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Heteromers Formed by GPR55 and Either Cannabinoid CB₁ or CB₂ Receptors Are Upregulated in the Prefrontal Cortex of Multiple Sclerosis Patients

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Abstract: Multiple sclerosis (MS) is an autoimmune, inflammatory, and neurodegenerative disease of the central nervous system for which there is no cure, making it necessary to search for new treatments. The endocannabinoid system (ECS) plays a very important neuromodulatory role in the CNS. In recent years, the formation of heteromers containing cannabinoid receptors and their up/downregulation in some neurodegenerative diseases have been demonstrated. Despite the beneficial effects shown by some phytocannabinoids in MS, the role of the ECS in its pathophysiology is unknown. The main objective of this work was to identify heteromers of cell surface proteins receptive to cannabinoids, namely GPR55, CB₁ and CB₂ receptors, in brain samples from control subjects and MS patients, as well as determining their cellular localization, using In Situ Proximity Ligation Assays and immunohistochemical techniques. For the first time, CB₁R-GPR55 and CB₂R-GPR55 heteromers are identified in the prefrontal cortex of the human brain, more in the grey than in the white matter. Remarkably, the number of CB₁R-GPR55 and CB₂R-GPR55 complexes was found to be increased in MS patient samples. The results obtained open a promising avenue of research on the use of these receptor complexes as potential therapeutic targets for the disease.



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ultimately result in the typical symptomatology of the disease [3–6]. Symptoms include, but are not limited to, fatigue, tremors, spasticity, pain, bladder dysfunction, visual impairment, and cognitive deficits [7]. The heterogeneous clinical course delineates up to four MS types, i.e., clinically isolated syndrome (CIS), relapsing-remitting MS (RRMS), primary progressive MS (PPMS), and secondary progressive MS (SPMS). An early diagnosis of the initial episode is instrumental in prescribing individualized treatments and preventing multiple relapses [8]. To date, all attempts to identify a panel of biomarkers applicable to the disease have failed [3,9,10]. The exact cause of MS is unknown, but a variety of genetic and environmental factors, such as lifestyle and viral infections, may contribute to the onset of the disease [3,11]. While there is no cure for MS, translational research has provided effective treatments focused on reducing symptoms (e.g., glucocorticoids) or modifying the natural course of the disease (e.g., monoclonal antibodies and immunomodulatory agents) [12–14]. Despite symptomatic relief and, in some cases, the slowing of disease progression, all of these therapies fail in the long term and seriously compromise patients' quality of life, both physically and cognitively. Cumulative clinical evidence has demonstrated that certain natural cannabinoids such as Δ^9 -tetrahydrocannabinol (THC) and cannabidiol (CBD) in a 1:1 mixture available and approved in the form of an oromucosal spray, Sativex® (nabiximols), reduce MS-related spasticity [15]. Therefore, cannabinoids have aroused great interest since they may offer potential new treatment options for patients suffering from MS [16–18].

Endogenous cannabinoids acting through cannabinoid receptors, CB₁ and/or CB₂, are relevant neuromodulators. They participate in several events occurring in the CNS, from neural development to neurotransmission and synaptic plasticity [19–21]. The CB₁ receptor (CB₁R) is the most abundant G-protein-coupled receptor (GPCR) superfamily member in mammalian CNS, it is particularly abundant in the olfactory bulb, hippocampus, basal ganglia, and cerebellum [22–24]. Moderate/low levels of this receptor have been found in the cerebral cortex, amygdala, hypothalamus, some areas of the brainstem, and the spinal cord [22,25]. Regarding CB₂ receptor (CB₂R) expression in the CNS, it is primarily identified in microglia but also in some neurons in diverse brain areas including the cerebral cortex, hippocampus, and globus pallidus [23,26–31]. At the neuronal level, these two receptors are preferentially located in the plasma membrane of pre- and post-synaptic terminals, but they can appear in the soma and dendrites of glutamatergic and serotonergic neurons, as well as in GABAergic interneurons [25,29,32,33]. Cannabinoids, endogenous, natural, or synthetic, may limit excitotoxic damage and enhance synaptic plasticity via CB₁R or may exert anti-inflammatory and immunomodulatory actions via CB₂R [17,34]. Despite responding to cannabinoids, GPR55 (G protein-coupled receptor 55) is still considered an orphan receptor. Although early studies proved the binding of CP55940, a synthetic cannabinoid, to GPR55 and activation of this receptor by anandamide [35], it is now assumed that the binding of cannabinoids to the receptor occurs at an allosteric site [36–38]. GPR55 is abundant in various regions within the CNS, both in neurons and glial cells; it has been described in the hippocampus, thalamic nuclei, and basal ganglia of rodent, primate, and human brains [25,30,39,40]. While CB₁R and CB₂R couple primarily to the adenylyl cyclase-inhibiting heterotrimeric G_{i/o} protein [41–43], GPR55 likely couples to G_{q/G11} and

This study aimed to explore the possibility that CB₁R-GPR55 and CB₂R-GPR55 heteromers are expressed in the human prefrontal cortex. Taking into account that cannabinoids can ameliorate clinical signs of MS by exerting their effects through cannabinoid receptors [52,53], this study also determined whether the expression of these heteromers was altered in prefrontal cortex samples from patients affected by MS.

2. Results

2.1. Expression of CB₁R-GPR55 Heteromers in Controls and MS Patients

The In Situ Proximity Ligation Assay (PLA) is the most appropriate technique to detect the presence of complexes formed by two different receptors in a native system. Using specific antibodies against the CB₁R and the GPR55 coupled to PLA oligonucleotide probes, a punctate fluorescent red signal was observed surrounding DAPI-counterstained nuclei, reflecting the formation of CB₁R-GPR55 heteromers in the plasma membrane of cells in all prefrontal cortex samples analyzed (see Video S1), both in GM and WM (Figure 1A). Data analysis demonstrated that the number of red dots per cell, which reflects the number of CB₁R-GPR55 heteromers, was significantly higher in the GM compared to the WM in the prefrontal cortex samples from the controls and MS patients ($F_{1,596} = 8.23$; $p < 0.05$) (Figure 1B).

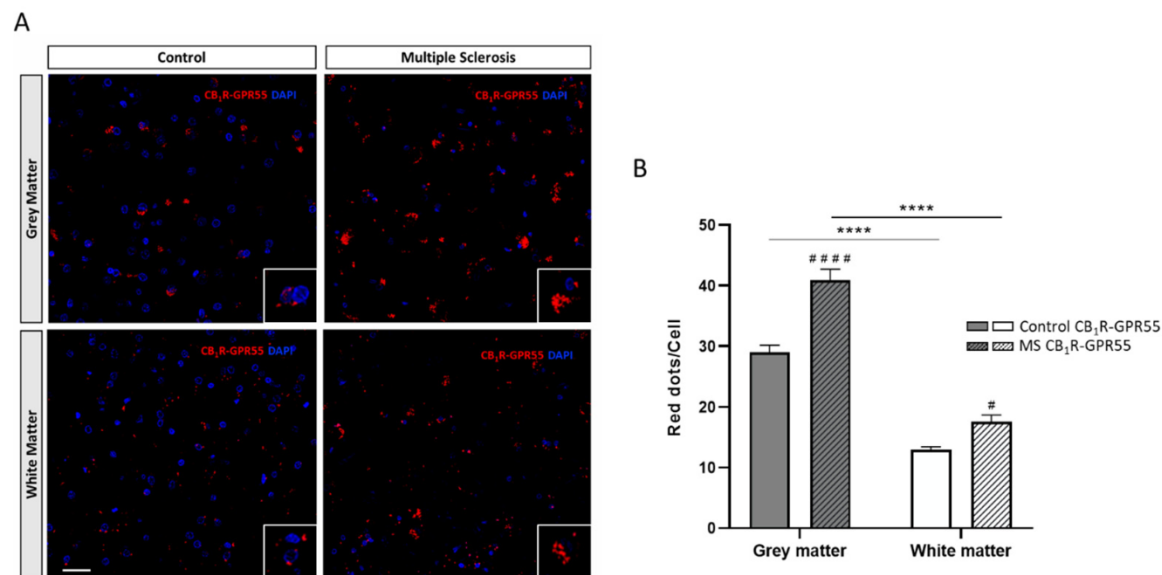


Figure 1. CB₁R-GPR55 heteromers detected using the In Situ Proximity Ligation Assay (PLA) in the grey (GM) and white matter (WM) of the prefrontal cortex of samples from the controls and multiple sclerosis (MS) patients. Representative confocal images (40×) showing PLA label for CB₁R-GPR55 heteromers as red dots in cells with DAPI-stained nuclei. Scale bar 50 μm (A). Quantification of CB₁R-GPR55 heteromers as the number of red dots per cell in the GM and WM in the prefrontal

2.2. Expression of CB₂R-GPR55 Heteromers in Controls and MS Patients

Similar findings were found when analogous assays addressing CB₂R-GPR55 heteromer formation were performed using the same brain samples. Representative images and quantitative data from PLA assays (Figure 2A) revealed that CB₂R and GPR55 formed heteromeric complexes in the plasma membrane of cells (see Video S2) in all brain areas and samples analyzed. When considering the total number of CB₂R-GPR55 heteromers by assessing the number of red dots per cell, data analysis demonstrated higher expression in the GM of the prefrontal cortex compared to the WM, both in the control and MS patient samples ($F_{1,572} = 4.63$; $p < 0.05$) (Figure 2B).

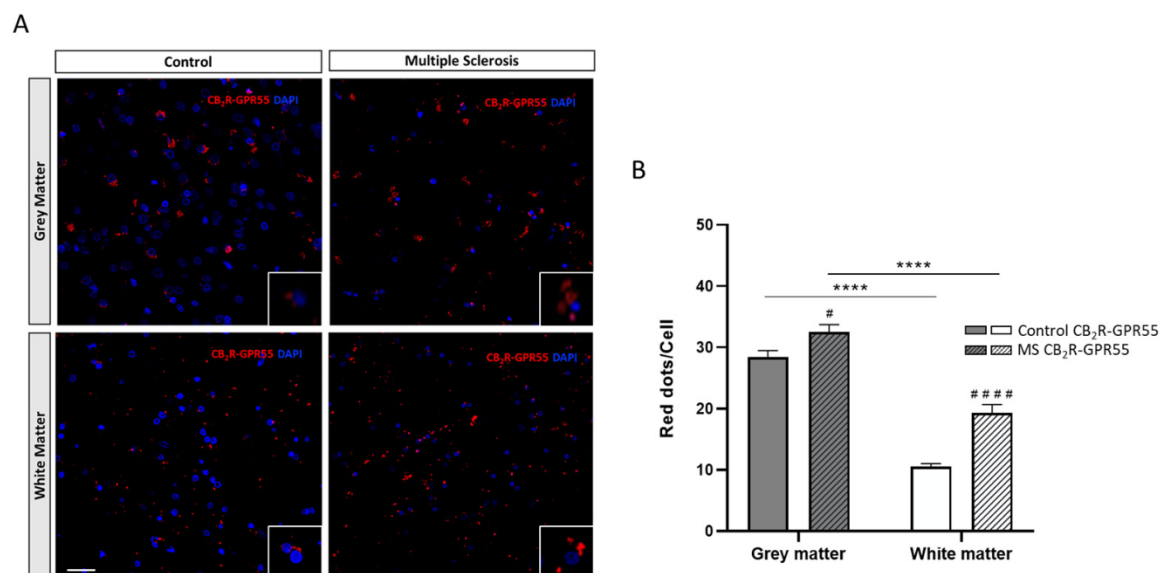


Figure 2. CB₂R-GPR55 heteromers detected using the In Situ Proximity Ligation Assay (PLA) in the grey (GM) and white matter (WM) of the prefrontal cortex of samples from the controls and multiple sclerosis (MS) patients. Representative confocal images (40×) showing the PLA label for CB₂R-GPR55 heteromers as red dots in cells with DAPI-stained nuclei. Scale bar 50 μm (A). Quantification of CB₂R-GPR55 heteromers as the number of red dots per cell in the GM and WM in the prefrontal cortex samples of the controls and MS patients. Data are the mean ± SEM (40 fields per section) (B). Significant differences were analyzed via two-way ANOVA followed by post hoc Bonferroni's test. **** $p < 0.0001$ compared with GM; # $p < 0.05$, #### $p < 0.0001$ compared with the control.

Once again, significant differences were found when comparing the red fluorescent dots in the controls and individuals diagnosed with MS; the prefrontal cortex of patients showed a greater amount of CB₂R-GPR55 heteromers compared to that observed in the controls (Figure 2A,B).

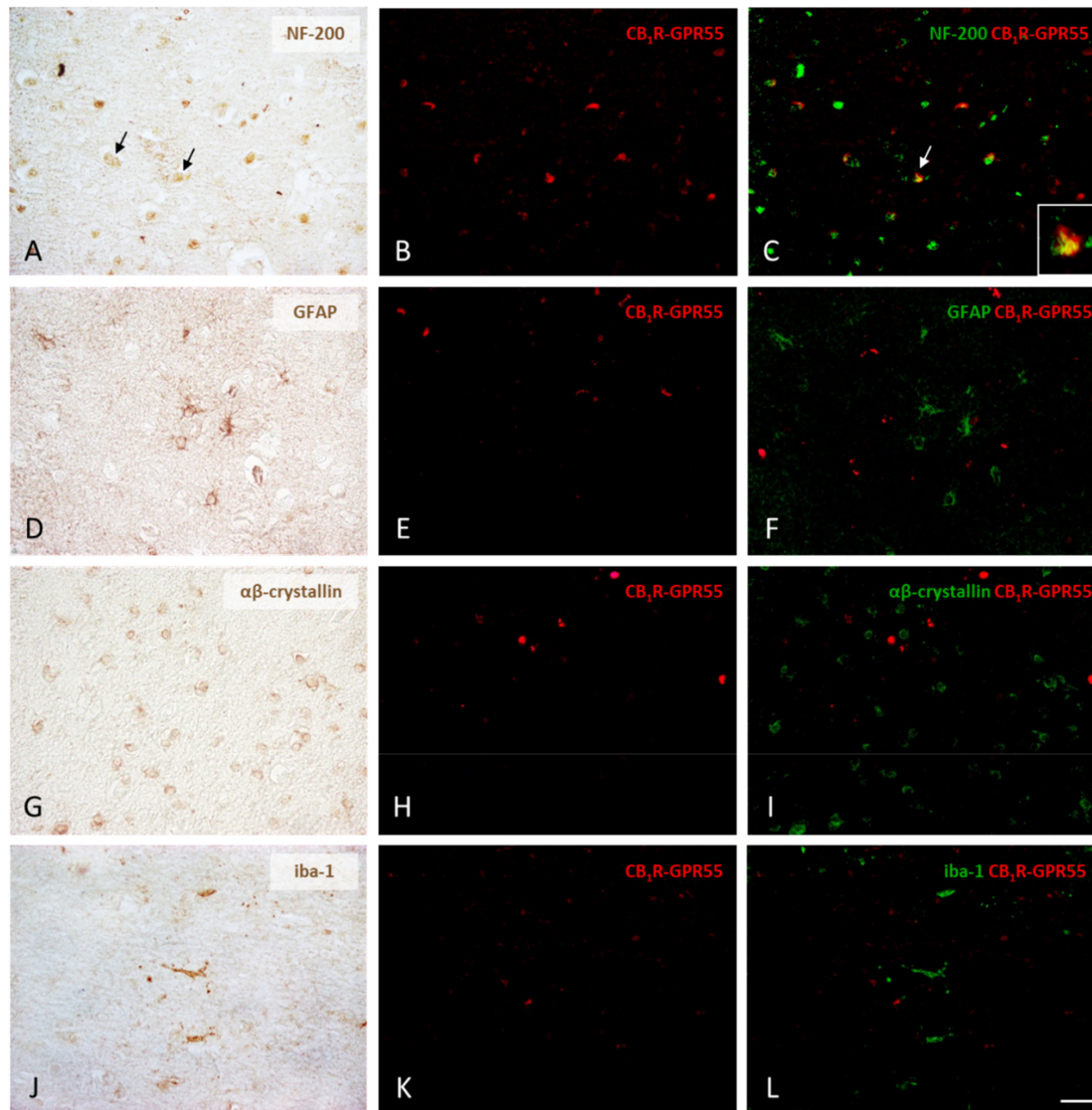


Figure 3. Expression of CB₁R-GPR55 heteromers in neurons, astrocytes, oligodendrocytes, and microglia in the prefrontal cortex of the control subjects. Following the chromogenic immunohistochemical detection of the four different types of cells, those positive for NF-200, GFAP, αβ-crystallin, and iba-1 (A,D,G,J), CB₁R-GPR55 heteromers were identified as red dots in cells with DAPI-stained

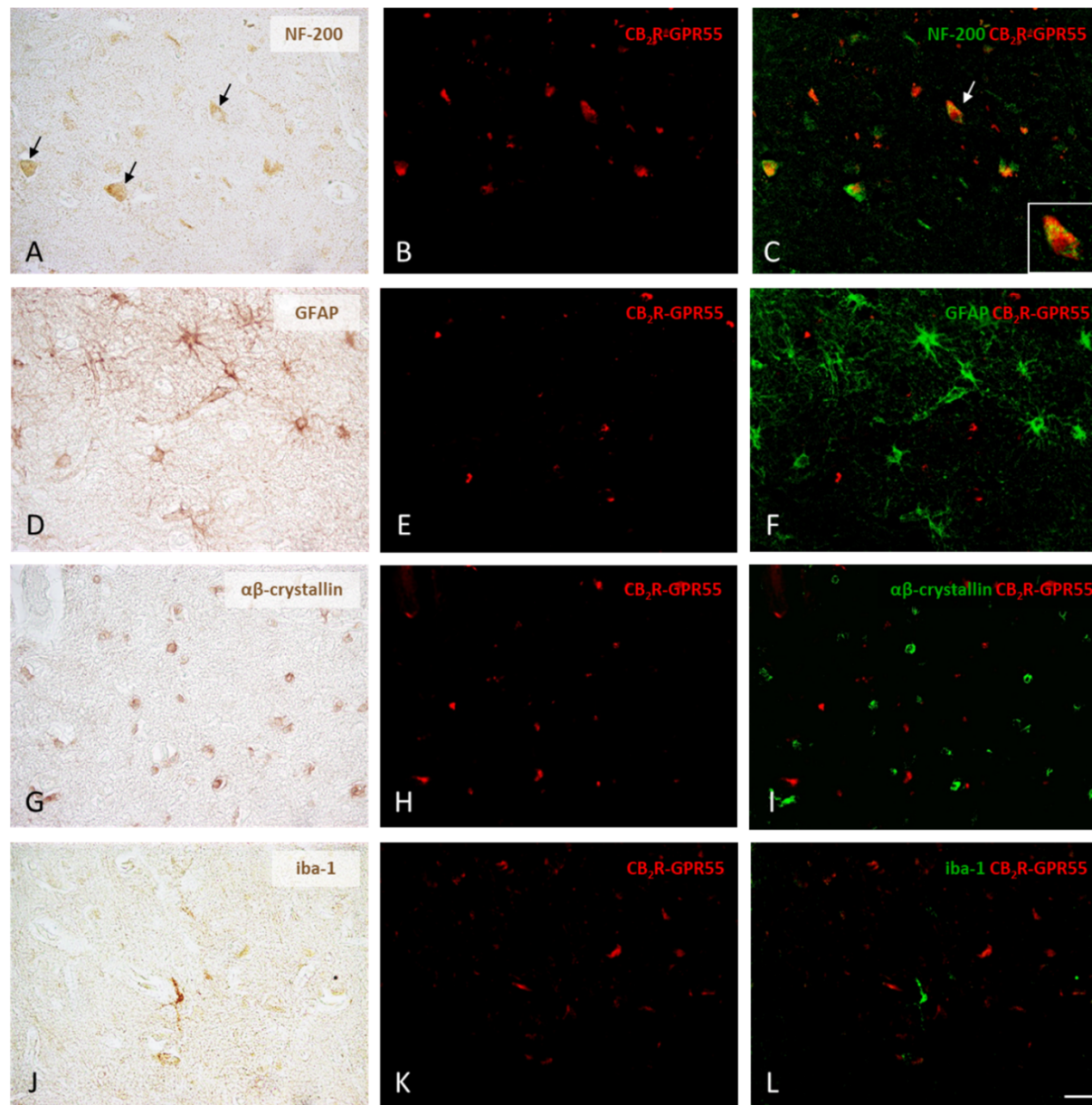


Figure 4. Expression of CB₂R-GPR55 heteromers in neurons, oligodendrocytes, astrocytes, and microglia in the prefrontal cortex of the control subjects. Following the chromogenic immunohistochemical detection of the four different types of cells, those positive for NF-200, GFAP, αβ-crystallin, and Iba-1 (A,D,G,J), CB₂R-GPR55 heteromers were identified as red dots in cells with DAPI-stained

through the activation of cannabinoid receptors [54]. Preclinical studies in the experimental autoimmune encephalomyelitis (EAE) mouse model demonstrated that treatment with CBD and THC decreases axonal damage, inflammation, microglial activation, and T-cell recruitment, leading to a symptomatic improvement that seems to be related to direct action on the CB₁R [55,56]. Spasticity increases rapidly after the administration of a CB₁R antagonist, rimonabant, suggesting that alleviation of hind limb alteration is CB₁R dependent [57]. A more recent study reported in a cuprizone-induced mouse model of MS that reducing the global amount of CB₁R limits myelin repair potential [52]. Regarding CB₂R, some studies have shown that JWH-133, a selective receptor agonist, ameliorates tremor and spasticity in EAE mice by promoting autophagy and inhibiting NLRP3 inflammasome activation [53]. Similarly, treatment with another CB₂R agonist, HU-308, attenuated the development of the pathological condition. Consistent with these observations, CB₂R^{-/-} mice displayed greater vulnerability to neurofilament degeneration, inflammation, apoptosis, and axonal damage, common pathological features of the EAE [54]. Moreover, it is increasingly recognized that CBD and THC, administered together in controlled doses (Sativex®), reduce muscle spasms and spasticity in MS patients and even induce analgesia [15]. However, the pharmacology behind the receptor-mediated neuroprotective effects exerted by cannabinoids is not straightforward; it involves multiple targets and mechanisms that, collectively, unfold a particular pattern of cellular events. In this sense, dimer/oligomerization is now considered a relevant mechanism to induce diverse functional selectivity in signaling mediated by GPCRs [47]. Considering the ability of cannabinoid receptors to form heteromers that may constitute therapeutic targets, as already postulated for neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [30,58], this work aimed to adequately characterize the formation of complexes between CB₁ or CB₂ and GPR55 receptors in the CNS and to evaluate whether the expression of these receptor complexes is affected in MS.

The results presented herein constitute the first description of CB₁R-GPR55 and CB₂R-GPR55 heteromers in the human prefrontal cortex of control individuals and patients with MS. By taking advantage of the PLA technique and immunohistochemistry using antibodies against neuronal and glial markers, the expression of these receptor complexes was confirmed in neurons of the cerebral cortex, both in GM and WM, but not in glial cells labeled with antibodies against αβ-crystallin, GFAP or Iba-1. The possibility of direct interaction between CB₁ or CB₂ and GPR55 receptors has previously been demonstrated in cell cultures using energy transfer techniques [49,50] and cell biology techniques using brain samples of rats and non-human primates [25,30,51]. Indeed, CB₁R-GPR55 heteromers were identified on the cell surface and in intracellular locations of striatal neuronal subtypes [25]. The expression of CB₂R in the neurons of the CNS has been less well characterized, and the described changes in CB₂R-GPR55 heteromer levels in the striatum of the *Macaca fascicularis* model of Parkinson's disease were attributed to the upregulation of heteromers in activated microglia [30]. Interestingly, our data show that CB₁R-GPR55 and CB₂R-GPR55 heteromers are expressed almost exclusively at the level of the neuronal plasma membrane in the prefrontal cortex. Of note, the presence of these complexes is scarce in neuronal extensions

disease; it has been suggested that microglia in these animals display a neuroprotective phenotype that could explain why cognitive deficits do not appear until late in the life of the transgenic Alzheimer's disease model [58]. The relevance of increased heteromer appearance to the pathophysiology of MS is unclear but could be part of a compensatory mechanism to restore homeostasis and brain integrity in response to neuronal damage. Cannabinoids are important players in neuroinflammation by regulating the release of neuropeptides and the activation of microglia [59,60]. In addition, these compounds may affect cellular energy production via GPR55-containing receptor complexes; mitochondrial dysfunctions observed in the disease could be caused by functional changes derived from differential heteromer expression [50,61]. All of this evidence supports the neuroprotective effect attributed to cannabinoids acting on cannabinoid and GPR55 receptors [50]. In this context, endocannabinoids and natural/synthetic cannabinoids capable of activating CB₁R, CB₂R, and GPR55 appear to offer protection against excitotoxic damage [17,38]. Furthermore, some studies in murine models have described a significant increase in endocannabinoid levels i.e., anandamide, palmitoylethanolamide, and 2-arachidonoylglycerol, as a part of an anti-inflammatory response resulting from axonal damage [19]. The presence of heteromers at significant levels in MS also opens up new possibilities in drug discovery, that is, targeting them for therapeutic benefit. The study has a limitation which is the small sample size derived from the difficulty in obtaining samples from patients. Although it may take time, validating MS-related changes in heteromer expression requires research with larger human cohort samples.

The potential of cannabinoids as drugs to combat or delay the progression of a neurological disease such as MS has gained interest in recent years. In the field of Parkinson's disease research, it is increasingly recognized that targeting neuronal CB₁R-GPR55 and CB₂R-GPR55 heteromers with cannabinoids can be a successful therapeutic approach to both manage symptoms and delay disease progression [62,63]. The beneficial effect of some phytocannabinoids on MS symptoms [15] may be due to multiple molecular mechanisms. Cannabinoids can not only drive individualized responses through CB₁, CB₂, or GPR55 receptors but also, as this work suggests for the first time, act on functional units consisting of receptor heteromers. What is crucial to designing effective approaches is to consider the particular properties of the heteromers in terms of signaling. GPR55-mediated signaling is complex and this issue is delaying the discovery of selective compounds and the development of drugs targeting it. In contrast, the sustained interest in CB₂R as a therapeutic target for neuroprotection has gained momentum over the last decade; agonists, antagonists, and allosteric modulators have been designed that, unlike CB₁R targeting, lack unwanted psychotropic side effects [17,64]. Also interesting for the design of therapies to combat MS is the finding that CB₁R-GPR55 and CB₂R-GPR55 heteromers are expressed in neurons. This piece of information related to the CB₂R-GPR55 heteromer is both intriguing and relevant since it is considered that the CB₂R in the CNS is expressed more in the glia than in neurons. Finally, it should be noted that the existence of differences in the number of heteromers when comparing samples from control individuals and patients with MS offers a way forward for future research. In this sense, correlating changes in

demyelination consistent with MS, properly confirmed by a neuropathologist. Detailed information on the subjects and samples is given in Table 1.

Table 1. Demographic data of cases.

Case	Sex	Age	Type of MS	Brain Area	Post-Mortem Time	Brain Bank
1	Male	58	MS	Prefrontal cortex	6–12 h	Central Nervous Tissue Bank Madrid (CIEN Foundation)
2	Male	38	SPMS	Prefrontal cortex	6–12 h	University Central Hospital of Asturias (HUCA)
3	Female	47	PPMS	Prefrontal cortex	6–12 h	Center for Biomedical Research of Navarra (NAVARRABIOMED)
4	Female	48	MS	Prefrontal cortex	6–12 h	Southern Galicia Health Research Institute (IISGS)
5	Male	38	Control	Prefrontal cortex	6–12 h	University Central Hospital of Asturias (HUCA)
6	Male	66	Control	Prefrontal cortex	6–12 h	Center for Biomedical Research of Navarra (NAVARRABIOMED)
7	Male	62	Control	Prefrontal cortex	6–12 h	Center for Biomedical Research of Navarra (NAVARRABIOMED)
8	Female	52	Control	Prefrontal cortex	6–12 h	University Central Hospital of Asturias (HUCA)

MS, multiple sclerosis; PPMS, primary progressive MS; SPMS, secondary progressive MS.

Following retrieval, the brain specimens were fixed by immersion in 10% buffered formalin, dehydrated, cleared in butyl acetate, and embedded in paraffin. Tissue blocks containing the prefrontal cortex were sectioned at 7 μ m, mounted on SuperFrost Plus (Mentzel-Glasse) slides, dried at 36 °C, and stored at room temperature until processed.

The ethics committees of each participating biobank reviewed and approved the study protocol. Moreover, all research procedures involving the manipulation of human samples were approved by the *Comité de Ética de la Investigación del Principado de Asturias (CEImPA 23-174)* and are in accordance with the ethical principles and guidelines established in the Declaration of Helsinki and in the Spanish laws: *Ley de Investigaciones Biomédicas (ley 14/2007)* and *Ley de Protección de Datos Personales y Garantías de los Derechos Digitales (Ley Orgánica 3/2018)*.

4.2. In Situ Proximity Ligation Assay (PLA)

In Situ PLA, a technique instrumental for detecting receptor–receptor interactions and their precise anatomical localization, was used to test for the presence of CB₁R-GPR55 and CB₂R-GPR55 heteromers in the prefrontal cortex of brain samples from control individuals and patients diagnosed with MS. For this purpose, the tissue sections were incubated for 1 h at 37 °C with the blocking solution, followed by overnight incubation at 4 °C with the PLA probe-linked antibodies (at a final concentration of 75 μ g/mL). Proximity probes consist of affinity-purified antibodies modified by covalent attachment of the 5' end of various nucleotides to each primary antibody. In this case, PLA probes were prepared by conjugating a rabbit anti-CB₁ antibody (PA1-743, Invitrogen, Paisley, UK) and a rabbit anti-CB₂ antibody (101550, Cayman Chemical, Ann Arbor, MI, USA) with a PLUS oligonucleotide

negative control assays were carried out to ensure that there was a lack of non-specific labeling and amplification.

The quantification of PLA signals and cell nuclei was performed in images generated from a Leica sTCS-SP8X Spectral Confocal Laser Microscope (Leica Microsystems, Mannheim, Germany). Regarding selected regions of interest (ROIs), and for each field of view, a stack of two channels (one per staining) and 9–15 Z stacks with a step size of 1 μm were acquired with the 63 \times oil-immersion lens. Statistical analysis on the receptor heteromer densities was conducted according to a modification of the method of Tolivia et al. (see [65]). A quantification of cells containing one or more red spots versus total cells (blue nucleus) and the ratio r (number of red spots/cell) in cells containing spots were determined considering a total of 300–400 cells from ten different fields in both WM and GM for each prefrontal cortex section. The experiments were performed on a blind basis; the experimenter was not aware of the label and the conditions (control or MS) when images were taken. Moreover, the experimenter who made the analysis did not know the exact nature of the analyzed samples.

4.3. Co-Staining Combining PLA and Immunohistochemistry

Identification of the specific cell types (neurons, oligodendrocytes, astrocytes, and microglia) expressing CB₁R-GPR55 and CB₂R-GPR55 heteromers was carried out using immunohistochemistry followed by PLA. First, chromogenic immunodetection was performed using specific neuronal and glial markers. The immunohistochemistry process was conducted as follows. Prefrontal cortex sections of the control subjects were sequentially treated with Triton X-100 (0.01%, 5 min), washed with distilled water, treated with H₂O₂ (3%, 5 min), washed with distilled water again, and treated with PBS (2 min). Non-specific binding was blocked via incubation with 1% BSA (30 min). Then, incubation with a specific monoclonal antibody against NF-200, a neuronal marker (1:100; N-0142, Sigma-Aldrich, St. Louis, MO, USA), rabbit antibody against the glial fibrillary acidic protein (GFAP), an astrocytic marker (1:500; z0334, DAKO Agilent, Santa Clara, CA, USA), goat antibody against Iba-1, a microglia marker (1:1000; ab107159, Abcam, Cambridge, UK), and rabbit antibody against $\alpha\beta$ -crystallin, a mature OLG marker (1:200; NCL-ABCrys-512, Novocastra, St. Gallen, Switzerland), was carried out overnight at 4 °C. After several washes in PBS, the sections were incubated at room temperature using a biotinylated horse universal antibody (1:40; PK-8800, Vector Laboratories Inc., Newark, NJ, USA) for 30 min. Afterward, the sections were treated with Extravidin labeled with horseradish peroxidase (HRP) (E2886, Sigma-Aldrich Extra-3, Sigma-Aldrich, St. Louis, MO, USA). Peroxidase activity was visualized with diaminobenzidine (DAB) (D4168, Sigma-Aldrich, St. Louis, MO, USA). After the immunohistochemical protocol, the PLA technique was used, as described in Section 4.2, to detect CB₁R-GPR55 and CB₂R-GPR55 heteromers.

The sections were observed using an Epi-Fl Nikon Eclipse E400 microscope (Nikon, Minato-ku, Tokyo, Japan) equipped with Plan Fluor objectives, and images were recorded using a digital camera (63 \times ; NikonDN-100, Nikon, Minato-ku, Tokyo, Japan). Final images were obtained through the digital superposition of the corresponding DAB (NF-200, GFAP,

were tested for normality of populations and homogeneity of variances. Differences were considered significant when $p < 0.05$.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms25084176/s1>.

Author Contributions: Conceptualization was agreed by E.M.-P. and R.F., who also participated in the design of the project and analyzed the results; C.M.-P. and E.M.-P. performed the majority of the experiments; R.R.-S. participated in a significant number of experiments; A.N., E.d.V. and J.T. participated in the data analysis and preparing the final figures; C.M.-P., E.M.-P. and R.R.-S. performed many of the imaging assays using the confocal microscope, took photographs, and participated in the data analysis; E.M.-P. and R.F. wrote the first draft of the manuscript which was further edited by all co-authors, who agreed with the final submission. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and the Spanish laws: *Ley de Investigaciones Biomédicas* (ley 14/2007) and *Ley de Protección de Datos Personales y Garantías de los Derechos Digitales* (Ley Orgánica 3/2018) and approved by the *Comité de Ética de la Investigación del Principado de Asturias* (CEImPA 23-174; 25 March 2023).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated and/or analyzed during the current study are not publicly available due to privacy/ethical restrictions but are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

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