



Targeting CB₁ and GPR55 Endocannabinoid Receptors as a Potential Neuroprotective Approach for Parkinson's Disease

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Abstract

Cannabinoid CB₁ receptors (CB₁R) and the GPR55 receptor are expressed in striatum and are potential targets in the therapy of Parkinson's disease (PD), one of the most prevalent neurodegenerative diseases in developed countries. The aim of this paper was to address the potential of ligands acting on those receptors to prevent the action of a neurotoxic agent, MPP⁺, that specifically affects neurons of the substantia nigra due to uptake via the dopamine DAT transporter. The SH-SY5Y cell line model was used as it expresses DAT and, therefore, is able to uptake MPP⁺ that inhibits complex I of the respiratory mitochondrial chain and leads to cell death. Cells were transfected with cDNAs coding for either or both receptors. Receptors in cotransfected cells formed heteromers as indicated by the in situ proximity ligation assays. Cell viability was assayed by oxygen rate consumption and by the bromide-based MTT method. Assays of neuroprotection using two concentrations of MPP⁺ showed that cells expressing receptor heteromers were more resistant to the toxic effect. After correction by effects on cell proliferation, the CB₁R antagonist, SR141716, afforded an almost full neuroprotection in CB₁R-expressing cells even when a selective agonist, ACEA, was present. In contrast, SR141716 was not effective in cells expressing CB₁/GPR55 heteromeric complexes. In addition, an agonist of GPR55, CID1792197, did not enhance neuroprotection in GPR55-expressing cells. These results show that neurons expressing heteromers are more resistant to cell death but question the real usefulness of CB₁R, GPR55, and their heteromers as targets to afford PD-related neuroprotection.

Keywords Cannabinoid receptors · GPCRs · Neurodegenerative diseases · Neuroprotection · MPP⁺

José Luis Lanciego and Rafael Franco contributed equally to this work.

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Introduction

Parkinson's disease (PD) is an unrelenting neurodegenerative disorder caused by the progressive degeneration of dopamine-producing neurons of the substantia nigra *pars compacta*. Dopamine loss triggers dysfunction of basal ganglia circuits, ultimately leading to the cardinal symptoms of the disease (reviewed in [1]). Although levodopa is still viewed as the gold-standard pharmacological treatment for PD, affording an effective symptomatic alleviation, chronic levodopa administration leads to abnormal involuntary movements known as dyskinesia (LIDs; [2–4]). High-frequency deep brain stimulation (HFS-DBS) is a neurosurgical procedure that provides good clinical management of LIDs, particularly when HFS-DBS electrodes are placed in the internal segment of the globus pallidus (GPi; [5]). Despite symptomatic relief, it is worth noting that both dopamine-replacement strategies and functional neurosurgical interventions do not have any effect on disease progression and, therefore, in the long run, PD patients often end up with a cognitive decline that has trends in common with those observed in Alzheimer's disease [6].

In the last two decades, extensive research has been carried out to elucidate the mechanisms underlying why dopaminergic neurons are particularly vulnerable to degenerate, as well as in pushing forward safe and efficacious approaches for neuroprotection, in an attempt to slow down (or ideally arrest) disease progression rates. Regarding neuroprotection, most studies performed so far have relied on neurotoxin-induced mice and non-human animal models of PD [7, 8]. A number of *in vitro* cellular models of PD-like neurodegeneration have been made available, such as those using neural-related cell lines exposed to neurotoxic compounds as MPP⁺ (C₁₂H₁₂N⁺) that inhibits complex I of the respiratory mitochondrial chain and leads to cell death. MPP⁺ by far is the most often used compound mimicking PD-related cell death in cellular models [9–11]. In what concerns suitable cell lines for MPP⁺ *in vitro* administration, SH-SY5Y human neuroblastoma cell line seems to be a convenient choice (reviewed in [12]).

When looking for novel drug candidates, cannabinoids rank among the compounds with a more potential as neuroprotective agents [13, 14]. They act via two specific receptors that belong to the superfamily of G protein-coupled receptors (GPCRs): cannabinoid CB₁ and CB₂. Within the central nervous system (CNS), CB₁ receptors (CB₁R) are mainly expressed in neurons, whereas CB₂ receptors (CB₂R) are mainly expressed in glial cells. Actually, CB₁R are considered as the most abundant GPCR in the CNS. Although not directly engaged in PD pathophysiology, polymorphisms in the receptor gene were linked to PD-associated depression [15]. Pharmacology of CB₁R is complex due to the hydrophobic nature of the natural ligands and the very particular positioning of the orthosteric site, which is hidden from the extracellular milieu in the 3D structures already reported [16–18]. The entrance of CB₁R ligands seems thus

possible via the lipid bilayer of the plasma membrane. These recent discoveries lead to the possibility of finding specific ligands that may fully activate, partially activate, partially deactivate, or fully block the receptor's response. Acting on CB₁R, endogenous agonists, 2-arachidonoylglycerol and anandamide, lack the psychotropic effects exerted by the phytocannabinoid, Δ⁹-tetrahydrocannabinol (THC) [19]. Moreover, it should be possible to develop structurally different antagonists showing less side effects than rimonabant, an anti-obesity drug that was withdrawn from the market due to suicide cases [20]. Cannabinoids are able to interact and affect the signaling of the orphan GPR55 receptor, which was considered as a third cannabinoid receptor. Despite the receptor may be activated by an endogenous molecule, L-α-lysophosphatidylinositol, IUPHAR (<http://www.guidetopharmacology.org>) did not consider GPR55 as de-orphaned because *in vivo* efficacy of L-α-lysophosphatidylinositol acting on GPR55 has not yet been reported [21–26]. GPR55 is expressed in different regions within the brain and has been proposed as a potential target for PD [27–29].

GPR55 may be coupled to diverse signaling pathways [30], and indeed the CB₁R may modulate GPR55-mediated signaling [31]. These results led to the search for potential CB₁R/GPR55 heteromeric complexes that were identified in *in vitro* models and also in the rat striatum [29, 31]. This paper aims to assess whether the expression of CB₁ or GPR55 receptors or of CB₁/GPR55 heteromeric receptor complexes may be engaged in response to the MPP⁺ toxic effects within neuroblastoma SH-SY5Y cells.

Materials and Methods

Drugs

The following drugs have been used here: N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA; ref. 1319, Tocris Bioscience) and 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide (SR141716; ref. 168273-06-1, Cayman Chemical). CID1792197 [21] was *in-house* synthesized (>99% purity). ACEA was supplied as pale yellow oil that was diluted in ethanol in a 10 mM concentration stock solution. SR141617 (10 mM) and CID1792197 (10 mM) stock solutions were prepared in DMSO. Aliquots of these stock solutions were kept frozen at -20 °C until use.

Fusion Proteins and Expression Vectors

The human cDNAs for the CB₁R and GPR55 were cloned in pcDNA3.1 and amplified without their stop codons using sense and antisense primers harboring either unique EcoRI or BamHI sites. The fragments were then

subcloned to be in-frame with EYFP into the BamHI and EcoRI restriction sites of an EYFP expressing vector (EYFP-N1; enhanced yellow variant of GFP; Clontech, Heidelberg, Germany), to generate the plasmids expressing CB₁R and GPR55 fused to YFP on the C-terminal end of the receptor (CB₁R-YFP or GPR55-YFP). Expression of receptors was tested by confocal microscopy and the receptor functionality was tested performing ERK1/2 activation assays.

Cell Line Culture and Transfection

Human neuroblastoma SH-SY5Y cell line was obtained from Sigma (ref 94030304; Sigma-Aldrich, St. Louis, MO, USA). Cells were grown in DMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, 1% nonessential amino acids, and 5% (v/v) heat inactivated fetal bovine serum (FBS) (all supplements were from Invitrogen, Paisley, Scotland, UK). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and were passaged when they were 80–90% confluent, i.e., approximately twice a week for no more than 20 passages.

SH-SY5Y cells growing in 10 cm dishes were transiently transfected with the corresponding fusion proteins cDNAs (see figure legend) using Lipofectamine 2000 as described by the manufacturer (ref 11668027; Invitrogen, Paisley, Scotland, UK) and were used 48 h post-transfection.

Cell Treatments

Cell treatments were performed 48 h post-transfection using 3000 cells per well in 96-well plates for MTT reduction assay (MTT is a water-soluble tetrazolium reagent in REDOX reactions involving mitochondrial components), 40,000 cells per well in 96-well plates for oxygen consumption assay and 30,000 cell/well in 8-well chambered cover glasses (Nunc® Lab-Tek® II, Sigma-Aldrich, St. Louis, MO, USA) for in situ proximity ligation assay (PLA). After 24 h, the culture medium was replaced by fresh serum-free DMEM and cells were cultured for another 24 h. In each experiment, cells were treated or not with the CB₁R agonist, ACEA (100 nM), and with the GPR55 agonist, CID1792197 (1 μM), for 30 min before freshly added MPP⁺ (ref D048, Sigma-Aldrich, St. Louis, MO, USA), or pre-treated for 20 min with CB₁R antagonist, SR141716 (rimonabant; 250 nM), prior to agonist treatment. If more than one compound was used, they were added simultaneously to the culture media. The drug concentrations and times of treatments used in these experiments were based on the bibliography related to pharmacology of the receptors and on our previous experience [21, 29].

Oxygen Consumption Rate (OCR) Assay

Oxygen consumption was monitored in oxygen-sensing microplates (Oxoprobics Biosciences S. L., Madrid, Spain). The probe is quenched in the presence of oxygen; as oxygen is consumed by cellular respiration, the fluorescence signal increases being directly related to cell metabolism [32]. Briefly, cells were seeded in oxygen-sensing plates and incubated for 24 h, the different treatments were added to the cells at the indicated final concentrations, and the wells were sealed from ambient oxygen by the addition of 100 μl/well of mineral oil. Plates were placed in a plate reader (Envision, Perkin-Elmer, Waltham, MA, USA) previously equilibrated at 37 °C and monitored using 340/665 nm excitation/emission filters, with a delay time of 70 μs for 24–48 h.

MTT Reduction Assay

Cell viability was also studied by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Once the treatments were completed, 10 μl of MTT [5 mg/ml in phosphate buffered saline (PBS)] (ref M5655; Sigma-Aldrich, St. Louis, MO, USA) were added to each well. Four hours later, 100 μl of lysis solution [20% sodium dodecyl sulfate (SDS); 50% dimethylformamide; pH 4] were added to the culture and incubated overnight at 37 °C. Absorbance at 570 nm was measured using a Multiskan EX Microplate Reader (ThermoFisher Scientific, Waltham, MA, USA). Values from blank dishes, containing only medium, were subtracted from the values of the samples. Viability was expressed as the percentage of the controls.

In situ Proximity Ligation Assay (PLA)

For PLA, SH-SY5Y transfected cells (SH-SY5Y-CB₁R, SH-SY5Y-GPR55, or SH-SY5Y-CB₁R/GPR55) were fixed in 4% paraformaldehyde for 15 min, washed with PBS containing 20 mM glycine to quench the aldehyde groups, and permeabilized with the same buffer containing 0.05% Triton X-100 (5 min treatment). To create our PLA probes we conjugated a rabbit anti-CB₁R (ref: PA1-745, Thermo Scientific, Rockford, USA) with a PLUS oligonucleotide (Duolink® In Situ Probemaker PLUS DUO92009, Sigma-Aldrich, St. Louis, MO, USA) and a rabbit anti-GPR55 (ref 10224, Cayman Chemical, Michigan, USA) with a MINUS oligonucleotide (Duolink® In situ Probemaker MINUS DUO92010, Sigma-Aldrich, St. Louis, MO, USA) following manufacturer's instructions. Fixed cells were incubated for 1 h at 37 °C with blocking solution (from PLA kit, see below), followed by overnight incubation (4 °C) with the PLA probe-linked antibodies described above. Specificity of antibodies was tested in non-transfected SH-SY5Y cells (data not shown).

Duolink II in situ PLA detection kit (Duolink® In situ Detection Reagents Red, DUO92008, developed by Olink Bioscience, Uppsala, Sweden; now distributed by Sigma-Aldrich as Duolink® using PLA® Technology) was used to detect the presence/absence of receptor clusters in the samples, which were incubated with the ligation solution for 1 h, washed and subsequently incubated with the amplification solution for 100 min (both steps at 37 °C in a humid chamber). Nuclei were stained with Hoechst (1/100; ref. 33258, Sigma-Aldrich, St. Louis, MO, USA). Mounting was performed using 30% MOWIOL™ (ref 9002-89-5; EMD Millipore Calbiochem™). Negative controls were performed by omitting the primary anti-GPR55 antibody.

Samples were observed in a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with an apochromatic 63X oil-immersion objective (N.A. 1.4), and 405 nm and 561 nm laser lines. For each field of view, a stack of two channels (one per staining) and five Z stacks with a step size of 1 µm were acquired. The number of cells containing one or more red spots versus total cells (blue nucleus) and, in cells containing spots, the ratio r (number of red spots/cell), were determined by means of the Duolink Image tool software.

Labeling of Mitochondria

SH-SY5Y cells transfected with CB₁R and GPR55, subjected or not to treatments, were labeled with MitoTracker® Red CMXRos probe (ref M7512; Invitrogen, Paisley, Scotland,

UK), according to the manufacturer's instructions. CB₁R or GPR55 fused to YFP protein was detected by its fluorescence properties. Samples were observed in a Zeiss 510 Meta confocal laser-scanning microscope.

Data Analysis

Data result from at least five independent experiments. The data in the graphs are presented as the mean ± SEM. Statistical analysis was performed with SPSS 18.0 software. The test of Kolmogorov-Smirnov with the correction of Lilliefors was used to evaluate the fit of the data to a normal distribution and the test of Levene to evaluate the homogeneity of variance. Significance was analyzed by one- or two-way ANOVA test followed by Tukey's test for multiple comparisons. Significant differences were considered when $p < 0.05$.

Results

CB₁ and GPR55 Receptor Cellular Localization

The heterologous expression of the human version of CB₁R-YFP and GPR55-YFP fusion proteins was analyzed in single-transfected SH-SY5Y neuroblastoma cells following the protocol described in the “Materials and Methods” section and with the MitoTracker® Red CMXRos probe, which labels mitochondria. As shown in Fig. 1, the two GPCRs were

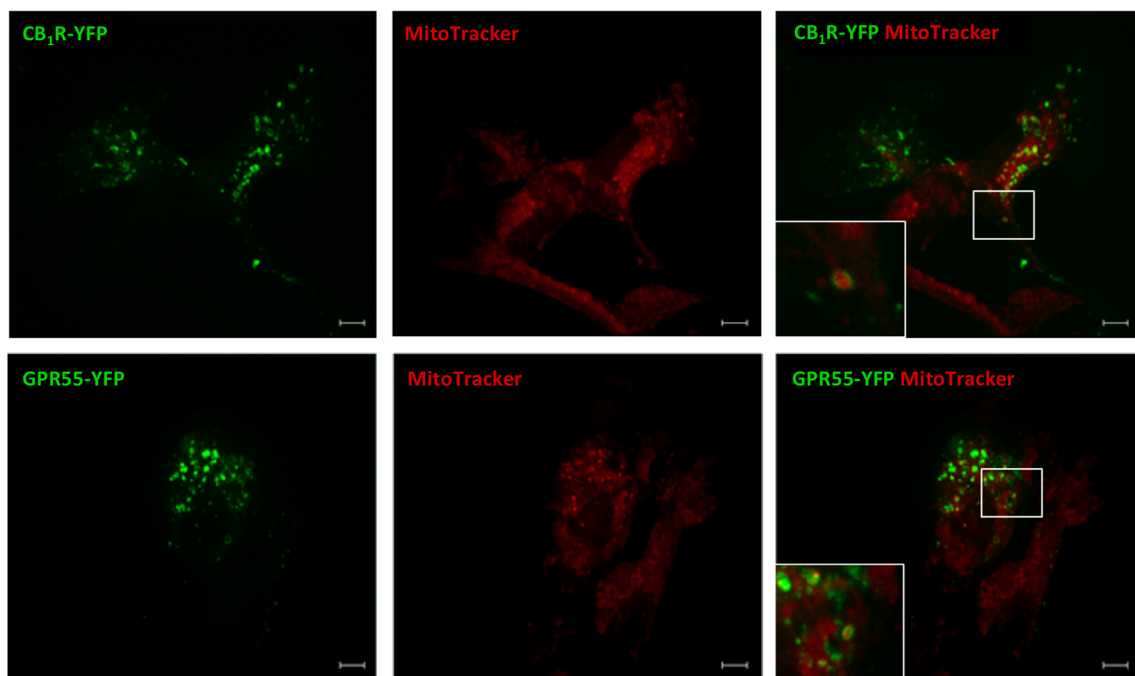


Fig. 1 Cellular localization of CB₁R and GPR55. SH-SY5Y cells, transfected with CB₁R (top left image) and GPR55 (bottom left image) fused to YFP protein and identified by its fluorescence properties (green signal), were labeled with MitoTracker® Red CMXRos probe (top and

bottom middle images) (red signal). Colocalization is shown in yellow in the merge images (top and bottom right images), where CB₁R and GPR55 are present in the surface of mitochondria, as it is shown in the inserts. Confocal microscopy images are shown (× 100). Scale bars 5 µm

expressed in different cell locations of SH-SY5Y-CB₁R-YFP and SH-SY5Y-GPR55-YFP cells; interestingly, there were receptors expressed in intracellular organelles (or “structures”) that mainly correspond to mitochondria. The presence of the CB₁R in mitochondria has been reported elsewhere [33, 34].

Neuroprotective Effect of Cannabinoid Receptor Expression in a MPP⁺ Cellular Model of Parkinson’s Disease

In order to evaluate the potential neuroprotective effect of cannabinoid receptors in cells treated with MPP⁺, the toxic metabolite of MPTP, we firstly performed a dose-response curve to elucidate the proper concentration of MPP⁺ to be used in subsequent experiments. A wide range of MPP⁺ concentrations, from 0.005 to 5 mM, was hence used to induce cytotoxicity in SH-SY5Y cells, and cell viability was assessed by two different approaches. As shown in Fig. 2a, b, cell viability assessed by the degree of MTT reduction decreased in a concentration-dependent manner 24 and 48 h after treatment with MPP⁺. Data analysis revealed a statistically significant decrease of circa 45% in cell viability upon 24 h treatment with 2 mM MPP⁺. In cells treated for 48 h under similar increasing concentrations, the effect of 2 mM MPP⁺ was too strong, within a 70–80% decreased viability range, and doses of 5 mM led to a virtually complete cell death. Based in these dose-response results, 1 mM and 2 mM MPP⁺ were the concentrations chosen for further assays. These findings were confirmed by measuring OCR (Fig. 3a, b). In fact, our data demonstrated a dose-response decrease in cellular respiration caused by MPP⁺ in SH-SY5Y cells at both 24 and 48 h of incubation. It should be noted that toxicity at low MPP⁺ concentrations may be better detected using the OCR than the MTT assay. However, the effect on OCR measurements has a narrow dynamic range and, for this reason, the MTT assay was preferred.

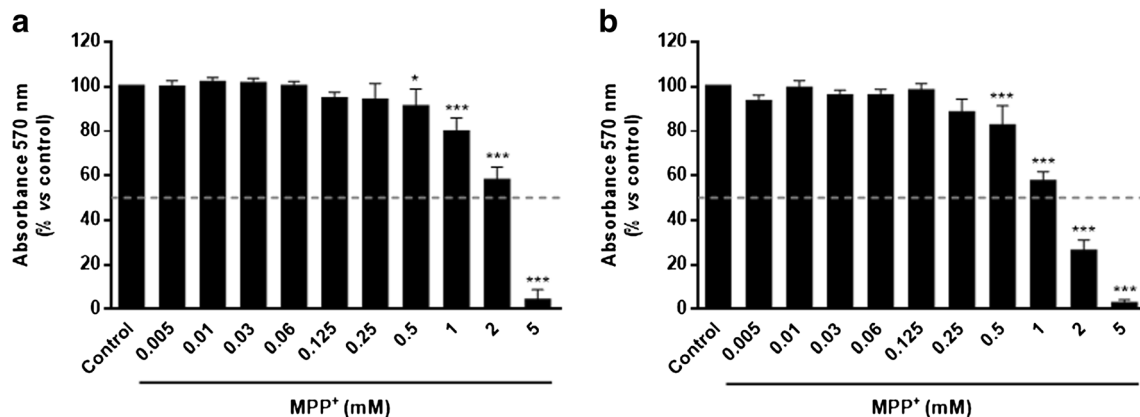


Fig. 2 MTT reduction assay in SH-SY5Y cells treated with increasing concentrations of MPP⁺ (0.005–5 mM) for 24 (a) and 48 h (b). Cell damage is represented as the p percentage of MTT reduction versus

The next step was to assess whether the expression of the cannabinoid receptor could be neuroprotective by itself. For this purpose, we tested cell viability of SH-SY5Y cells transiently expressing CB₁R and incubated with MPP⁺ for 24 h. We found that SH-SY5Y-CB₁R cells treated with 1 mM MPP⁺ did not show any reduction in the MTT signal while the toxic effect was noticeable in non-transfected cells (Fig. 4a, b). Interestingly, similar results were found in SH-SY5Y-GPR55 cells treated with 1 or 2 mM MPP⁺ (Fig. 4c). Due to the higher degree of toxicity over time, all these seemingly neuroprotective effects were not found at 48 h of treatment (Supplementary Fig. 1). Accordingly, we selected 24 h incubation for the majority of subsequent experiments.

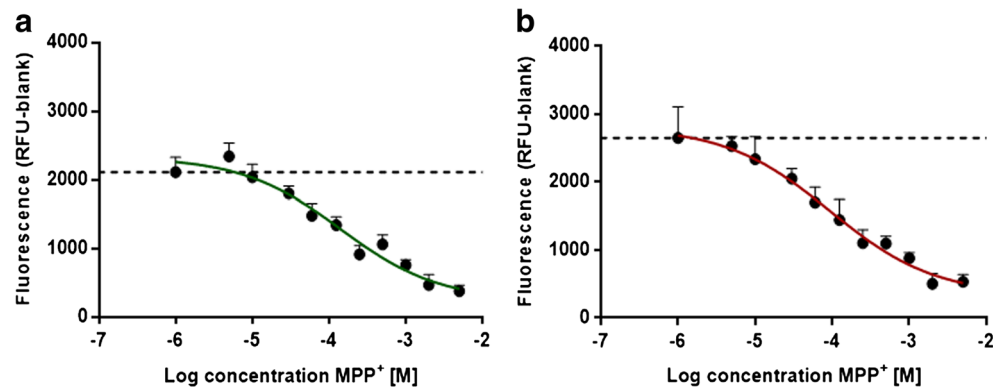
Neuroprotection in SH-SY5Y Cells Expressing CB₁/GPR55 Heteroreceptor Complexes

Cannabinoids, which may bind to both cognate receptors and non-cognate GPR55 receptors, are considered as neuroprotective agents. To better assess the underlying mechanism of neuroprotection, CB₁R and GPR55 were transiently coexpressed in SH-SY5Y cells. Of the two types of cannabinoid receptors, the CB₁R were selected because they are the GPCRs with the highest presence in CNS neurons [35–37]. In contrast, the CB₂R is expressed in fewer neuronal populations, at restricted CNS sites, and in glial cells [38, 39]. It is known that GPR55 is also expressed in neurons at different brain regions [27, 40–42].

CB₁R/GPR55 heteromers have been demonstrated in vitro and in vivo [29, 31]; in the CNS, the heteromers have been identified in the striatum [29]. Accordingly, we performed in situ PLA to identify direct receptor-receptor interactions in SH-SY5Y-CB₁R/GPR55 cells. This approach showed that CB₁R and GPR55 readily form receptor heteromeric complexes in cotransfected cells (Fig. 5). We found that cells expressing those receptors, i.e., which express receptor

control. Data are the mean ± SEM of five independent experiments. Significant differences were analyzed by a one-way ANOVA followed by post-hoc Tukey’s test. **p* < 0.05, ****p* < 0.001 compared with control

Fig. 3 OCR assay in SH-SY5Y cells treated with increasing concentrations of MPP⁺ (0.005–5 mM) for 24 (a) and 48 h (b). Oxygen consumption was monitored in real-time using 96-well plate oxygen-sensing plates during 24 or 48 h. Data are the mean \pm SEM of five independent experiments. RFU relative fluorescence units



heteromers, are less sensitive to the MPP⁺ insult. In fact, SH-SY5Y cells transfected with cDNAs for CB₁R and GPR55 were resistant to a 24 h treatment with 1 mM MPP⁺ (Fig. 6a, b). However, the protection was not afforded when cells were treated with 2 mM MPP⁺ or for 48 h with the toxic molecule. Interestingly, incubation with MPP⁺ for 48 h in cotransfected cells led to similar results that those encountered in cells expressing only one receptor (Supplementary Fig. 2), i.e., coexpression of receptors leads to a decrease in the neuroprotective potential of cells against the MPP⁺ insult.

Neuroprotective Effect of Ligands of CB₁ and GPCR55 Receptors in the MPP⁺-Based Cellular Model of Parkinson's Disease

A final aim was to assess the neuroprotective potential of ligands interacting with CB₁R or GPR55. As a control, the

effect of selective ligands on the growth of transiently single-transfected SH-SY5Y cells was tested (Fig. 7). Virtually, all assayed compounds affected cell growth after 24 h when compared with the results obtained in control. Differences in growth rate after transfection are expected and the effect of ACEA, the CB₁R agonist, and of CID1792197, the GPR55 agonist was significant. ACEA was able to reduce growth in CB₁R and GPR55-expressing cells but not in cotransfected cells. The GPR55 agonist was able to significantly affect growth but increasing it in untransfected cells, in cells expressing CB₁R or GPR55 but not in cotransfected cells (Fig. 7). These results indicate that cell growth is affected by ACEA and by CID1792197, which would not be exerting its effect via its cognate receptor but via unknown targets. The effect of the selective CB₁R antagonist, SR141617, increased growth only in cells expressing the cognate receptor (Fig. 7). This effect was remarkable as the antagonist not only reversed the

Fig. 4 MTT reduction assay in SH-SY5Y (a), SH-SY5Y-CB₁R (b), and SH-SY5Y-GPR55 (c) cells treated with MPP⁺ (1–2 mM) for 24 h. Cell damage is represented as the percentage of MTT reduction versus control. Data are the mean \pm SEM of five independent experiments. Significant differences were analyzed by a one-way ANOVA followed by post-hoc Tukey's test. ** p < 0.01, *** p < 0.001 compared with control

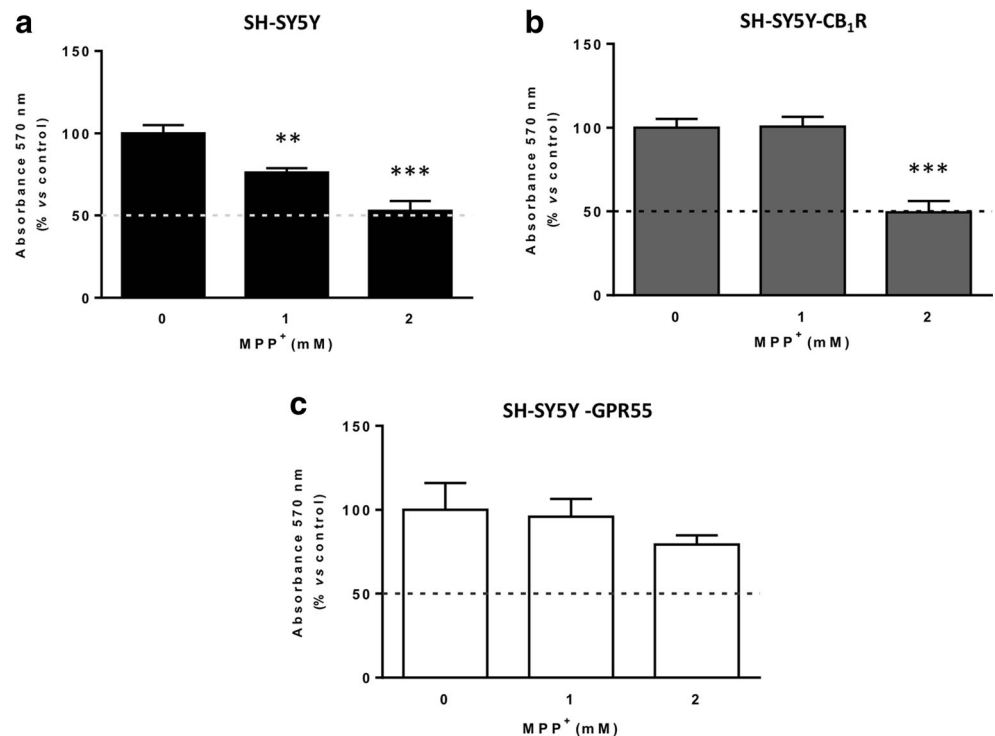
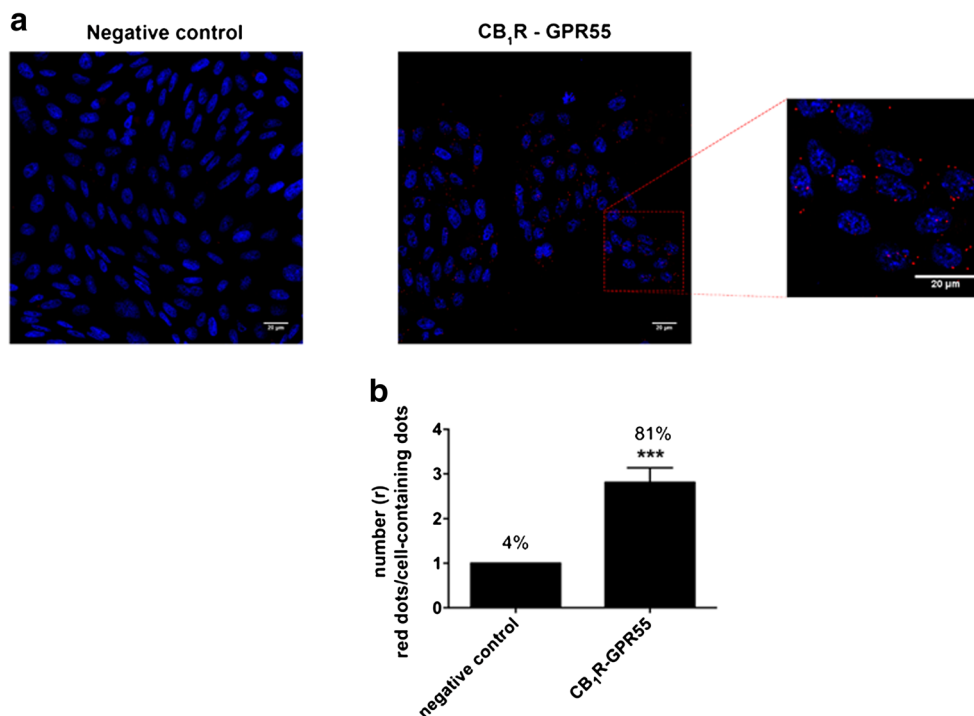


Fig. 5 In situ PLA assay performed as described in the “Materials and Methods” section in SH-SY5Y and SH-SY5Y-CB₁R/GPR55 cells using specific primary antibodies against these receptors. Panel a: representative PLA confocal images showing CB₁R/GPR55 complexes as red dots in cells with Hoechst-stained nuclei. Scale bars 20 μ m. Panel b: percentage of positive cells (containing one or more red dots; from cells in four–six different fields), and number (r) or red dots/cell-containing dots in cotransfected SH-SY5Y cells. Data are the mean \pm SEM of five independent experiments. Significant differences were analyzed by a one-way ANOVA followed by post-hoc Tukey’s test. *** $p < 0.001$ compared with control



effect of the agonist thus suggesting that CB₁R_s are already activated even in the absence of ACEA, i.e., they display a constitutive activity when heterologously expressed in HEK-293T cells. It should be noted that no validated GPR55 antagonist is available.

As above indicated, neuroprotection may be afforded by heterologous expression of receptors. We were subsequently interested in the neuroprotective potential of agonists acting on these receptors. For this purpose, SH-SY5Y cells were treated for 24 h with 1 mM MPP⁺ in the presence of agonists, ACEA (100 nM) for CB₁R and CID1792197 (1 μ M) for GPR55, or with the CB₁R antagonist, SR141716 (250 nM), in the absence or presence of receptor agonists. Compounds were added 30 min before the toxic. The assays were performed in non-transfected cells and in cells expressing either CB₁R, GPR55, or both. The growth of each cell type in

supplemented medium in the absence of the neurotoxic and of any receptor ligand was used as reference (100% viability) in Fig. 8. On the one hand, results confirmed that the simple presence of CB₁R or GPR55 was protective (Fig. 8). However, activation of the CB₁R by ACEA was not protective neither in CB₁R nor in CB₁R/GPR55-expressing cells. Actually, the compound blocked the protection due to the presence of the receptors. On the other hand, the agonist of GPR55, CID1792197, did not enhance neuroprotection in GPR55-expressing cells, and the result was, again, similar to that obtained in CB₁R-expressing cells. This is consistent with off-target effects of this compound. Agonists were neither effective in cotransfected cells. As it would be expected, the CB₁R antagonist, SR141716, did not affect the results obtained using the GPR55 agonists. However, this antagonist afforded an almost full neuroprotection in CB₁R-expressing

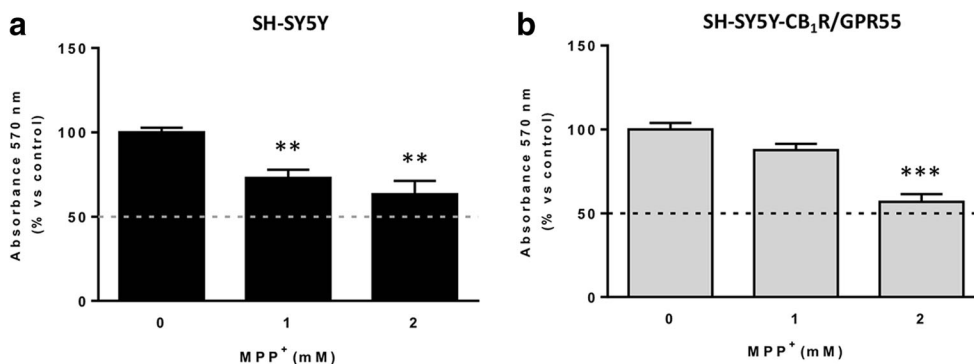
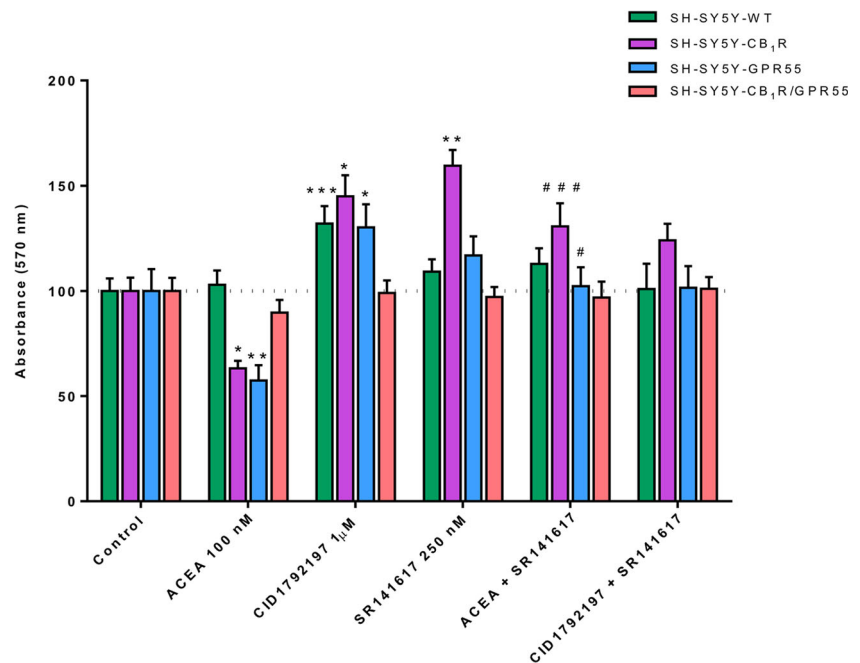


Fig. 6 MTT reduction assay in SH-SY5Y (a) and SH-SY5Y-CB₁R/GPR55 (b) cells treated with MPP⁺ (1–2 mM) for 24 h. Cell damage is represented as the percentage of MTT reduction versus control. Data are

the mean \pm SEM of five independent experiments. Significant differences were analyzed by a one-way ANOVA followed by post-hoc Tukey’s test. ** $p < 0.01$, *** $p < 0.001$ compared with control

Fig. 7 MTT reduction assay in SH-SY5Y transiently transfected with cDNA coding for CB₁R, GPR55 or both receptors treated with the CB₁R agonist, ACEA (100 nM), with the GPR55 agonist, CID1792197 (1 μM), and with the CB₁R antagonist, SR141716 (250 nM). Cell damage is represented as the percentage of MTT reduction versus control. Data are the mean ± SEM of five independent experiments. Significant differences were analyzed by a two-way ANOVA followed by post-hoc Tukey's test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the respective controls (vehicles); # $p < 0.05$, ### $p < 0.001$ compared with ACEA treatment



cells even when ACEA was present ($p < 0.05$). These results suggest that both the presence of CB₁R and of CB₁R antagonists results in further neuroprotection. Finally, the beneficial effect of the CB₁R antagonist was lost when cells expressed CB₁R/GPR55 heteromers (Fig. 8).

Discussion

Neuroprotection by cannabinoid compounds may be afforded by targeting either CB₁R or CB₂R. PD is usually diagnosed when a significant number of nigral dopaminergic neurons are

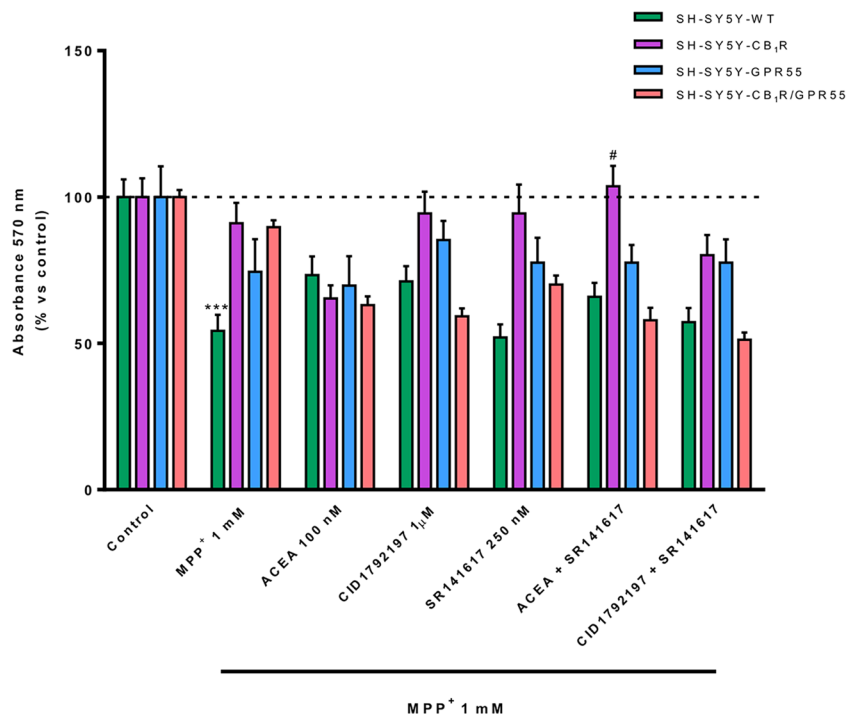


Fig. 8 MTT reduction assay in SH-SY5Y transiently transfected with cDNA coding for CB₁R, GPR55 or both receptors treated with the CB₁R agonist, ACEA (100 nM), and with the GPR55 agonist, CID1792197 (1 μM), in the presence or in the absence of the CB₁R antagonist, SR141716 (250 nM), before MPP⁺ (1 mM) addition for 24 h. Cell damage is represented as the percentage of MTT reduction

versus control (represented by each cell type as 100%, i.e., the growth in the absence of MPP⁺ and of receptor ligand). Data are the mean ± SEM of five independent experiments. Significant differences were analyzed by a two-way ANOVA followed by post-hoc Tukey's test. *** $p < 0.001$ compared with their respective control (vehicle). # $p < 0.05$ compared with ACEA treatment

already lost. Therefore, targeting neurons to prevent further death appears as the most promising choice for disease-modifying approaches. In that sense, CB₁R have been considered as a relevant target and receptor ligands as potentially neuroprotective candidates. On the one hand, success has been partial and the underlying mechanisms not fully elucidated until now. On the other hand, CB₁R may be targeted by compounds acting in the orthosteric center (agonists, antagonists or inverse agonists) or by cannabidiol, which has been reported as an allosteric modulator of CB₁R function [43]. Further work is needed to properly ascertain whether CB₁R ligands are neuroprotective for dopaminergic neurons and whether the neuroprotection requires agonists, antagonists, inverse agonists or allosteric modulators [44–49].

Despite the fact that GPR55 has been by far less studied in CNS than the CB₁R, its activation leads to increased neurotransmitter release in CNS synapses [41], and a recent report shows that hippocampal GPR55 mediates an increase of neural stem cell proliferation and of adult neurogenesis [50]. A further aspect that may be relevant is the finding of GPR55 in mitochondria. There are few reports showing GPCRs located in the mitochondrial membrane but, interestingly, it was described that CB₁R in this location may regulate the energy status of neurons [33, 51]. We also found that AT₁ and AT₂ angiotensin receptors could be expressed in the mitochondria of dopaminergic neurons; the data suggested that altered expression of these receptors impacts on aging-related neurodegeneration [52]. At present, the exact mechanisms by which GPCRs located in mitochondria regulate the activity of this organelle are unknown.

As previously described, our results confirmed that MPP⁺ is toxic for SH-SY5Y cells [12]. Interestingly, the expression of CB₁R was enough to almost completely prevent the toxic effect exerted by a moderate concentration of the neurotoxin. At higher concentrations, the MPP⁺-induced cell death is very high and virtually unsurmountable. Also noteworthy was the finding that the expression of GPR55 was also protective against the insult exerted by MPP⁺. Coexpression of the two receptors led to the formation of CB₁/GPR55 heteroreceptor complexes (as shown in Fig. 5) as it was reported in a heterologous expression system and in rat striatum [29].

Our initial hypothesis considering a neuroprotection enhancement in the presence of agonists was not confirmed. This study was designed using another reportedly GRP55 agonist, CID1792197, but the results indicate that it may be a biased agonist that uncouples GPR55 from any neuroprotective mechanisms and/or that it is acting in unknown targets. Activation of GPR55 by CID1792197 did not lead to any significant modification in the protective effect against MPP⁺. Furthermore, some of the results were similar in cells other than those expressing GPR55. We do not favor the idea that our findings might be due to a constitutive activation of the receptor (a fact that to our

knowledge has not yet been reported), although a CID1792197-mediated production of a significant amount of the putative endogenous agonist of the receptor (L- α -lysophosphatidylinositol) cannot be completely ruled out. The results found when using a well-characterized synthetic CB₁R agonist, ACEA, are difficult to interpret. On the one hand, ACEA by itself decreases the growth rate of cells (e.g., in the absence of MPP⁺). As ACEA reverses neuroprotection in CB₁R-expressing cells, it may be producing off-target effects leading to a decrease in the growth of cells that shades the neuroprotective effect due to CB₁R. In addition, it has been reported that ACEA may act via GPR55 receptors and that other presumably cannabinoid receptors ligands, such as AM251, may act as antagonist of CB₁R but agonist of GPR55 [53]. The results obtained using a very selective CB₁R antagonist, SR141716, were interesting as this compound afforded neuroprotection against the MPP⁺ insult. This effect may be due either to the reversal of CB₁R constitutive activity that decreases growth as when ACEA is used, or to the blockade of the action of endocannabinoids having a detrimental role in this cellular MPP⁺ model. Although technically challenging, we think that it would be very relevant to know whether CB₁R or GPR55 display any constitutive activity in their own (both in SH-SY5Y cells and in primary neuronal cultures), as well as whether SH-SY5Y cells are able to produce endogenous agonists of CB₁R, GPR55, or of both receptors. Also relevant was the finding of negative cross-talk when the two receptors were coexpressed and forming heteromers. The pharmacology and signaling of GPR55 are still under scrutiny in the CNS, and our previous results [29] indicate that functional interactions between these two receptors are complex and may depend on the nature of the compounds and on their potential to skew signal transduction events (biased agonism). In what concerns the resistance to the effects of MPP⁺, a neurotoxic used to produce models of parkinsonism, our results show benefits due to individual receptors but not to receptor heteromers. In summary, neuroprotection against MPP⁺ is likely prevalent in neurons expressing one of the receptors. In neurons expressing the two receptors, disrupting the heteromer may be a likely intervention to afford neuroprotection.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interests.

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