D). This pattern has also been observed previously (Ishikawa *et al.*, 1995; Seidel *et al.*, 2012). In addition to the loss of Purkinje neurons, there was also a marked degeneration of neurons located in the dentate nucleus in the cerebellum of SCA patients. These results were obtained using immunostaining with another marker such as neuron-specific enolase (data not shown), which was used to determine neuronal damage in other cerebellar disorders (Koeppen *et al.*, 2011).

We also observed significant microgliosis in the cerebellum of patients with SCAs (Figure 2D–F) compared with the control subjects (Figure 2A–C). The microglial activation was observed using immunolabelling with the microglial marker Iba-1 and was particularly evident in the white matter of the folia (Figure 2E; see inside the ellipse) and in that surrounding the dentate nucleus (Figure 2F), as well as in the granular layer (Figure 2D). The microglial cells identified in SCA patients presented a typical morphology of reactive microglia (i.e. reduction of processes and increase in the cell body size; see Figure 2D.I and F.I). There was also an increase in the perivascular microglia in SCA patients (Figure 2F.II).

Lastly, there was also marked astrogliosis, demonstrated by an increase in GFAP immunostaining, in the cerebellum of SCA patients compared to controls (see Figure 3). This was observed in the granular layer as well as in the white matter of the folia or that surrounding the dentate nucleus (data not shown), but it was particularly evident in the cerebellar cortex in which a notable difference in GFAP immunostaining was found in SCA patients (Figure 3B and B.I) compared to controls (Figure 3A and A.I). The increase in GFAP immunostaining in this structure in SCA patients may reflect the increase experienced by the Bergmann glia, a characteristic astrocyte subtype present in the cerebellum (marked with arrowheads in Figure 3B.I). The increase corresponded to both a higher number of these glial cells and also an increase in the number of cell processes. Bergmann gliosis was found close to the Purkinje cells, as these glial cells surround the Purkinje cells and their processes reach the molecular layer (see arrowheads in Figure 3C). In addition to Bergmann glial cells, other GFAP-positive cells were also identified close to the Purkinje cells. They corresponded to protoplasmic astrocytes that form a barrier between the granular layer and the Purkinje cells (marked with arrows in Figure 3C). These changes to astrocytes in SCA patients have been observed previously (Robitaille *et al.*, 1995).

Immunohistochemical study of CB₁ receptors in the cerebellum of SCA patients

As regards changes in endocannabinoid receptors, we first analysed the immunoreactivity for the CB₁ receptor, the major cannabinoid receptor present in neurons within the CNS. We found immunolabelling for this receptor in the Purkinje layer and also in the dentate nucleus (Figure 4). In control subjects, CB₁ receptors were apparent in Purkinje neurons; the immunolabelling was found in the basal region of these cells, in the so-called pinceau formations, which might indicate that the immunolabelling corresponds to the axons of basket cells that contact Purkinje cells (Figure 4A; see arrows in A.I). Similar findings have been obtained in the cerebellum of laboratory animals (Tsou *et al.*, 1998; Suárez *et al.*, 2008), although CB₁ receptor immunostaining has also been identified in the soma of Purkinje cells in the rat cerebellum (Moldrich and Wenger, 2000). In contrast, in all the SCA patients indicated the immunolabelling was much more evident in these pinceau formations but also in the remaining cell body of Purkinje cells (Figure 4B; see arrows in B.I). CB₁ immunostaining was also extremely high in neurons of the dentate nucleus in all SCA patients (Figure 4D; see arrows in D.I), whereas it was rather low in control subjects (Figure 4C; see arrows in C.I). As an additional measure to

determine the relevance of these observations, we quantified the CB₁ receptor immunolabelling in the Purkinje cells and in neurons of the dentate nucleus following the procedure described in Methods. These results confirmed that CB₁ receptor immunolabelling was strongly elevated in each surviving neuron of SCA patients compared to controls and this occurred in both structures (Figure 4), although it is important to note that, given the marked neuronal loss found in the Purkinje layer and the dentate nucleus, the immunolabelling for the CB₁ receptor in SCA patients would be lower than controls if the whole structure is considered rather than the labelling in individual cells. The quantified results also confirmed that the increase seen in individual cells occurred in all patients, even though they corresponded to different SCA types, and that the differences among the different SCA patients were not significant in either structure (Figure 4).

Double-labelling studies using calbindin as marker of Purkinje neurons indicated that CB₁ receptors and calbindin were co-localized in SCA patients (see Figure 5E, F, G, H and H.I); this was confirmed by orthogonal reconstruction (see Figure 5H.II). This strongly supports the presence of CB₁ receptors in Purkinje cells in SCA patients, but not, apparently, in the control subjects (see Figure 5A, B, C, D and D.I), probably due to the extremely low CB₁ receptor immunofluorescence detected in the controls' samples. In this sense, our data confirmed the increase in CB₁ receptors in the Purkinje layer of SCA patients found with DAB immunostaining (Figure 4), as it was evident that the CB₁ receptor immunolabelling was lower in cells containing high calbindin immunoreactivity (poorly degenerated cells), while it was higher when calbindin immunoreactivity was lower (highly degenerated cells) (data not shown).

Using DAB immunostaining, we also identified CB₁ receptors in the granular layer and in the white matter areas (folia and dentate nucleus) (see Figure 6). We found that the immunostaining was much more marked in the granular layer in all SCA patients (Figure 6C and C.I) compared to control subjects (Figures 6A and A.I). We also found a stronger immunostaining in the white matter of the cerebellar folia of SCA patients (Figure 6D and D.I) compared with controls (Figure 6B and B.I), and similarly with the white matter surrounding the dentate nucleus, although the differences in labelling of SCA patients (Figure 6F and F.I) compared with the controls (Figure 6E and E.I) were much more moderate in this structure. As in the case of the Purkinje layer and the dentate nucleus, we also quantified the CB₁ receptor immunolabelling in these structures (this time using a defined area, not labelling in individual cells) as an additional measure to strengthen the relevance of the above observations. Again, we confirmed that immunolabelling was higher in SCA patients compared to controls in the three structures, and that this increase occurred in all SCA patients despite the fact that they corresponded to different SCA types (Figure 6). However, there was one exception, the white matter of the folia of one of the SCA patients (subject #8) showed a significantly higher value compared to the others, which explains the high SEM found in the quantification of SCA patients in this structure (Figure 6). On the other hand, the CB₁ receptor-positive cells located in the white matter of the folia in SCA patients have a similar morphology to microglial cells (Figure 6D.II). To investigate this, we also conducted double-labelling experiments in

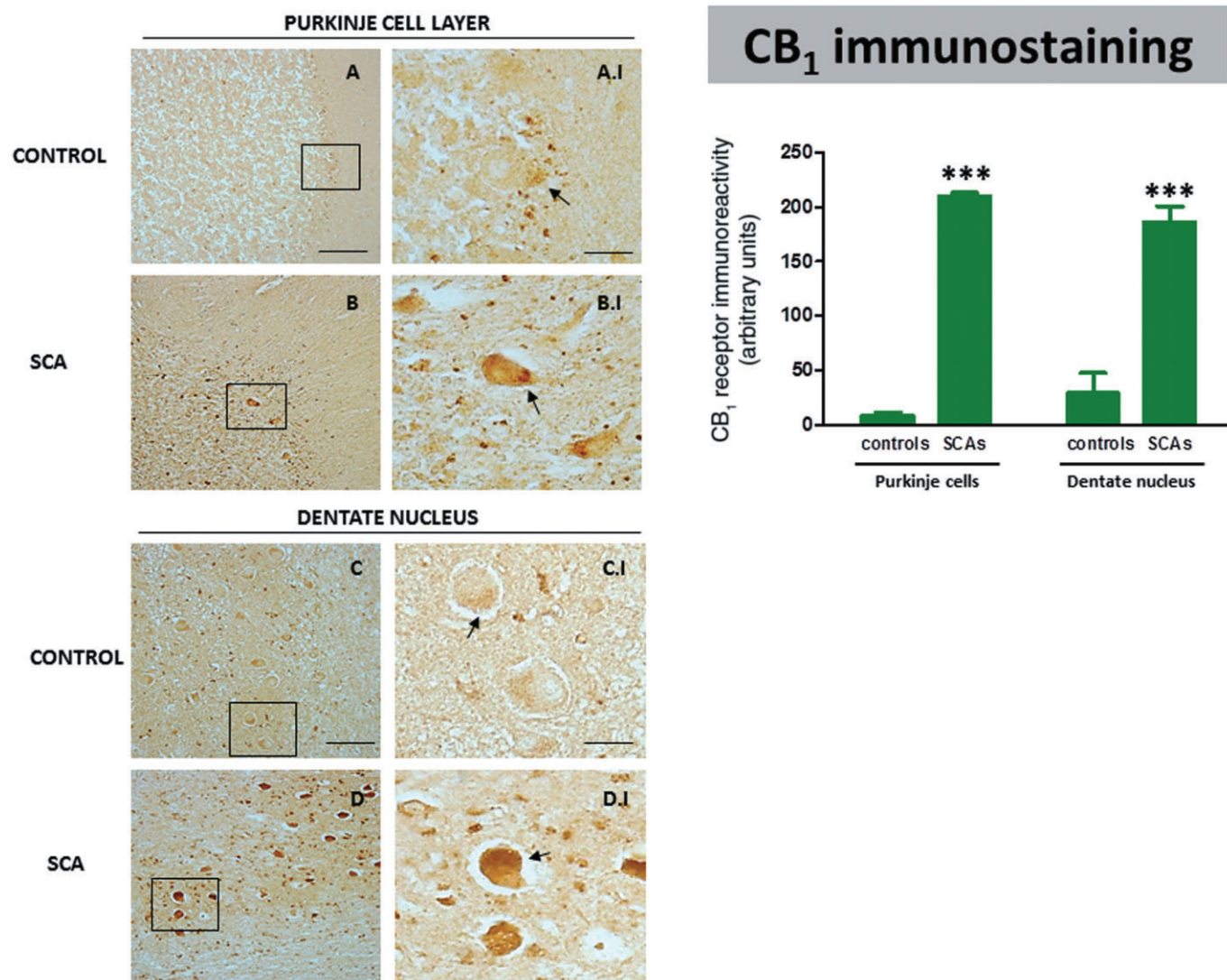


Figure 4

DAB immunostaining for the CB₁ receptor in the Purkinje layer and dentate nucleus of the cerebellum of SCA patients (B, B.I, D and D.I) and control subjects (A, A.I, C and C.I). The arrows indicate the CB₁ receptor-positive cells (scale bars: A–D = 100 µm; A.I–D.I = 25 µm). The microphotographs of SCA cases correspond to the subject #9 (SCA7) for the Purkinje layer and to subject #10 (SCA6) for the dentate nucleus. The bar panel corresponds to the quantification of CB₁ receptor immunostaining in the two structures of SCA patients and control subjects. Values correspond to the mean density of labelling in individual cells and are expressed as means ± SEM of five subjects per group. Data were assessed by Student's *t*-test (****P* < 0.001).

these structures, using specific markers for glial elements (i.e. Iba-1 for microglia and GFAP for astrocytes) as well as for infiltrated macrophages (i.e. Cd68). Our data indicated the presence of CB₁ receptors in Iba-1- and GFAP-positive cells in both the granular layer (Figure 7A–H) and the white matter structures (Figure 7I–P), while the presence of CB₁ receptors in Cd68-positive cells was found only in the white matter areas (Figure 7Q–T) but not in the granular layer (data not shown).

Immunohistochemical study of CB₂ receptors in the cerebellum of SCA patients

CB₂ receptor expression in the CNS is, in general, relatively limited and frequently associated with glial elements, in particular, those recruited to lesioned structures in pathological

conditions (Fernández-Ruiz *et al.*, 2007). Our data indicated that in SCAs this receptor is up-regulated, as found previously for other neurodegenerative disorders (see specific references in the Introduction). Thus, we observed a more marked immunostaining for the CB₂ receptor in the granular layer (Figure 8C; see arrows in C.I), in the white matter surrounding the dentate nucleus (Figure 8F; see arrows in F.I) and, in particular, in the white matter of the folia (Figure 8D; see arrows in D.I) of all SCA patients compared to control subjects (see Figure 8A and A.I, B and B.I, and E and E.I, respectively). Given the frequent problems observed with the anti-CB₂ receptor antibodies used so far (reviewed in Atwood and Mackie, 2010), we were extremely careful to choose an appropriate antibody for these immunostainings as well as

CB₁/calbindin double immunofluorescence

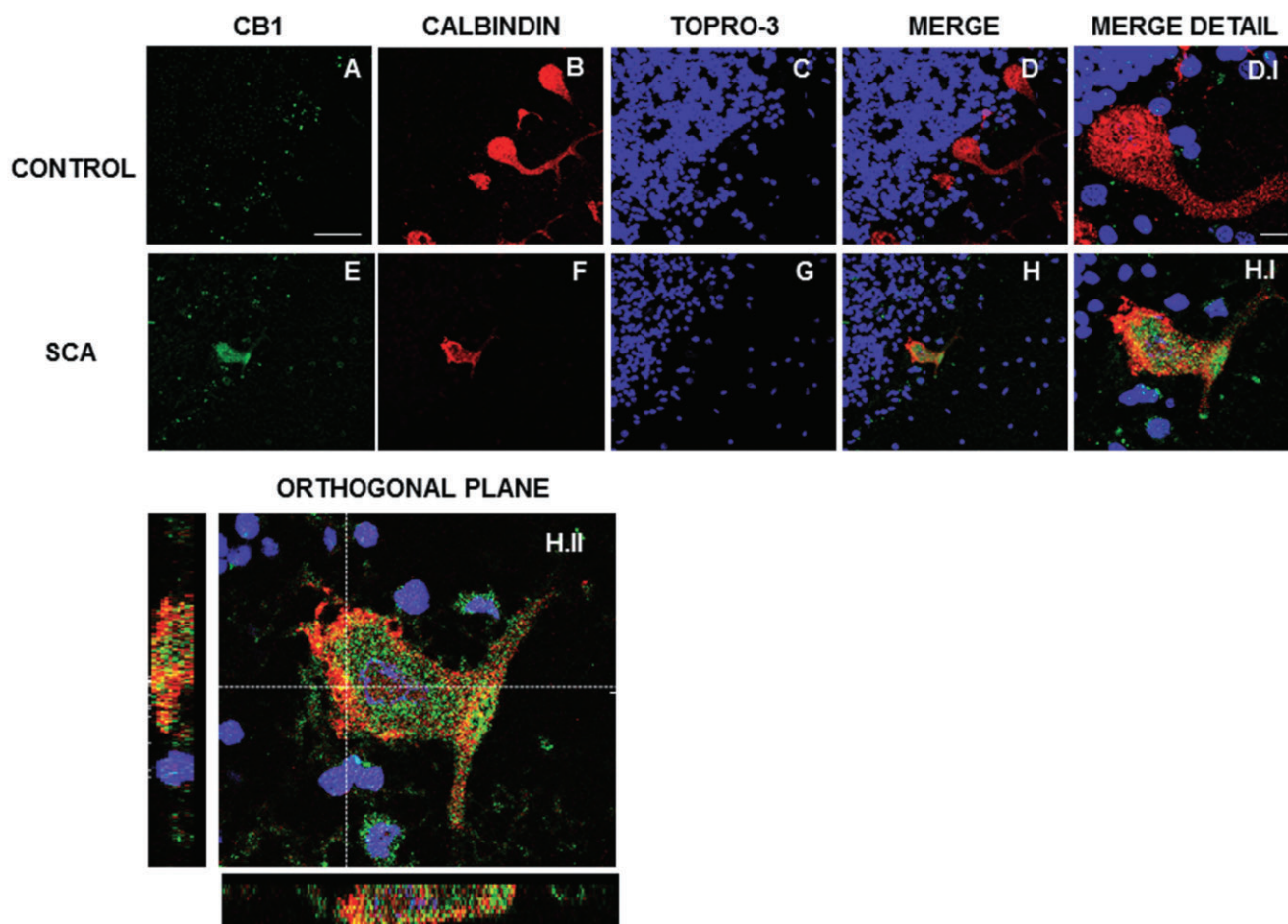


Figure 5

Double-labelling immunofluorescence using antibodies for the CB₁ receptor and calbindin, and TOPRO-3 staining, in the Purkinje layer of the cerebellum of SCA patients (E–H and H.II) and control subjects (A–D, D.I), showing co-localization of CB₁ receptors and calbindin in SCA patients but not in control subjects (scale bars: A–H = 50 µm; D.I and H.II = 10 µm). The H.II panel corresponds to the orthogonal reconstruction from confocal z-series in x-z (below) and y-z (left) planes. This reconstruction confirms the co-localization of CB₁ receptors and calbindin in SCA patients (scale bar: H.II = 10 µm). The microphotographs of SCA cases correspond to subject #7 (SCA2).

using appropriate internal controls (by omitting the primary antibody or by using a blocking peptide; the antibody is only for human tissue, so the option to check its specificity in CB₂ receptor knockout mice is not valid as it does not label tissues from wild-type mice) to ensure the specificity of the signal found. Again, the quantification of this immunolabelling confirmed it was higher in SCA patients compared to controls in the three structures, and that this increase occurred in all patients despite the fact that they corresponded to different SCA types. The quantification data also confirmed that the differences among the different SCA patients were not significant in these structures, with the exception of the white matter of the folia, in which the SCA3 patient showed a significantly lower value compared to the others; again this explains the high SEM found in this structure for SCA patients (Figure 8).

Our DAB immunostaining results indicate that the increase in CB₂ receptors in SCA patients occurs in reactive microglial cells and possibly in astrocytes. The astrocytes observed in these structures in SCA patients did not develop the same hypertrophic profile as those located in the Purkinje and granular layers (see below). In the white matter areas, the most evident change experienced by these astrocytes was the increase in the number of processes (data not shown). Double-labelling studies using antibodies against CB₂ receptors and GFAP confirmed that these receptors were located in astrocytes in white matter areas (see Figure 9I–L), while similar studies using Iba-1 to identify microglial cells also supported the presence of CB₂ receptors in these cells in both the granular layer (see Figure 9A–D) and areas of the white matter (see Figure 9E–H). Lastly, DAB immunostaining also revealed the presence of immunoreactivity for the CB₂ receptor in perivas-

CB₁ immunostaining

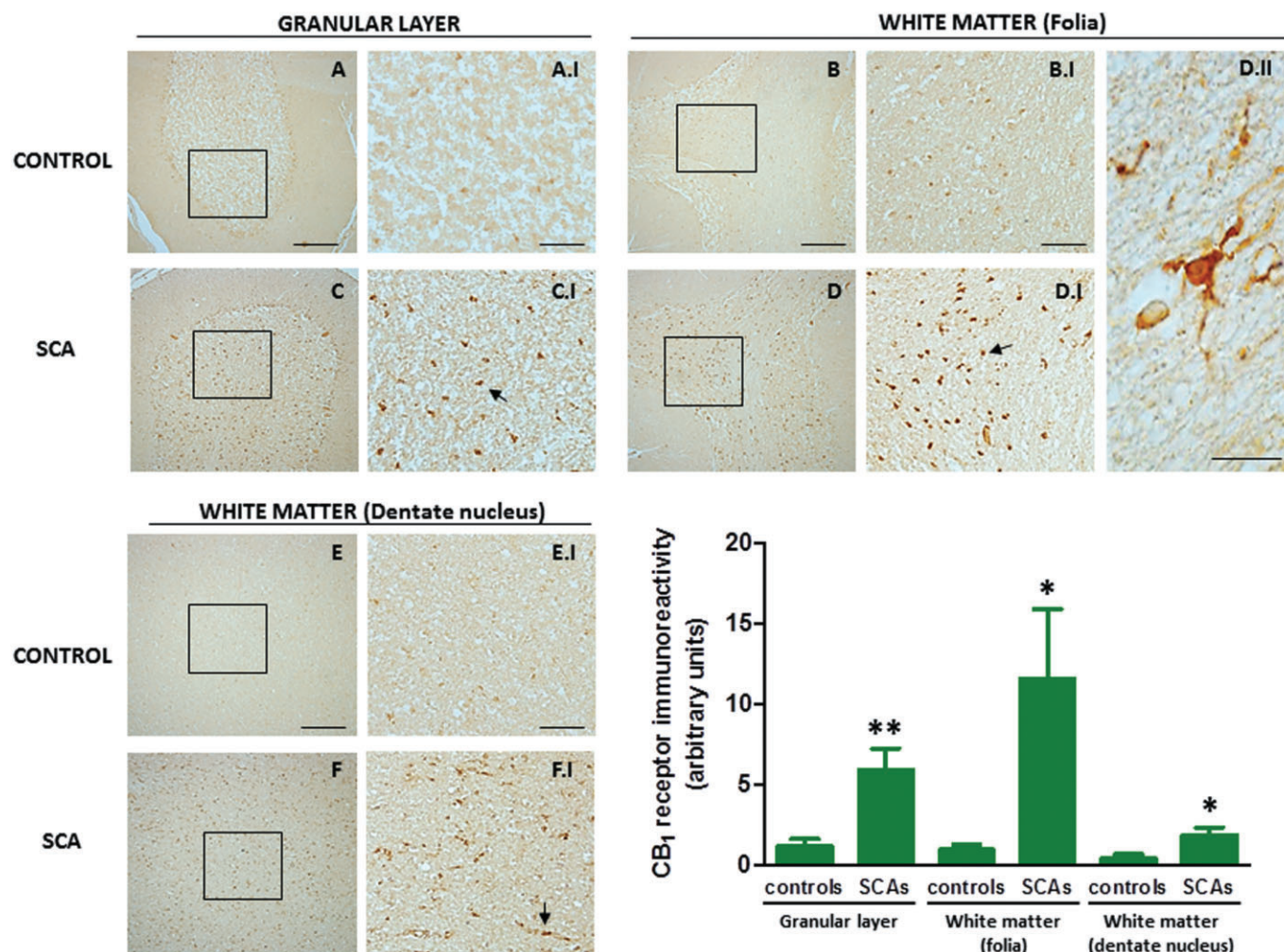


Figure 6

DAB immunostaining for the CB₁ receptor in the granular layer and the white matter areas of the cerebellum of SCA patients (C, C.I, D, D.I, F and F.I) and control subjects (A, A.I, B, B.I, E and E.I). The arrows indicate the CB₁ receptor-positive cells. The microphotograph shown in D.II corresponds to a detail of the CB₁ receptor-positive cells in the white matter of the folia of SCA patients (scale bars: A–F = 200 μ m; A.I–F.I = 50 μ m; D.II = 10 μ m). The microphotographs of SCA cases correspond to subject #9 (SCA7) for the granular layer and the white matter of the folia, and to subject #10 (SCA6) for the white matter surrounding the dentate nucleus. The bar panel corresponds to the quantification of CB₁ receptor immunostaining in the three structures of SCA patients and control subjects. Values correspond to the mean density of labelling in a selected area and are expressed as means $\pm$ SEM of five subjects per group. Data were assessed by Student's *t*-test (**P* < 0.05; ***P* < 0.01).

cular zones, although this only occurred in SCA patients (see Figure 8G). Double-labelling studies, using antibodies for GFAP and the CB₂ receptor, demonstrated that the labelling corresponds to astrocytes (see Figure 9M–P). This co-localization did not exist in control subjects (data not shown).

However, unexpectedly, the CB₂ receptor was identified not only in glial cells but also in neurons, particularly in Purkinje cells (Figure 10A and B), and, as in the case of the CB₁ receptor, this was apparent in all SCA patients but not in control subjects. In controls, the immunoreactivity for the CB₂ receptor was found only in a few Purkinje cells (marked

with arrows in Figure 10A.I), and, in the stained-cells, the labelling was found only in the basal region of the cell (marked with arrows in Figure 10A.I). This observation suggests that this labelling corresponds to terminals of basket cells rather than to Purkinje cells, as has been proposed in previous studies (Suárez *et al.*, 2008). However, the immunoreactivity for the CB₂ receptor seen in all SCA patients in Purkinje cells was much more intense, with most of the cells being immunolabelled and the immunoreactivity occupying most of the cell body (see Figure 10B.I). We next conducted double-labelling studies using antibodies against the CB₂

Double immunofluorescence with CB₁ receptors and glial markers

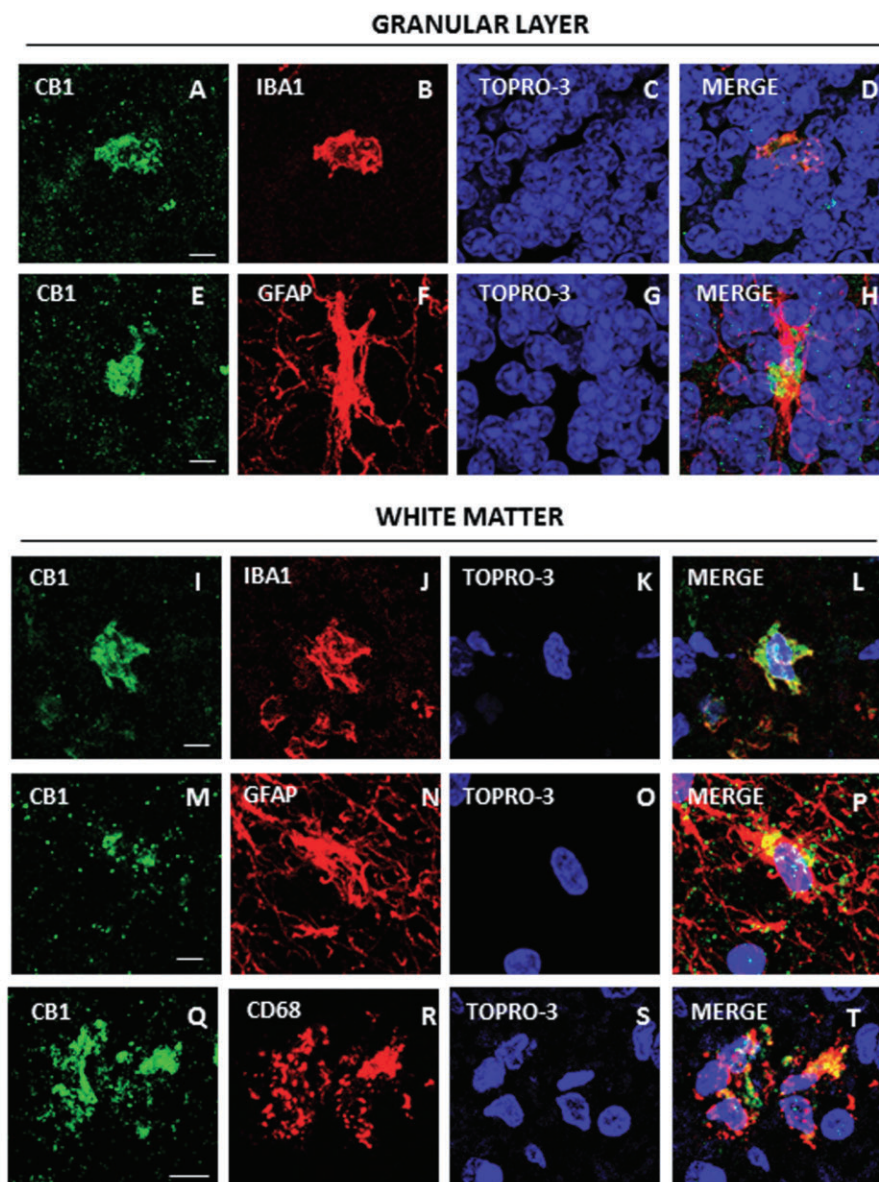


Figure 7

Double-labelling immunofluorescence using antibodies for the CB₁ receptor and markers of glial elements (Iba-1 for microglia and GFAP for astrocytes) or infiltrated macrophages (Cd68), and TOPRO-3 staining, in the granular layer and white matter of the cerebellum of SCA patients. The microphotographs showed co-localization of CB₁ receptors with Iba-1 in the granular layer (A–D) and the white matter (I–L), with GFAP in the granular layer (E–H) and the white matter (M–P), and with Cd68 in the white matter (Q–T) (scale bars: A–T = 20 µm). The microphotographs of SCA cases correspond to subject #8 (SCA), except in the case of Cd68 immunostaining that corresponds to subject #9 (SCA7).

receptor and calbindin to label Purkinje cells. We found that the CB₂ receptors strongly co-localized with calbindin in all SCA patients (see Figure 11E, F, G, H and H.I), and this was confirmed by orthogonal reconstruction (see Figure 11H.II).

CB₂ receptor immunolabelling was also found in the Purkinje layer in cells that do not appear to be neurons and that presumably could correspond to Bergmann glial cells (see Figure 10A.I and B.I). This immunoreactivity was found

in control subjects (Figure 10A.I; indicated by arrowheads) but to a greater extent in all SCA patients (Figure 10B.I; indicated by arrowheads). As mentioned above, the Bergmann glial cells are activated (hypertrophic) in SCA patients (Robitaille *et al.*, 1995); we also observed this (see Figure 3). We tried to identify these CB₂ receptor-positive cells using double-labelling studies with L1-NCAM, vimentin or GFAP as glial markers, but our results were not conclusive (data not

CB₂ immunostaining

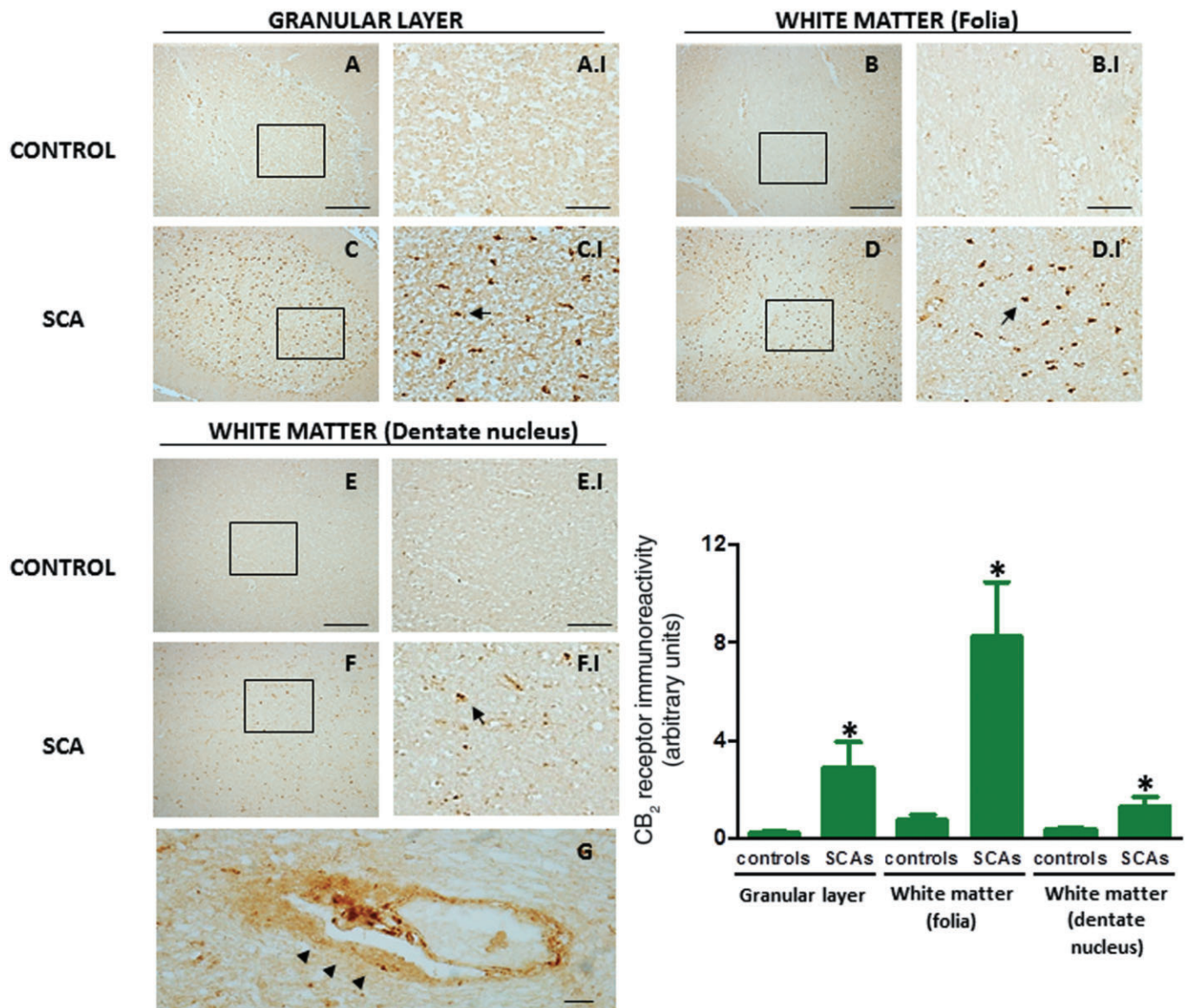


Figure 8

DAB immunostaining for the CB₂ receptor in the granular layer and the white matter areas of the cerebellum of SCA patients (C, C.I, D, D.I, F and F.I) and control subjects (A, A.I, B, B.I, E and E.I). The arrows indicate the CB₂ receptor-positive cells. The microphotographs of SCA cases correspond to subject #10 (SCA6). The microphotograph shown in G represents a detail of the CB₂ receptor-positive cells (marked with arrowheads) in perivascular zones of the white matter of SCA patients (scale bars: A–F = 200 µm; A.I–F.I = 50 µm; G = 10 µm). It corresponds to the subject #8 (SCA). The bar panel corresponds to the quantification of CB₂ receptor immunostaining in the three structures of SCA patients and control subjects. Values correspond to the mean density of labelling in a selected area and are expressed as means ± SEM of five subjects per group. Data were assessed by the Student's t-test (*P < 0.05).

shown). Lastly, CB₂ receptor immunoreactivity was also identified in neuronal elements in the dentate nucleus (Figure 10C and D), and, as with the CB₁ receptors, the immunoreactivity was much more intense in all SCA patients (Figure 10D; see arrows in D.I) than in control subjects

(Figure 10C; see arrows in C.I). We also quantified the CB₂ receptor immunolabelling in this nucleus and in the Purkinje cells and, in both cases, we confirmed that the immunolabelling was higher in SCA patients compared to controls, and that this increase occurred in all patients despite them corre-

Double immunofluorescence with CB₂ receptors and glial markers

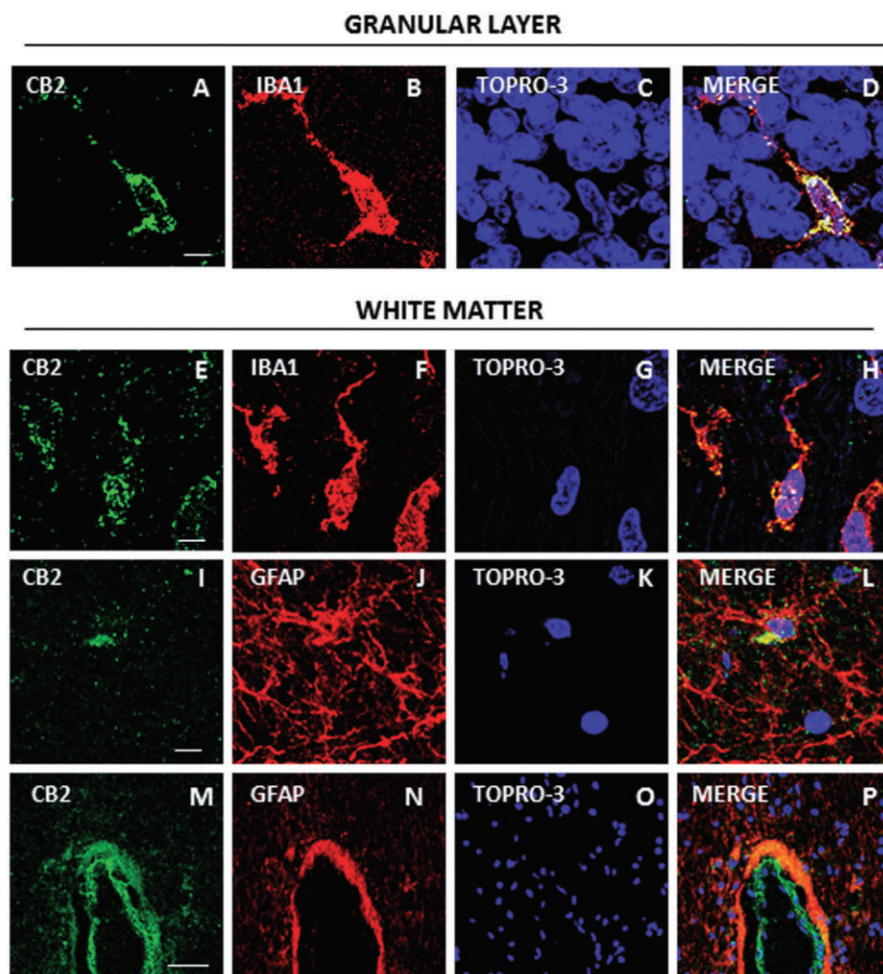


Figure 9

Double-labelling immunofluorescence using antibodies for the CB₂ receptor and markers of glial elements (Iba-1 for microglia and GFAP for astrocytes), and TOPRO-3 staining, in the granular layer and white matter of the cerebellum of SCA patients. The microphotographs showed co-localization of CB₂ receptors with Iba-1 in the granular layer (A–D) and the white matter (E–H), and with GFAP in the white matter (I–L) and also in perivascular zones (M–P) (scale bars: A–L = 20 µm; M–P = 50 µm). The microphotographs of SCA cases correspond to subject #9 (SCA7) for Iba-1 immunostaining and to subject #10 (SCA6) for GFAP immunostaining.

sponding to different SCA types, and that the differences among the different SCA patients were not significant in either structure (Figure 10). As with the CB₁ immunostaining, this increase was seen in surviving neurons when they were analysed individually, but the labelling would be lower in SCA patients compared to controls if the whole structure was considered instead of the labelling in individual cells, due to the marked loss of neurons seen in these two structures in SCA patients (Figure 10).

Discussion

The data obtained in this study support the idea that key elements of the endocannabinoid system, that is the cannabi-

noid CB₁ and CB₂ receptors, are significantly altered in the cerebellum of SCA patients compared to control subjects, as has been found in other neurodegenerative disorders (reviewed in Fernández-Ruiz *et al.*, 2010). Our data were obtained from post-mortem samples from patients with different types of SCAs. However, it is important to note that, due to the difficulties in recruiting a sufficient number of samples for each type of SCA (see Methods), our experimental group was formed using only one subject from each of the most representative types of SCAs. *A priori* this might represent a problem with reliability of findings, given that these SCAs have different pathologies with regard to the specific neuronal subpopulation that is most affected. However, losses of Purkinje cells, as well as of other cerebellar neuronal subpopulations, have been documented in all the SCA types

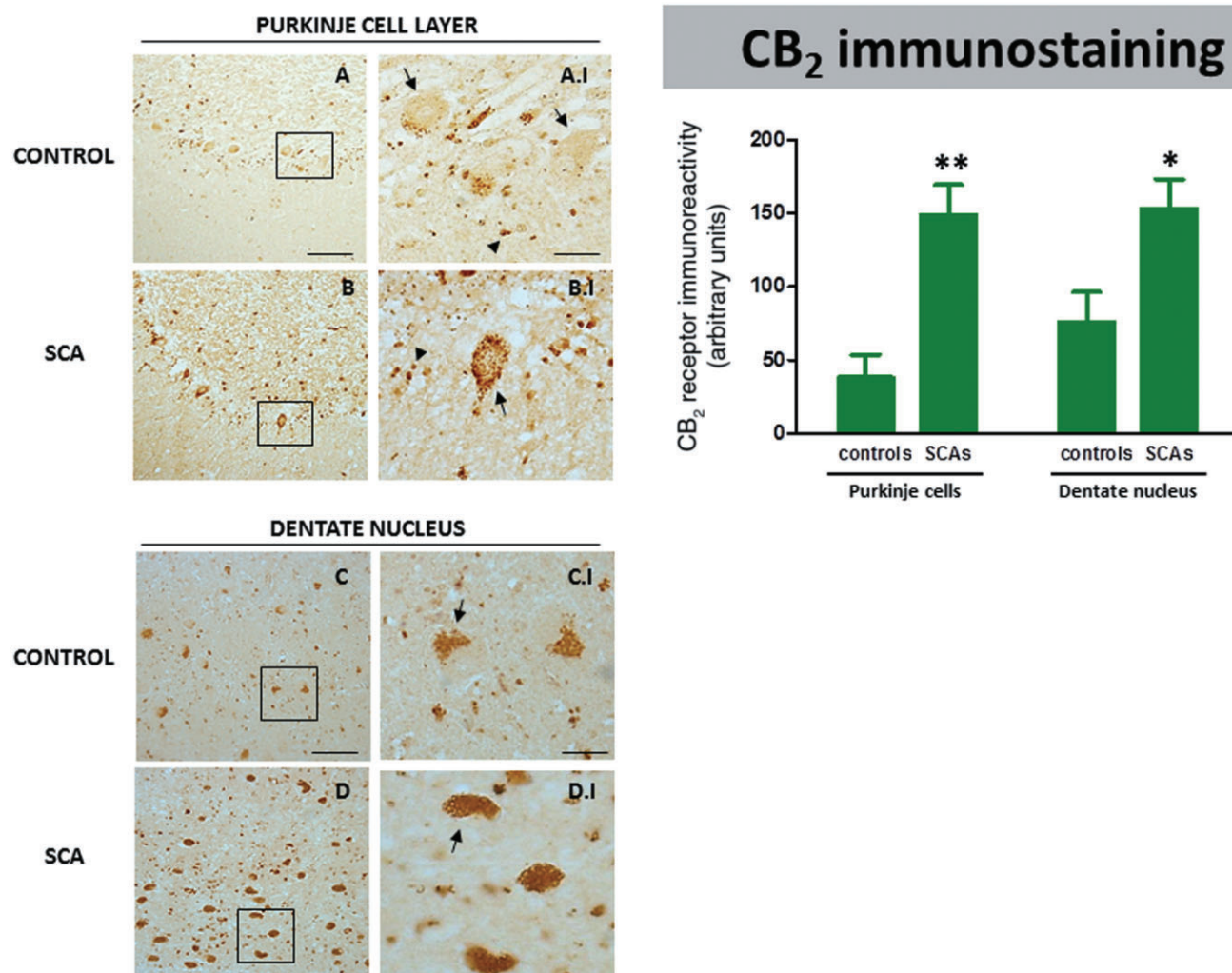


Figure 10

DAB immunostaining for the CB₂ receptor in the Purkinje layer and dentate nucleus of the cerebellum of SCA patients (B, B.I, D and D.I) and control subjects (A, A.I, C and C.I). The arrows indicate the Purkinje cells that are CB₂ receptor-positive, whereas the arrowheads indicate other CB₂ receptor-positive cells that presumably are Bergmann glial cells (scale bars: A–D = 100 µm; A.I–D.I = 25 µm). The microphotographs of SCA samples correspond to subject #9 (SCA7) for the Purkinje layer and to subject #10 (SCA6) for the dentate nucleus. The bar panel corresponds to the quantification of CB₂ receptor immunostaining in the two structures of SCA patients and control subjects. Values correspond to the mean density of labelling in individual cells and are expressed as means ± SEM of five subjects per group. Data were assessed by Student's *t*-test (**P* < 0.05; ***P* < 0.01).

included in this study (Matilla-Dueñas *et al.*, 2012), and the neuropathological information provided by the biobank and our preliminary examination of all the post-mortem samples received allowed us to confirm these neuronal losses. In addition, despite the specific characteristics of each type of SCA, we found the same responses for CB₁ and CB₂ receptors and, in general, to a similar extent in all SCA types included in our study, which support the validity of our experimental approach. Of course, it would have been better to concentrate on only one SCA type but, at present, it is difficult to collect a sufficient number of subjects of the same SCA type that are appropriately matched for gender, age and post-mortem delay, or that do not have to be excluded as a result of their medication and intoxication histories. We were extremely careful in the selection of SCA cases and control subjects and,

in fact, only 50% of the samples available were used (see Methods). In this regard, we excluded all cases of cannabis, alcohol or tobacco addiction and confirmed the SCA group contained samples from patients that had received standard medications for this condition. Within the cases selected, we found two potential problems, a patient that had been treated with nabilone, and the use of morphine in all SCA patients days or hours before death. However, we concluded that these two factors had no significant effect on our results; the nabilone treatment occurred 6 years before death in this patient and palliative treatment with morphine (or fentanyl) was also administered to patients from the control group that had died from colon carcinoma, and no changes in the cerebellar levels of CB₁ and CB₂ receptors were observed in samples from the control group.

CB₂/calbindin double immunofluorescence

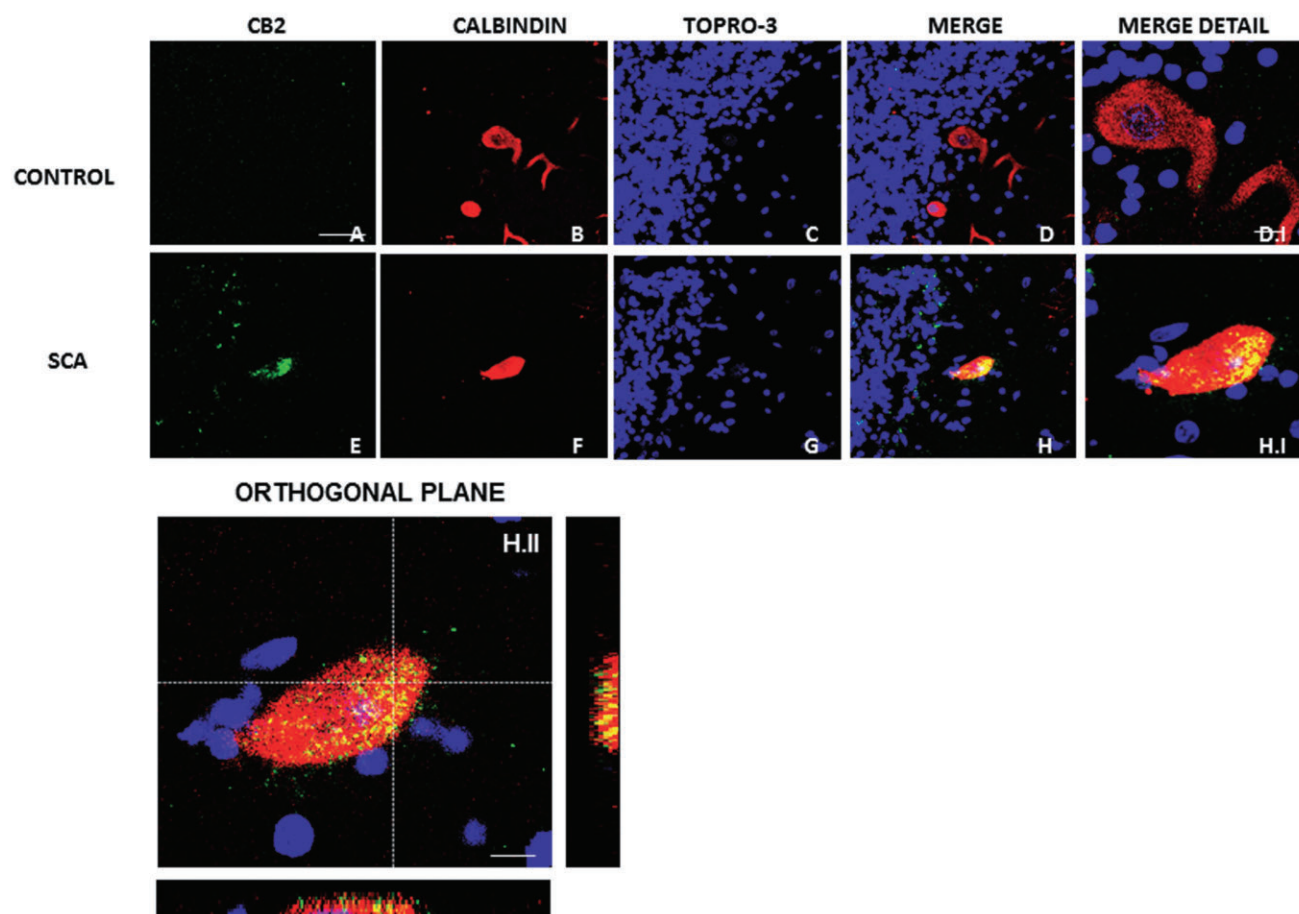


Figure 11

Double-labelling immunofluorescence using antibodies for the CB₂ receptor and calbindin, and TOPRO-3 staining, in the Purkinje layer of the cerebellum of SCA patients (E–H and H.I) and control subjects (A–D, D.I), showing co-localization of CB₂ receptors and calbindin in SCA patients but not in control subjects (scale bars: A–H = 50 µm; D.I and H.I = 10 µm). The H.II panel corresponds to the orthogonal reconstruction from confocal z-series in x-z (below) and y-z (right) planes. This reconstruction confirms the co-localization of CB₂ receptor and calbindin in SCA patients (scale bars: H.II = 10 µm). The microphotographs of SCA samples correspond to subject #9 (SCA7).

Our immunohistochemical analyses revealed that the level of CB₁ receptors was increased in the SCA group in all the cerebellar structures investigated, that is the Purkinje and granular layers, dentate nucleus and white matter areas. However, the physiopathological significance of this up-regulatory response deserves some comments. For example, the increase in CB₁ receptor immunolabelling observed in neurons, that is Purkinje cells and neurons of the dentate nucleus, of SCA patients was seen in those cells that survived the degeneration, but, as the number of neurons in these structures was significantly lower than the controls, the labelling in the whole structures was rather reduced compared to control subjects. As this up-regulation of CB₁ receptors was seen in those neurons that had survived the degeneration, it is possible that this overexpression of CB₁ receptors may serve as a marker of those cells that are going to degenerate or even that this response may contribute to the cerebellar

dysfunction/degeneration during SCA pathogenesis. Indeed, activation of CB₁ receptors has been associated with ataxia in some animal species (DeSanty and Dar, 2001; Patel and Hillard, 2001), so it is possible that the increase in CB₁ receptors observed in the cerebellum of SCA patients, in particular in Purkinje cells, may be related to the occurrence of ataxia in these subjects. This would be similar to the scenario in Parkinsonian patients where an increase in CB₁ receptor binding and signalling occurs in parallel with onset of rigidity and bradykinesia in these patients (Fernández-Ruiz, 2009). However, in Parkinson's disease the up-regulation of neuronal CB₁ receptors occurs in neurons that do not degenerate and as a consequence of an adaptive response (Fernández-Ruiz, 2009). In contrast, in SCAs the overexpression of neuronal CB₁ receptors occurs in those subpopulations that degenerate, thus making it unlikely that these CB₁ receptor changes are the cause of the ataxic gait.

Alternatively, as it has been well-demonstrated that cannabinoid receptors can have a neuroprotective function (Fernández-Ruiz *et al.*, 2010), therefore, it is possible that the overexpression of CB₁ receptors in degenerating cerebellar neurons may represent a protective response elicited to attenuate the cerebellar dysfunction. This may also apply to the response of CB₁ receptors in the other cerebellar areas studied. The increase in CB₁ receptors observed in the granular layer and white matter areas in SCA patients was not influenced by losses in cells containing this receptor, as, in these regions the CB₁ receptor is located in glial elements and its increase is probably related to the reactive states exhibited by these cells during the pathology of SCA. If the overexpression of CB₁ receptors is associated with a protective response in SCA, then, this could be pharmacologically exploited to develop possible novel disease-modifying therapies based on the pharmacological activation of this receptor type (see below).

We also studied the response of the other major receptor type for the endocannabinoid system, the CB₂ receptor, in the cerebellum of SCA patients. Similar to the CB₁ receptors, an overexpression of CB₂ receptors was apparent in all the cerebellar structures investigated, that is the Purkinje and granular layers, dentate nucleus and white matter areas. An overexpression of this receptor typically occurs in glial elements, such as astrocytes and, in particular, reactive microglia, in most neurodegenerative disorders (see the Introduction) and we obtained similar results in the granular layer and the white matter regions of our SCA patients. As with the other pathologies (reviewed in Fernández-Ruiz *et al.*, 2007; 2010; Benito *et al.*, 2008), these findings suggest that the CB₂ receptor may act as a controller of the glial influences on neurons, in particular, by enhancing the trophic support by astrocytes and by reducing the cytotoxic effects exerted by reactive microglial cells. However, we also observed an overexpression of CB₂ receptors in Purkinje neurons, as well as in neurons of the dentate nucleus, in all SCA patients similar to the results found for the CB₁ receptor. In fact, this overexpression also occurred in neurons that had survived the degenerative process, so as with the CB₁ receptor, the levels of this receptor in the whole structures were reduced in the SCA patients compared to controls due to the loss of these neurons during the pathogenesis. Therefore, the same issues that have been discussed with regard to the physiopathological meaning of the overexpression of neuronal CB₁ receptors (i.e. is it a marker of those neurons that are going to degenerate, does it contribute to the cerebellar dysfunction/degeneration, or is it a part of a protective response?) may be also applied to the overexpression of the neuronal CB₂ receptor in those cells that degenerate in SCAs. The therapeutic implications of these responses are particularly challenging, not only for SCAs but also for other neurodegenerative disorders, such as Parkinson's disease where CB₂ receptors have also been identified in degenerating neurons (unpublished data). If these observations are confirmed, the pharmacological scenario may be relatively similar in both of these disorders, and this should be further investigated by targeting the two key cannabinoid receptors located in the various types of neural cells.

In conclusion, our study demonstrates that the endocannabinoid system is significantly altered in the cerebellum of

SCA patients. These changes included an increase in CB₁ and CB₂ receptors in glial elements in the granular layer and the white matter areas, which may be part of an endogenous protective response and could be used to develop novel pharmacological therapies based on targeting these receptors to control glial influences on cerebellar neurons. However, we also demonstrated that CB₁ and CB₂ receptors are overexpressed in those neurons that degenerate in this disease, in particular in Purkinje neurons, a fact that, in addition to its therapeutic implications, may be related to the pathogenesis of SCA. We are presently engaged in additional experiments aimed at confirming that the changes observed in the cerebellum of SCA patients can be reproduced in experimental models of SCAs (i.e. Tg-SCA3 mice) and to determine the timing/sequence of these alterations. We also plan to develop additional pharmacological experiments aimed at identifying the pharmacological profile of those cannabinoid compounds able to arrest/delay disease progression in these transgenic mouse models.

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Conflicts of interest

Authors declare that they have no conflict of interest.

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