Cannabinoid CB1 and CB2 Receptors, and Monoacylglycerol Lipase Gene Expression Alterations in the Basal Ganglia of Patients with Parkinson's Disease

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Abstract

Previous studies suggest that the endocannabinoid system plays an important role in the neuropathological basis of Parkinson's disease (PD). This study was designed to detect potential alterations in the cannabinoid receptors CB1 (CB₁r) and CB2 (A isoform, CB₂Ar), and in monoacylglycerol lipase (MAGL) gene expression in the substantia nigra (SN) and putamen (PUT) of patients with PD. Immunohistochemical studies were performed to identify precise CB₂r cellular localization in the SN of control and PD patients. To ensure the validity and reliability of gene expression data, the RNA integrity number (RIN) was calculated. CB₁r, CB₂Ar, and MAGL gene expressions were evaluated by real-time polymerase chain reaction (real-time PCR) using Taqman assays. Immunohistochemical experiments with *in situ* proximity ligation assay (PLA) were used to detect the precise cellular localization of CB₂r in neurons, astrocytes, and/or microglia. All RIN values from control and PD postmortem brain samples were > 6. CB₁r gene expression was unchanged in the SN but significantly higher in the PUT of patients with PD. CB₂Ar gene expression was significantly increased (4-fold) in the SN but decreased in the PUT, whereas MAGL gene expression was decreased in the SN and increased in the PUT. Immunohistochemical analyses revealed that CB₂r co-localize with astrocytes but not with neurons or microglial cells in the SN. The results of the present study suggest that CB₁r, CB₂r, and MAGL are closely related to the neuropathological processes of PD. Therefore, the pharmacological modulation of these targets could represent a new potential therapeutic tool for the management of PD.

Keywords Parkinson's disease · CB1 and CB2 receptor · MAGL · Gene expression · Immunohistochemistry · Basal ganglia

Introduction

The search for new biomarkers to further understand the neurobiological basis of Parkinson's disease (PD) and to achieve an early diagnosis and better treatment are important challenges [1, 2]. In this respect, there is an increasing amount of data relating to the endocannabinoid system with regard to the

pathophysiology of PD [3, 4]. Indeed, changes in cannabinoid CB1 receptor (CB₁r) gene expression were found in PD animal models [5] and human postmortem brain tissue [6]. Furthermore, regional alterations of CB₁r *in vivo* availability have been described in patients with PD [7]. These results strongly support the idea that this receptor may play an important role in the treatment of PD [8–12].

Since the identification of the cannabinoid CB2 receptor (CB₂r) in the brain under non-pathological conditions [13], several studies suggest its participation in the regulation of different neurobiological processes. Interestingly, some authors reported the anti-inflammatory and neuroprotective potential of CB₂r [14, 15], suggesting a role of this receptor in neurodegenerative diseases such as PD. In addition, CB₂r gene expression was decreased in the cerebellum and hippocampus of patients with PD compared with healthy controls [16]. Furthermore, the overexpression of CB₂r in mice markedly

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reduced the dopaminergic lesion induced by the 6-hydroxydopamine (6-OHDA) dopaminergic toxin, decreasing the motor impairment, the dopaminergic neuronal loss, and the recruitment of astrocytes and microglia by the lesioned regions [17]. Moreover, special attention has been paid to the role of the most abundant endocannabinoid ligand, 2-arachidonylglycerol (2-AG), which presents with high affinity to CB₂r activation [18], owing to its modulatory and neuroprotective effect [19, 20]. Indeed, the inhibition of the 2-AG metabolizing enzyme monoacylglycerol lipase (MAGL) with the antagonist URB602 induced significant neuroprotective effects [21]. In addition, a recent study showed that MAGL inhibition produces a neuroprotective effect in an animal model of PD through restorative astroglia and microglia activation and the release of neuroprotective and anti-inflammatory molecules [22].

Several studies have shown that glial cells play a crucial role in the neuropathogenesis of PD. In the first stages of the disease it has been suggested that astrocytes take up altered α -synuclein from axon terminals [23], leading to neurodegenerative processes through different proposed mechanisms such as the production of proinflammatory cytokines and chemokines [24] and microglial activation [25]. Next, activated phagocytic microglia gain an important role in the midbrain dopaminergic neuronal loss during the progression of the disorder [26, 27]. Interestingly, there is evidence for the presence of CB₂r in reactive microglia and activated astrocytes [28], suggesting a relevant modulatory role in the neuroinflammatory and neurodegenerative processes. Indeed, it has been determined that CB₂r activation decreases the *in vitro* production of proinflammatory molecules in different neural cell types, such as rat microglial cells [29, 30], primary mouse astrocytes [31, 32], human microglial cells [33], and human astrocytes [34]. Furthermore, Price et al. [9] showed an up-regulation of CB₂r in microglia recruited to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned brain regions. A recent study also reveals a significant increase of CB₂r gene expression after the intra-striatum injection of 6-OHDA or bacterial lipopolysaccharide (LPS). This effect was also accompanied by an increase of microglial activation, suggesting that targeting CB₂r could be a useful tool with which to modify neuroinflammation in PD [35].

Despite the available literature about the pivotal role of the endocannabinoid system in the neuropathological processes of PD and the potential therapeutic usefulness of its pharmacological modulation, additional studies are needed to clarify precisely how it participates in the nigrostriatal neurodegenerative process. Thus, the aim of this study was to evaluate further the potential involvement of the endocannabinoid system in PD through the identification of CB₁r, CB₂r brain-specific A-isoform (CB_{2A}r) [36], and MAGL gene expression alterations in the substantia nigra (SN) and putamen (PUT) of patients with PD. In addition, immunohistochemical analyses were performed to determine the precise co-localization of

CB₂r with tyrosine hydroxylase (TH)-positive neurons, astrocytes, and/or microglia in the SN of healthy controls and patients with PD.

Materials and Methods

Subjects

Frozen brain samples from patients with PD and their respective controls were obtained from Fundación CIEN Brain Bank (Madrid, Spain), Fundación Alcorcón University Hospital Brain Bank (Madrid, Spain), London Neurodegenerative Diseases Brain Bank (London, UK), and Parkinson's UK Brain Bank (London, UK). Fixed brain samples were taken from the Navarrabiomed Brain Bank (Pamplona, Spain). A declaration of intent regarding the responsible use of supplied postmortem tissue and clinical information from brain donors was approved by all the brain banks. All patients with PD fulfilled the diagnostic criteria for advanced disease (stages 4–5) [37] and were matched with control subjects without neuropathological disease. The neuropathological data provided by the brain banks and the immunohistochemical analysis of brain samples clearly showed a notable neurodegeneration of dopaminergic neurons in the SN of patients with PD. Demographic and postmortem data of all the subjects included in the study are shown in Table 1. SN and PUT were dissected from controls ($n = 16$ and $n = 24$, respectively) and patients with PD ($n = 25$ and $n = 28$, respectively). The cases included in the present study were male and female and were matched as closely as possible for age (controls: 70.8 ± 14.2 years; patients with PD: 75.8 ± 7.8 years) and postmortem interval (controls: 20.2 ± 13.9 h; patients with PD: 19.7 ± 19.9 h). Other factors such as additional diseases, cause of death, or medication were analyzed for the brain tissue selection to avoid interference with the studied parameters. The collection and analysis of postmortem brain tissue was approved by the ethics committees of Miguel Hernandez University and University of Navarra.

RNA Integrity Number Evaluation

Total RNA was isolated from SN and PUT snap-frozen tissue. Integrity of the RNA samples was analyzed by the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) detecting RNA ribosomal bands (18S and 28S). Depending on the ratio 28S/18S, a value (1–10), named the RNA integrity number (RIN), was calculated, suggesting the degree of RNA degradation (1 = completely degraded; 10 = intact sample). All RNA samples (SN and PUT) from controls and patients with PD presented a mean RIN value > 6 [controls: 6.2 ± 0.7 (range 5.0–7.1); patients with PD: 6.4 ± 0.7 (range 5.3–7.7)],

Table 1 Data from patients with Parkinson's disease (PD) and healthy controls (C)

Subject ID	Age	Gender	PMI (hours)	Classification	Biobank
C1	68	M	8	C	CIEN-BB
C2	56	M	8	C	CIEN-BB
C3	68	M	17	C	CIEN-BB
C4	58	F	6	C	CIEN-BB
C5	80	M	8	C	HUFA-BB
C6	80	F	7	C	HUFA-BB
C7	81	M	18	C	MRC-BB
C8	79	M	47	C	MRC-BB
C9	79	M	24	C	MRC-BB
C10	71	M	5	C	MRC-BB
C11	86	M	6	C	MRC-BB
C12	85	M	42	C	MRC-BB
C13	60	M	35	C	MRC-BB
C14	59	M	50	C	MRC-BB
C15	57	M	26	C	MRC-BB
C16	92	M	13	C	PUK-BB
C17	90	F	15	C	PUK-BB
C18	35	M	22	C	PUK-BB
C19	88	M	22	C	PUK-BB
C20	68	M	30	C	PUK-BB
C21	65	M	12	C	PUK-BB
C22	78	F	23	C	PUK-BB
C23	80	F	23	C	PUK-BB
C24	82	M	48	C	PUK-BB
C25	77	M	4	C	NBM-BB
C26	53	M	7	C	NBM-BB
C27	63	M	29	C	NBM-BB
C28	46	M	11	C	NBM-BB
PD1	69	F	13	PD	CIEN-BB
PD2	67	M	5	PD	CIEN-BB
PD3	55	M	6	PD	CIEN-BB
PD4	75	M	8	PD	CIEN-BB
PD5	85	F	3	PD	CIEN-BB
PD6	66	F	6	PD	CIEN-BB
PD7	75	M	40	PD	MRC-BB
PD8	70	M	49	PD	MRC-BB
PD9	84	M	34	PD	MRC-BB
PD10	74	M	10	PD	MRC-BB
PD11	59	M	23	PD	MRC-BB
PD12	79	M	89	PD	MRC-BB
PD13	72	M	29	PD	MRC-BB
PD14	76	M	66	PD	MRC-BB
PD15	73	M	40	PD	MRC-BB
PD16	78	M	22	PD	PUK-BB
PD17	79	M	21	PD	PUK-BB
PD18	75	M	22	PD	PUK-BB
PD19	89	M	16	PD	PUK-BB
PD20	77	M	6	PD	PUK-BB

Table 1 (continued)

Subject ID	Age	Gender	PMI (hours)	Classification	Biobank
PD21	80	M	16	PD	PUK-BB
PD22	86	M	3	PD	PUK-BB
PD23	70	M	13	PD	PUK-BB
PD24	82	M	10	PD	PUK-BB
PD25	75	M	15	PD	PUK-BB
PD26	75	M	3	PD	PUK-BB
PD27	72	M	9	PD	PUK-BB
PD28	79	M	22	PD	PUK-BB
PD29	88	F	1.5	PD	NBM-BB
PD30	87	M	7	PD	NBM-BB
PD31	79	F	2	PD	NBM-BB

Brain samples were obtained from Fundación CIEN Brain Bank, Spain (CIEN-BB); Fundación Alcorcón University Hospital Brain Bank, Spain (HUFA-BB); London Neurodegenerative Diseases Brain Bank, United Kingdom (MRC-BB); Parkinson's UK Brain Bank, United Kingdom (PUK-BB); and Navarrabiomed Brain Bank, Spain (NBM-BB).

PMI = postmortem interval; M = male; F = female

suggesting an appropriated RNA quality to perform gene expression analyses.

Gene Expression Analyses: Real-time Polymerase Chain Reaction (real-time PCR)

Relative gene expression analyses of CB₁r, CB₂Ar, and MAGL in the SN and PUT were carried out between controls and patients with PD. Total RNA was obtained from brain sections and reverse transcription to cDNA was carried out. Taqman Gene Expression Assays (Life Technologies, Carlsbad, CA, USA) were used to measure CB₁r (Hs00275634_m1), CB₂Ar (forward primer GGAAGAAA GAGAATATTGTTTCAGTTGATT, reverse primer GCTGGCCTTGAGAGTGACA, and Taqman probe CCAGATGCAGCCGC), and MAGL (Hs000200752_m1) relative gene expression on the StepOne© Sequence Detector System (Life Technologies). All reagents were obtained from Applied Biosystems (Life Technologies) and the manufacturer protocols were followed. All primer–probe combinations were optimized and validated for relative quantification of gene expression. In this study, 2 housekeeping genes, cyclophilin (*PPIA*, Hs99999904_m1) and synaptophysin (*SYP*, Hs00300531_m1), were used to ensure the validity and reproducibility of results. Data for each target gene were normalized with the endogenous reference genes, and the fold change in target gene abundance was determined using the $2^{-\Delta\Delta C_t}$ method [38]. Briefly, samples were run in duplicate for both the target and the housekeeping genes, in different 96-well plates (a maximum of 8 samples per group—C or PD—in each plate), and the result was expressed as the mean value

for both endogenous housekeeping genes (*PPIA* and *SYP*). Thus, sample values were considered when standard deviation (SD) between duplicates was less than ± 0.3 in order to avoid inhomogeneous pipetting.

Immunohistochemistry

For immunohistochemical analyses, fixed brain samples were taken from 4 patients with PD (stages 4–5) [37] and 3 age-matched controls (Navarrabiomed Brain Bank, Pamplona, Spain). The postmortem interval was between 1.5 and 9 h in all cases. Briefly, 4-mm-thick coronal blocks through the mesencephalon comprising the entire SN were fixed for 24 h in a buffered solution of 4% paraformaldehyde at 4°C under gentle shaking. Next, blocks were cryoprotected in a solution of 2% dimethyl sulfoxide and 20% glycerin in 0.1 M phosphate buffer pH 7.4 for 48 h. Frozen coronal sections (40 μ m-thick) were obtained in a sliding microtome and collected in 0.1 M phosphate buffer (pH 7.4). Free-floating sections were incubated in a blocking solution containing 5% of normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h. Next, sections were incubated in a cocktail solution of primary antisera comprising 1:1000 rabbit anti-glial fibrillary acidic protein (GFAP) or 1:500 rabbit anti-ionized calcium-binding adapter molecule (Iba1), and 1:1000 goat anti-tyrosine hydroxylase (TH) for 60 h at 4°C. Subsequently, sections were incubated for 2 h in a cocktail solution made of 1:500 Alexa488 donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) and 1:500 Alexa633 donkey anti-goat IgG (Invitrogen). Once these immunofluorescent stains were completed, then the *in situ* proximity ligation assay (PLA) was started by incubation for 1 h at 37°C with the blocking solution (Duolink® In Situ Probemaker PLUS; Sigma, St. Louis, MO, USA) in a preheated humidity chamber, followed by overnight incubation with a rabbit anti-CB₂r primary antibody (1:200; Cayman, Ann Arbor, MI, USA). This primary antibody was diluted in the antibody diluent provided by Sigma within the PLA kit described below. Sections were then incubated with the secondary antibodies known as PLA probes (Duolink® In Situ PLA Probe anti-rabbit MINUS and Duolink® In Situ PLA Probe anti-rabbit PLUS; both Sigma) diluted 1:5 in the antibody diluent provided by the manufacturer of the PLA kit. The presence of CB₂r in these samples was detected using the Duolink II *in situ* PLA detection kit (Duolink® In Situ Detection Reagents Red; Sigma). Following the detection protocol as described by the supplier, the sections were washed with buffer A at room temperature and incubated with the ligation solution for 1 h at 37°C, in a humidity chamber. After washing with buffer A, samples were incubated with the amplification solution for 100 min at 37°C and then washed with buffer B (Sigma), and finally mounted using an aqueous mounting medium.

Stained samples were inspected under a Zeiss 510 Meta confocal laser-scanning microscope. To ensure appropriate visualization of the labeled elements and to avoid false-positive results, the emission from the argon laser at 488 nm (GFAP- or Iba1-immunostained structures) was filtered through a band pass filter of 505 to 530 nm and color-coded in green. The emission following excitation with the helium laser at 543 nm (CB₂r) was filtered through a band pass filter of 560 to 615 nm and color-coded in blue. Finally, a long-pass filter of 650 nm was used to visualize the emission from the helium laser at 633 nm (TH⁺ neurons) and color-coded in red.

Statistical Analyses

Statistical analyses of gene expression in results were performed using the Student's *t* test when control and PD groups were compared. Differences were considered significant when the probability of error was < 5%. SigmaPlot v11.0 (Systat Software, San Jose, CA, USA) was employed.

Results

Evaluation of Total RNA Integrity in Frozen Tissue from SN and PUT of Controls and Patients with PD

The results showed that the mean RIN value was > 6 [SN: controls 6.2 ± 0.2 , PD 6.2 ± 0.1 ; PUT: controls 6.1 ± 0.3 , PD 6.6 ± 0.1 (Fig. 1)], which was considered a sufficiently good RNA quality level [39]. No significant difference was observed between controls and patients with PD in any of the brain regions studied (PUT: $t = -1.503$, $p = 0.014$, 49 df; SN: $t = 0.404$, $p = 0.689$, 36 df).

CB₁r, CB₂Ar, and MAGL Gene Expression Changes in the SN and PUT Between Controls and Patients with PD

CB₁r gene expression in the SN of controls and patients with PD was not significantly different ($t = -1.140$, $p = 0.265$, 36 df; Fig. 2A), whereas a significant increase in CB₁r mRNA levels was evident in the PUT of patients with PD compared with controls (205%; $t = -4.418$, $p < 0.001$, 49 df; Fig. 2B). CB₂Ar gene expression significantly increased in the SN (395%; $t = -5.762$, $p < 0.001$, 36 df; Fig. 2C) and decreased in the PUT (−42%; $t = 5.429$, $p < 0.001$, 49 df; Fig. 2D) of patients with PD compared with controls. In contrast, patients with PD presented a significant reduction in MAGL gene expression in the SN (−28%; $t = 2.978$, $p = 0.005$, 36 df; Fig. 2E) and a significant increase in the PUT (222%; $t = -3.295$, $p = 0.002$, 49 df; Fig. 2F) compared with controls.

Fig. 1 Total RNA integrity number (RIN) evaluation in the substantia nigra (SN) and putamen (PUT) of healthy controls ($n = 16$ and $n = 23$, respectively) and patients with Parkinson's disease ($n = 25$ and $n = 28$, respectively). (A, B) Representative electropherogram images of mean RIN values from SN and PUT brain regions, respectively. (C) RIN values summary table; gray boxes contain global mean values

Fig. 2 Cannabinoid CB1 receptor (CB_{1r}), cannabinoid CB2 receptor A isoform (CB_{2Ar}), and monoacylglycerol lipase (MAGL) gene expression analyses by real-time polymerase chain reaction (real-time PCR). Gene expression in patients with Parkinson's disease (PD) is expressed relative to healthy controls. Data are expressed as mean $\pm$ SEM of $2^{-\Delta\Delta Ct}$. CB_{1r}, CB_{2Ar}, and MAGL gene expression in the substantia nigra (SN) (A, C, and E, respectively) and PUT (B, D, and F, respectively). *Significantly different ($p < 0.05$) from controls

Immunohistochemical Localization of CB₂r in the SN Pars Compacta of Controls and Patients with PD

Dopaminergic neurons throughout the SN pars compacta were clearly identified by their immunoreactivity for TH (TH⁺ neurons), as well as for the frequent presence of intracytoplasmic-pigmented neuromelanin. As expected, PD brain samples exhibited a smaller number of TH⁺ neurons than those samples obtained from control specimens. TH⁺ neurons were surrounded by astrocytes in all cases, although the astroglial reaction was by large much more intense in PD brain samples (Fig. 3). CB₂r expression was clearly observed in astroglial processes instead of in dopaminergic neurons. Compared with expression levels in control cases, there was a higher immunoreactivity for CB₂r in the astrocytes from patients with PD (Fig. 3). Moreover, CB₂r were never observed within

microglial cells as observed with immunohistochemical stains for Iba1 (Fig. 4).

Discussion

The results of the present study provide further evidence of the role of the endocannabinoid system, specifically CB₁r, CB₂r, and MAGL targets, in the nigrostriatal dopaminergic neurodegenerative process of PD. This assumption is based on the following results: 1) CB₁r gene expression was significantly increased in the PUT of patients with PD, whereas no changes were present in the SN; 2) CB₂r gene expression was significantly upregulated in the SN (4-fold) and downregulated in the PUT of patients with PD; 3) MAGL gene expression was reduced in the SN of patients with PD, and increased in the

Fig. 3 Localization of cannabinoid CB₂ receptor (CB₂r) in the substantia nigra (SN) pars compacta. Dopaminergic neurons were identified by tyrosine hydroxylase (TH) immunostain and color-coded in blue. Astrocytes were evidenced following glial fibrillary acidic protein (GFAP) immunostain and are shown in green. The observed glial reaction was far more intense in brains from patients with Parkinson's disease (PD) than in the corresponding control samples. Furthermore, the

presence of CB₂r (as assessed with the proximity ligation assay (PLA) stain, illustrated in red) was mostly seen in pericellular astrocytial processes surrounding TH⁺ neuronal somata. It seems clear that the intensity of CB₂r immunostaining was always higher in samples coming from PD brains. The presence of self-fluorescent neuromelanin pigment was often observed in the cytoplasm of TH⁺ neurons. Scale bar is 10 μ m in all panels

Fig. 4 Localization of the cannabinoid CB₂ receptor (CB₂r) in the substantia nigra (SN) pars compacta. Dopaminergic neurons were identified by tyrosine hydroxylase (TH) immunostain and color-coded in blue. Microglial cells were evidenced following ionized calcium-binding adapter molecule 1 (Iba1) immunostain and color-coded in green

(arrows), whereas the proximity ligation assay (PLA) stain was done to elucidate the presence of CB₂r (red channel). CB₂r was abundantly found in extracellular locations other than dopaminergic and microglial cells. Scale bar is 10 μ m in all panels. LB = Lewy body

PUT of patients with PD; and 4) only astrocytes, but not TH⁺ or microglial cells, expressed CB₂r in the SN of patients with PD.

Little information has been provided about the regulation of CB₂r in the brains of patients with PD. Only two previous studies have analyzed CB₂r protein expression and its cellular localization through immunostaining experiments in a reduced number of postmortem brain samples from controls ($n = 5$) and patients with PD ($n = 5$). García et al. [40] showed that CB₂r was present in TH-immunopositive neurons in the SN of patients with PD and controls, accompanied by a highly significant reduction in CB₂r protein expression in patients with PD compared with controls. This result is intriguing considering the previous literature supporting the association between neurodegeneration and CB₂r upregulation, and especially the results from animal models of PD. However, in the same year another study was published by Gómez-Gálvez

et al. [41], in which CB₂r was evaluated by immunohistochemical analyses in the same set of brain samples. In this study, CB₂r protein expression was significantly upregulated in the SN of patients with PD. In addition, CB₂r was present in the microglia (cells immunopositive for Iba-1) and possibly in macrophages and astrocytes. In the present study, the gene expression of a brain-specific isoform (A) of CB₂r was evaluated in a large sample of postmortem brain tissue from controls ($n = 24$) and patients with PD ($n = 28$). In accordance with the results of Gómez-Gálvez et al. [41], in the current study CB_{2a}r gene expression was markedly upregulated (4-fold) and immunohistochemical analyses clearly demonstrated an increase of CB₂r-immunopositive cells (see Figs 3 and 4, red coded) in the SN of the analyzed patients with PD.

The activation of astrocytes and microglial cells under neuroinflammatory and neurodegenerative conditions leads to an up-regulation of CB₂r. This phenomenon has been

replicated under different experimental conditions in animal models of pain [42], inflammation [43], ischemia-induced hypoxia [44], Huntington's disease [45], and PD. Previous evidence has shown that in mice treated intraperitoneally with the neurotoxin MPTP there was significant up-regulation of CB₂r gene expression in the SN [9]. In addition, Concannon et al. [35, 46] studied different neurotoxic and neuroinflammatory animal models of PD, demonstrating that the expression of CB₂r is significantly increased, although this effect was more pronounced in those inflammation-driven animal models. Interestingly, studies in postmortem brain tissue from patients with a neurodegenerative disorder confirmed this result by also finding an up-regulation of CB₂r [47–49]. It has been suggested that this up-regulatory response in the microglia is aimed at controlling the production of neurotoxic factors such as nitric oxide, pro-inflammatory cytokines, and reactive oxygen species. In the case of astrocytes it is thought that the increase of CB₂r contributes to the neuroprotective process by generating pro-survival factors or metabolic substrates for neurons, although there is a lack of information about the precise function of CB₂r in these effects [50].

The presence of CB₂r was recently documented in hippocampal neuronal populations [51] and dopaminergic neurons located in the ventral tegmental area (VTA) [52] in mice, as well as in basal ganglia output neurons in macaques [53]. Moreover, the presence of CB₂r immunoreactivity was also recently found in the dopaminergic neurons of the SN in post-mortem human brains from controls and patients with PD, as previously mentioned [40]. Here, we failed to observe CB₂r immunoreactivity in TH⁺ nigral neurons, neither in controls brains nor in those of patients with PD. Immunohistochemical expression of CB₂r was only found in astrocytes, with a total lack of CB₂r staining in dopaminergic and microglial cells. Indeed, a clear increase in CB₂r staining intensity was observed within astrocytes in brain samples from patients with PD, which is in agreement with CB₂a_r gene expression results (see Fig. 2). These apparent discrepancies between both studies might be explained by the different methods applied during tissue processing. The procedure followed by García et al. [40] included a deparaffination step, followed by a microwave antigen-retrieval procedure. None of these procedures was done here, as in our experience the use of paraformaldehyde fixation followed by the preparation of frozen sections minimizes the presence of false positive results and enhances antibody penetration. Indeed, it is worth noting that by taking advantage of the PLA technique, a 1000-fold amplification of the signal is obtained, an important procedure when detecting antigens expressed at very low concentrations such is the case for CB₂r. Consequently, if a lack of CB₂r stain is found in dopaminergic and microglial cells, it is highly probable that CB₂r is not expressed within any of those cellular populations. Therefore, these data suggest that the CB₂r-mediated regulation of astrocytes function in the SN could play a pivotal role

in the neuropathogenesis of PD. This assumption is partially supported by previous reports that shed light about the role of CB₂r in astrocytes. These studies suggested that CB₂r participates in the modulation of the production of different inflammatory mediators [34, 54], and its activation presents a neuroprotective effect [55]. Furthermore, it is relevant to note that in the study performed by Gómez-Gálvez et al. in the SN brain tissue of controls and patients with PD, the authors stated that CB₂r immunofluorescence was also present in cells that were not labeled with Iba-1, probably astrocytes [41].

Therefore, taking into consideration that the presence of CB₂r was previously described in neurons from rodents and macaques, but in this study there was no signal in the SN of patients with PD employing a highly sensitive immunohistochemical technique, it might be hypothesized that these differences could be related to several factors, such as 1) the prominent neurodegenerative state of this brain region in aged patients with PD; or 2) species differences between animals and humans. However, additional analyses are warranted to shed light on this question.

In the PUT of patients with PD, CB₂a_r gene expression is significantly lower compared with controls. This result, in comparison with the notable increase detected in the SN, could be attributed, at least in part, to the different regional regulation of the endocannabinoid system that has been proposed to depend on the dopaminergic activity [56]. A previous study evaluating CB₁r availability by positron emission tomography (PET) and magnetic resonance imaging (MRI) showed a significant decrease in CB₁r availability in the SN of patients with PD, whereas an increase was found in nigrostriatal dopaminergic projection areas, such as PUT [7]. Indeed, in the present study CB₁r gene expression also showed a significant increase in the PUT of patients with PD, although there were no noticeable changes in the SN. Therefore, it can be hypothesized that the differences in the neuropathological state and the regulatory projections of both regions in PD might account for the CB₂a_r gene expression data [57], although additional studies are necessary to elucidate the underlying neurobiological mechanisms involved.

MAGL gene expression changes were also evaluated in the SN and PUT of controls and patients with PD to obtain an indirect measure of the 2-AG-mediated endocannabinoid tone in the nigrostriatal pathway. Evidence suggests that the release of 2-AG during neuronal injury could be closely associated with a neuroprotective reaction in different experimental animal models [19, 58–61]. In addition, previous studies have shown a relevant role of 2-AG in animal models of PD. Indeed a 7-fold increase of 2-AG was found in the globus pallidus of reserpine-treated rats [62], and an elevation of 2-AG in the striatum of monkeys lesioned with MPTP [63]. Interestingly, in a recent study employing MPTP-lesioned mice the inhibition of MAGL reduced degradation of 2-AG, improved MPTP-induced motor impairment and induced the release of neuroprotective and anti-inflammatory molecules [22]. Another study evaluated the

mechanisms involved in the neuroprotective effect induced by the MAGL inhibitor JZL184. The results revealed that CB₂r plays a relevant role as the selective CB₂r antagonist AM630 fully blocked the JZL184-mediated effects, JWH133 administration mimicked them, and the CB₁r antagonist rimonabant was without effect [64].

In accordance with the previous reports, it is possible to hypothesize that a significant and sustained elevation of 2-AG levels may be found in the PUT of patients with PD accounting for the compensatory reduction of CB₂aR and the elevation of MAGL gene expression (see Fig. 2). However, the marked neuronal loss occurring in the SN of patients with PD, and a different functional regulation of the endocannabinoid system, may entail a different scenario, probably with a lesser release of 2-AG. This decrease of 2-AG would be related to the remarkable increase of CB₂aR gene expression and the significant reduction of MAGL gene expression as compensatory mechanisms. This hypothetical assumption is based mainly on preclinical data and does not coincide with a recent study measuring 2-AG levels by mass spectrometry in mice following subchronic MPTP treatment. The results showed an increase in 2-AG levels in the ventral midbrain and a reduction in the striatum [65]. Therefore, additional studies are required to find out the precise regulatory mechanisms of the endocannabinoid system that could explain better these contrasting results between nigral dopaminergic body cells and its striatal terminals.

In conclusion, the results of the present study suggest that CB₁r, CB₂r, and MAGL play a relevant role in PD. This premise is based on the gene expression alterations that were found in the SN and PUT of patients with PD, and on the exclusive and higher presence of CB₂r in the astrocytes located in the SN pars compacta of patients with PD in comparison with controls. These data provide important clues for the development of mechanism-based therapeutics through the functional modulation of the endocannabinoid system, highlighting CB₁r, CB₂r, and MAGL as potential pharmacological targets that need further consideration for the treatment of PD.

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