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Neuroprotective Effect of JZL184 in MPP^+-Treated SH-SY5Y Cells Through CB_2 Receptors

María S. Aymerich [1,2,3] · Estefanía Rojo-Bustamante [1] · Carmen Molina [2] · Marta Celorrio [2] · Juan A. Sánchez-Arias [4] · Rafael Franco [2,5]

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Abstract Growing evidence suggests that the endocannabinoid system plays a role in neuroprotection in Parkinson's disease. Recently, we have shown the neuroprotective effect of monoacylglycerol lipase (MAGL) inhibition with JZL184 in the chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model. However, further investigation is needed to determine the neuroprotective mechanisms of the endocannabinoid system on the nigrostriatal pathway. The aim of this work was to investigate whether the neuroprotective effect of JZL184 in mice could be extended to an in vitro cellular model to further understand the mechanism of action of the drug. The SH-SY5Y cell line was selected based on its dopaminergic-like phenotype and its susceptibility to 1-methyl-4-phenylpyridinium iodide (MPP^+) toxicity. Furthermore, SH-SY5Y cells express both cannabinoid receptors, CB_1 and CB_2. The present study describes the neuroprotective effect of MAGL inhibition with JZL184 in SH-SY5Y cells treated with MPP^+. The effect of JZL184 in cell survival was blocked by AM630, a CB_2 receptor antagonist, and it was mimicked with JWH133, a CB_2 receptor agonist. Rimonabant, a CB_1 receptor antagonist, did not affect JZL184-induced cell survival. These results demonstrate that the neuroprotective effect of MAGL inhibition with JZL184 described in animal models of Parkinson's disease could be extended to in vitro models such as SH-SY5Y cells treated with MPP^+. This represents a useful tool to study mechanisms of neuroprotection mediated by MAGL inhibition, and we provide evidence for the possible involvement of CB_2 receptors in the improvement of cell survival.

Keywords Neuroprotection · JZL184 · Monoacylglycerol lipase · Endocannabinoid · Cannabinoid receptor · 2-AG

Abbreviations

2-AG	2-Arachidonoyl glycerol
AEA	Anandamide
CB_1	Cannabinoid type 1
CB_2	Cannabinoid type 2
ECS	Endocannabinoid system
FAAH	Fatty acid amide hydrolase
MAGL	Monoacylglycerol lipase
MPP^+	1-Methyl-4-phenylpyridinium iodide
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Introduction

The endocannabinoid system (ECS) regulates a broad range of physiological and pathological processes including anxiety, depression, neurogenesis, reward, motor control, cognition, learning, and memory [1] and constitutes an attractive therapeutic target for the treatment of different disorders. The principal ligands of the ECS are 2-arachidonoyl glycerol (2-AG) [2] and *N*-arachidonoyl-ethanolamine (anandamide, AEA) [3] which activate the two major cannabinoid receptors, type 1

✉ María S. Aymerich
maymerich@unav.es

[1] Department of Biochemistry and Genetics, School of Science, University of Navarra, Pamplona 31008, Spain

[2] Program of Neurosciences, Center for Applied Medical Research (CIMA), University of Navarra, Pío XII 55, 31008 Pamplona, Spain

[3] IdiSNA, Navarra Institute for Health Research, Pamplona, Spain

[4] Small Molecules Group, Center for Applied Medical Research (CIMA), University of Navarra, 31008 Pamplona, Spain

[5] Present address: Department of Biochemistry and Molecular Biology, University of Barcelona, 08028 Barcelona, Spain

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(CB_1) [4] and type 2 (CB_2) [5]. Endocannabinoid signaling pathways are terminated by enzymatic hydrolysis of 2-AG and AEA primarily by monoacylglycerol lipase (MAGL) [6] and fatty acid amide hydrolase (FAAH) [7], respectively. The endogenous endocannabinoid signaling and exogenous cannabinoids elicit diverse effects that could be expected when considering the widespread expression of CB_1 receptors in the brain [8]. To minimize the problems associated with CB_1 receptor agonists, inhibiting MAGL and FAAH activity to increase the endogenous levels of 2-AG and AEA has emerged as a potential strategy to exploit the ECS for medicinal purposes. Pharmacological inhibition of MAGL and FAAH was found to reduce pain, inflammation, anxiety, depression, and to be neuroprotective in rodent models without the undesirable effects in motility and behavior observed with direct CB_1 agonists [9–11]. CB_2 receptors are expressed primarily by immune cells, including microglia in the central nervous system [12]. There is evidence that they are involved in some neurological processes such as anxiety and addiction [13] and their expression has been recently described in the dopaminergic neurons from the human *substantia nigra pars compacta* [14].

Growing evidence suggests that the ECS plays a role in neuroprotection in Parkinson's disease. Δ^9-Tetrahydrocannabinol and cannabidiol protect nigrostriatal dopaminergic neurons in the 6-hydroxydopamine rat model [15], while WIN55, 212-2, a non-selective CB_1 and CB_2 receptor agonist, exerts neuroprotective effects in nigrostriatal neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model, ameliorating Parkinson's disease-like motor symptoms [16]. Recently, we have shown the neuroprotective effect of MAGL inhibition with JZL184 in the chronic MPTP mouse model [11].

Further investigation is needed to characterize the neuroprotective mechanisms of the endocannabinoid system on the nigrostriatal pathway. The aim of this work was to investigate whether JZL184 could have a neuroprotective effect by acting directly on neuron-like cells to further understand the mechanism of action of the drug. Based on their dopaminergic-like phenotype, their susceptibility to 1-methyl-4-phenylpyridinium iodide (MPP^+) toxicity [17] and the expression of all elements of the ECS system [18], SH-SY5Y cells were selected for this purpose. We found that JZL184 exerts a neuroprotective effect on SH-SY5Y cells treated with MPP^+. This effect is specific of MAGL inhibition, since blockade of FAAH does not affect cell survival, and may be mediated by CB_2 receptors, which are expressed at low levels in SH-SY5Y cells.

Materials and Methods

Materials

Human neuroblastoma SH-SY5Y CRL-2266 cells were obtained from ATCC (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin (P/S), non-essential amino acids, phosphate-buffered saline (PBS) pH 7.4, and Trypsin/EDTA solution were purchased from GIBCO (Grand Island, NY, USA); 1-methyl-4-phenylpyridinium iodide (MPP^+), dimethyl sulfoxide (DMSO), Trizol reagent, and triton X-100 from Sigma Aldrich (St. Louis, MO, USA); JZL184 and Rimonabant from Cayman Chemical (Ann Arbor, MI, USA); URB597 from Merck (Darmstadt, Germany); JWH133 and AM630 from Tocris Bioscience (Bristol, UK); PCR Master Mix solution from Promega Corporation (Fitchburg, WI, USA); iQ Syber Green Super mix from Bio-Rad Laboratories (Hercules, CA, USA); Cytotoxicity Detection kit assay from Roche Diagnostics (Indianapolis, IN, USA). Oxygen-sensing plates were from Oxoprobics Biosciences (Madrid, Spain).

Cell Culture and Treatments

SH-SY5Y cells were maintained at 37 °C in 5 % CO_2 in DMEM supplemented with 10 % fetal bovine serum, 10,000 U/ml penicillin and 10 mg/ml streptomycin, and 1× non-essential amino acids. Cells were subcultured after reaching 80 % confluence for no more than 20 passages. Experiments were performed using 1×10^4 cells/well into 96-well plates for cell viability assays and 6.25×10^5 cells/well into 6-well cultured plates for RNA extraction. After 24 h, the culture medium was replaced by fresh serum-free DMEM and the cells were cultured for another 24 h. In each experiment, the different compounds (JZL184, URB597, Rimonabant, AM630, or JWH133) were added 30 min after the MPP^+ treatment. If more than one compound was used, they were added simultaneously to the culture media. In the oxygen consumption experiment, MPP^+ and JZL184 were added at the same time.

Cell Viability Assays

Cell death was monitored by quantifying lactate dehydrogenase (LDH) release into the cell media with the Cytotoxicity Detection Kit. The level of LDH released from damaged cells was measured 24 h after cells were treated with different agents. Cell-free culture supernatants were collected from each well, diluted 1:2, and incubated with the appropriate reagent mixture during 30 min according to the supplier's instructions. The intensity of the color formed in the assay was measured at 490 nm in a Multiskan EX Microplate Reader (ThermoFisher Scientific, Waltham, MA, USA). LDH activity was calculated by subtraction of the control value (cells incubated in the absence of MPP^+). Data were normalized to the activity of LDH released from cells treated with 5 mM MPP^+ (100 %) and expressed as a percent of control mean±S.E.M. established from 4 to 5 independent experiments performed in triplicates.

Oxygen consumption was monitored in 96-well plates using a phosphorescent water-soluble oxygen probe. 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 [19]. 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 during 5 h.

RNA Isolation and PCR

Total RNA from SH-SY5Y cells was extracted using Trizol and DNAase I (Roche Applied Science, Penzberg, Germany), according to the manufacturer's instructions. Reverse transcription was performed using 2 μg of total RNA, M-MLV Reverse Transcriptase (Promega) and random oligodeoxyribonucleotides hexamers (Invitrogen). Expression of messenger RNA (mRNA) was assessed by conventional and real-time polymerase chain reaction (PCR and RT-PCR) and using the following specific primers: CB_1 receptor forward-TTTCGTTCTAGCGG ACAACC, CB_1 receptor reverse-TGACTGAGAAAGTGAC CCACA, CB_2 receptor forward-TCATGGGATGGACTTGCT GT, CB_2 receptor reverse-CGGAAAAGAGGAAGGCGATG, MAGL forward-AGCGTGCTCTCTCGGAATAA, MAGL reverse-GCCCCTTTGCTGTCACATAG, FAAH forward-CTGG TTCCCTTCTTGCCAAG, FAAH reverse-CAGCCGAACG AGACTTCATG, GAPDH forward-TGAGAACGGGAAGC TTGTCA, GAPDH reverse-ATCGCCCCACTTGATTTTGG. Conventional PCR was performed in a 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA) and RT-PCR in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The RT-PCR values were normalized with the expression of GAPDH.

Statistical Analysis

Quantitative data were assessed by one-way ANOVA followed by Tukey's post hoc test using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). The criterion for statistical significance was $p<0.05$. All values are expressed as means±S.E.M.

Results

Neuroprotective Effect of MAGL Inhibition with JZL184 in MPP^+-Induced Neurotoxicity

To evaluate the neuroprotective effect of JZL184 in cells treated with MPP^+, we first determined the right concentration of MPP^+ for neuroprotection studies. A broad range, between 0.1 and 10 mM, of MPP^+ dosage has been used to induce cytotoxicity in SH-SY5Y cells [20, 21]. Thus, a concentration response curve for MPP^+ was performed, and cell death was estimated with the LDH assay (Fig. 1). MPP^+ increased LDH activity in a concentration-dependent manner. Doses above 2 mM were sufficient to induce a significant release of LDH to the culture medium. Based on these observations, 5 mM MPP^+, which induced a cell death in the 20–40 % range, was considered the optimal dose to test neuroprotection.

Next, we evaluated the effect of MAGL inhibition in cell survival with JZL184. JZL184 is a potent and selective inhibitor of MAGL that displays an IC_{50} value of 8 nM in murine brain membranes, and it is 300-fold more selective for MAGL than for FAAH [22]. First, the expression of MAGL was confirmed by PCR in the SH-SY5Y cells (Fig. 2a) and then the effect of JZL184 on MPP^+-treated SH-SY5Y cells was tested. JZL184 significantly reduced (35 %) the release of LDH to the culture medium at doses of 100 nM or above, indicating a decrease in MPP^+-induced cell damage (Fig. 2b). A lack of toxicity was detected by the administration of JZL184 to the cells (Fig. 2c). This effect was confirmed by measuring oxygen consumption rates in the SH-SY5Y cells (Fig. 2d). The observed decrease in cellular respiration caused by MPP^+ was reversed by 100 nM JZL184 (Fig. 2e). The effect of FAAH inhibition on neuroprotection was also studied. Expression of FAAH in SH-SY5Y cells was checked by PCR (Fig. 3a). SH-SY5Y cells were incubated with MPP^+ and increasing doses of URB597, an irreversible inhibitor with an IC_{50} value of 4.6 nM for inhibition of FAAH in murine membranes [23]. URB597 added to the cells together with 5 mM MPP^+ during 24 h did not modify the cell death (Fig. 3b) and did not show cytotoxicity (Fig. 3c). These results suggest that neuroprotection after MPP^+ damage is achieved by the specific inhibition of MAGL.

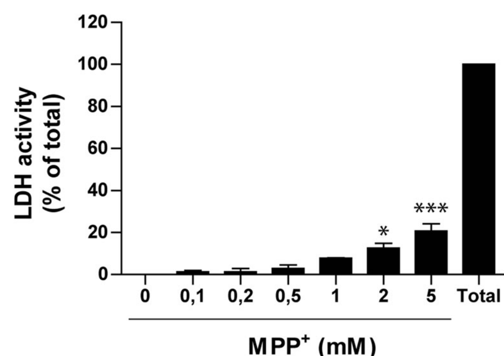

Fig. 1 Concentration response curve for MPP^+. LDH activity in culture medium from SH-SY5Y cells incubated with increasing concentrations of MPP^+ during 24 h. Increase in LDH release to the medium was calculated by subtraction of the control (cells incubated in the absence of MPP^+). Total LDH activity was determined after lysis of cells in 1 % Triton X-100. Values are represented as % of total LDH content. Values are mean +/− S.E.M. from three independent experiments. Significance versus control is indicated by *$p<0.05$; *** $p<0.001$

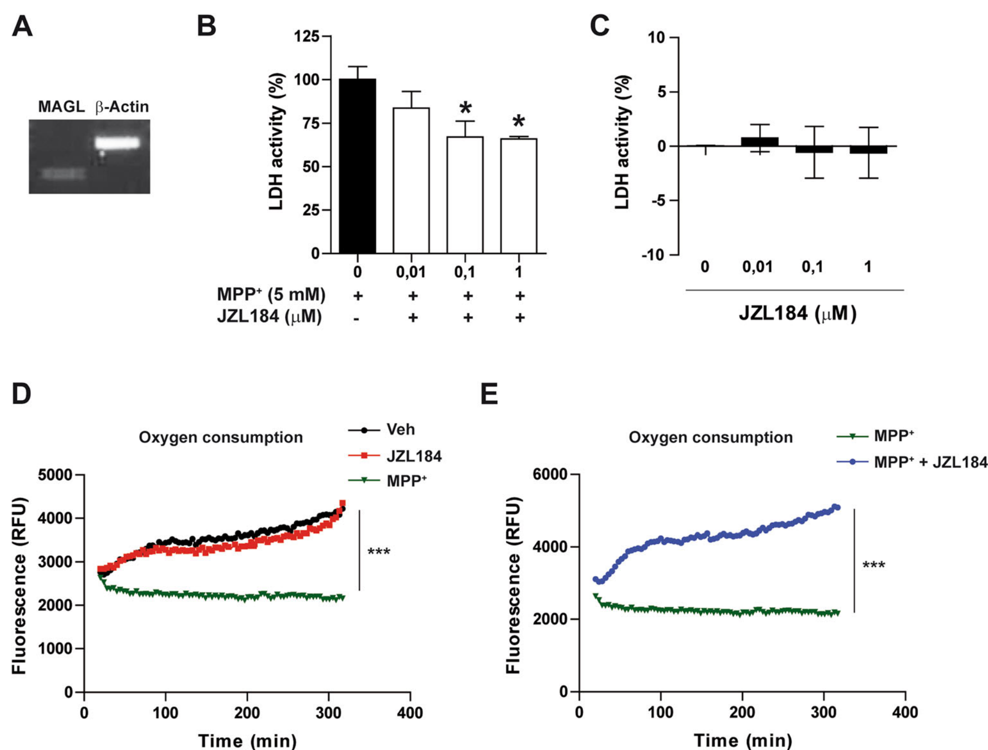

Fig. 2 Evaluation of the neuroprotective effect of MAGL inhibition in MPP^+-induced neurotoxicity in SH-SY5Y cells. **a** PCR for MAGL mRNA expression in SH-SY5Y cells. **b** SH-SY5Y cells were incubated during 24 h with 5 mM MPP^+ and increasing concentrations of JZL184, a potent and specific MAGL inhibitor. The amount of LDH released by the treatment with 5 mM MPP^+ was considered 100 %, and the results were normalized against this value. Values are mean +/− S.E.M. from four independent experiments. Significance versus MPP^+-induced cell death is indicated by $*p<0.05$. **c** LDH activity in culture medium from SH-SY5Y cells incubated with increasing concentrations of JZL184 during 24 h. Changes in LDH release to the medium were calculated by subtraction of the control value obtained from cells incubated in the absence of JZL184. No significant changes were detected under these conditions. **d** Oxygen consumption was monitored in real-time using 96-well plate oxygen-sensing plates during 5 h. Cells were incubated with MPP^+ and without MPP^+ in the presence and absence of JZL184 (100 nM). Data are shown as relative fluorescence units (RFU). **e** Oxygen consumption was monitored in the presence of MPP^+ with or without JZL184. Significance is indicated by $***p<0.001$

Involvement of CB_2 Receptors in the Neuroprotective Effect of JZL184

2-AG is an endogenous ligand for CB_1 and CB_2 receptors [2]. We checked and confirmed the expression of CB_1 and CB_2 receptors in SH-SY5Y cells by real-time PCR under the different experimental conditions. JZL184 did not induce changes in CB_1 or CB_2 receptors mRNA expression in control cells (Fig. 4a, b). Compared with the housekeeping gene, CB_1 receptor mRNA expression was higher in cells treated for 24 h

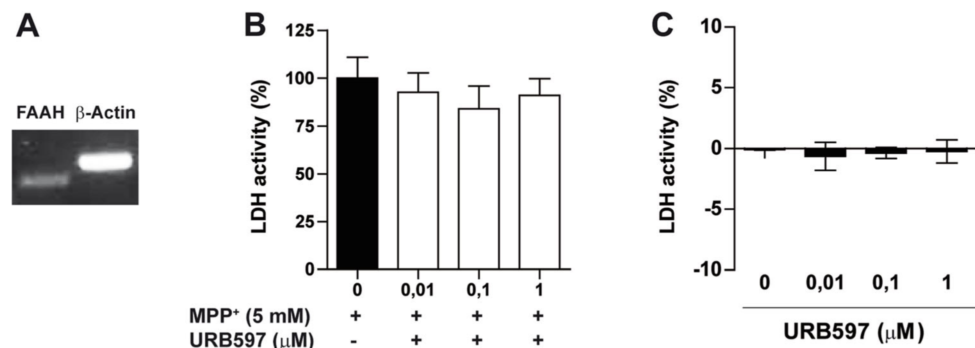

Fig. 3 Evaluation of the effect of FAAH inhibition in MPP^+-induced neurotoxicity in SH-SY5Y cells. **a** PCR for FAAH mRNA expression in SH-SY5Y cells. **b** SH-SY5Y cells were incubated during 24 h with 5 mM MPP^+ and increasing concentrations of URB597, a potent and specific FAAH inhibitor. The amount of LDH released by the treatment with 5 mM MPP^+ was considered 100 %, and the results were normalized against this value. No significant differences were detected between cells treated with MPP^+ alone or with URB597. Values are mean +/− S.E.M. from triplicates in five independent experiments. **c** LDH activity in culture medium from SH-SY5Y cells incubated with increasing concentrations of URB597 during 24 h. Changes in LDH release to the medium were calculated by subtraction of the control value obtained from cells incubated in the absence of URB597. No significant changes were detected under these conditions

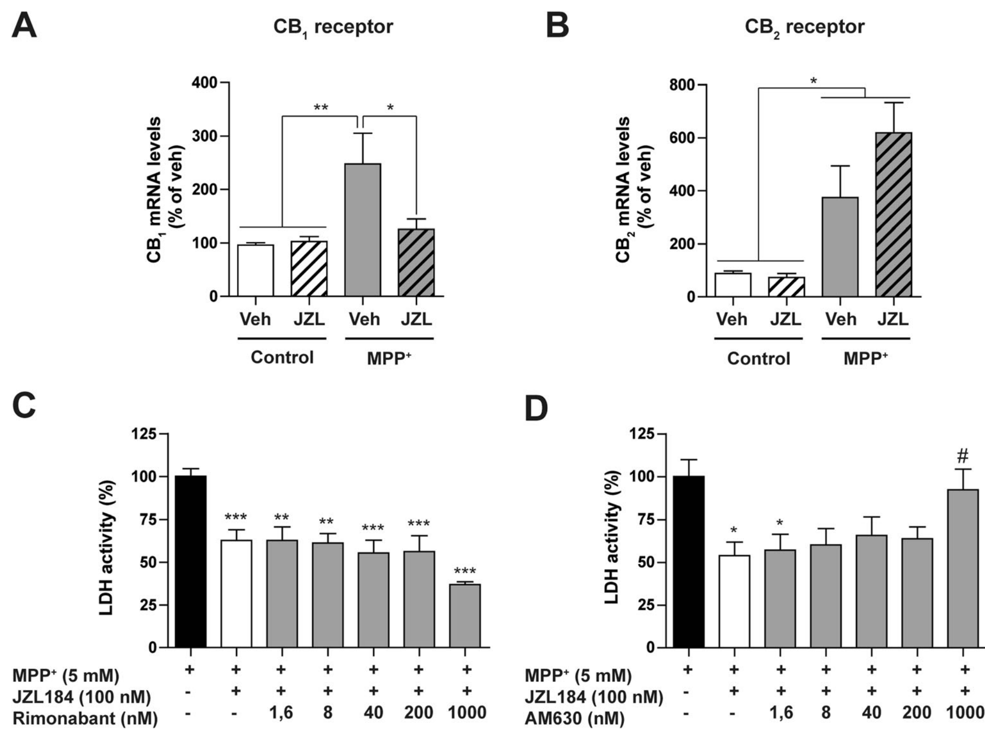

Fig. 4 Effect of CB_1 and CB_2 receptors inverse agonists in the neuroprotection-induced by JZL184. **a** Real-time PCR to detect the expression of CB_1 receptor mRNA transcripts under the different experimental conditions. SH-SY5Y cells were incubated with or without MPP^+ (5 mM), in the absence or presence of JZL184 (100 nM) during 24 h. **b** Real-time PCR to detect the expression of CB_2 receptor mRNA transcripts in SH-SY5Y cells with or without MPP^+, incubated with or without JZL184 during 24 h. Values are mean +/− S.E.M. from two independent experiments. Significance is indicated by $*p<0.05$; $**p<0.01$. **c** SH-SY5Y cells were incubated during 24 h with 5 mM MPP^+, 100 nM JZL184 and increasing concentrations of rimonabant, a CB_1 receptor inverse agonist. **d** SH-SY5Y cells were incubated during 24 h with 5 mM MPP^+, 100 nM JZL184 and increasing concentrations of AM630, a CB_2 receptor inverse agonist. The activity of LDH released to the culture medium was assayed. Data were normalized against the amount of LDH released in the presence of 5 mM MPP^+ (100 %). Values are mean +/− S.E.M. from four independent experiments. Significance versus MPP^+-induced cell death is indicated by $*p<0.05$; $**p<0.01$; $***p<0.001$. Significance versus MPP^+ and JZL184 treated cells is indicated by $\#p<0.05$

with 5 mM MPP^+ but returned to a similar level of expression by the administration of JZL184 (Fig. 4a). MPP^+ also increased significantly CB_2 mRNA levels (respect to those of the housekeeping gene) and continued high in cells treated with MPP^+ and JZL184 (Fig. 4b). Antagonists of CB_1 and CB_2 receptors were used to study the involvement of these receptors in the improvement in cell viability induced by JZL184 administration. SH-SY5Y cells were incubated with 5 mM MPP^+, 100 nM JZL184, and increasing concentrations of rimonabant ranging from 1.6 to 1000 nM (Fig. 4c). Blockade of CB_1 receptors with rimonabant did not have any significant effect on the improved cell viability induced by JZL184 treatment. However, the incubation with AM630, a selective CB_2 receptor antagonist, fully prevented the JZL184 neuroprotective effect at a dose of 1 μM (Fig. 4d). To confirm the involvement of CB_2 receptors in neuroprotection, the SH-SY5Y cells were incubated with JWH133, a selective CB_2 receptor agonist with a Ki of 3.4 nM and 200-fold selectivity over CB_1 receptors. Increasing concentrations of JWH133 did not have a cytotoxic effect on SH-SY5Y cells (Fig. 5a). Addition of JWH133 to SH-SY5Y cells treated with MPP^+ (5 mM) resulted in a significant decrease in LDH activity at 1.6 and 8 nM concentrations, indicating a neuroprotective effect of the CB_2 receptor agonist (Fig. 5b). This effect was not observed with higher doses of JWH133, probably due to other non-specific interactions of the drug. Addition of 200 nM AM630 to SH-SY5Y cells incubated with 5 mM MPP^+ and 8 nM JWH133 reversed the neuroprotective effect of JWH133 (Fig. 5c). Altogether, these results indicate that CB_2 receptors are involved in the neuroprotective effect of JZL184 in MPP^+-treated SH-SY5Y cells.

Discussion

The present study describes the neuroprotective effect of the MAGL inhibitor, JZL184, in human neuroblastoma SH-SY5Y cells incubated with the neurotoxin MPP^+. We demonstrate that CB_2 receptors are involved in the mechanism of neuroprotection of JZL184, as the use of JWH133 reproduces such effect and a CB_2 receptor antagonist at relatively high concentrations reverses it.

Recently, we have demonstrated the neuroprotective effect of MAGL inhibition with JZL184 in the chronic MPTP mouse

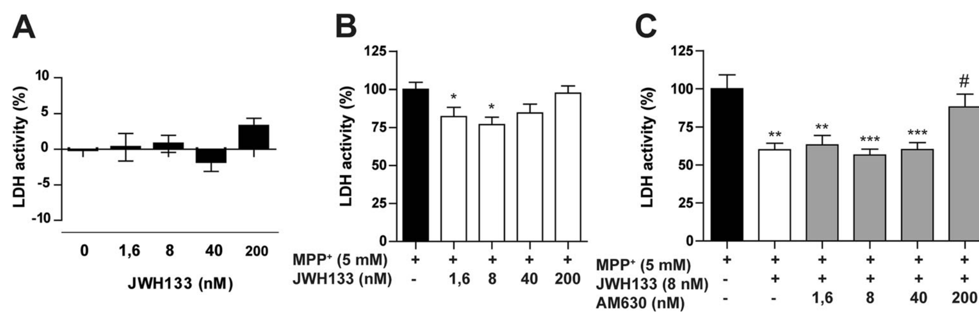

Fig. 5 Evaluation of the effect of a CB_2 receptor agonist in MPP^+-induced neurotoxicity in SH-SY5Y cells. **a** Effect of the administration of increasing concentrations of JWH133, a CB_2 receptor agonist, in the viability of SH-SY5Y cells. No significant changes were detected at the different concentrations of JWH133 assayed. Values are mean +/− S.E.M. from three independent experiments. **b** SH-SY5Y cells were incubated during 24 h with 5 mM MPP^+ and increasing concentrations of JWH133. Values are mean +/− S.E.M. from triplicates in five independent experiments. Data were normalized against the amount of LDH released in the presence of 5 mM MPP^+ (100 %). **c** SH-SY5Y cells were incubated during 24 h with 5 mM MPP^+, 8 nM JWH133 and increasing concentrations of AM630. Values are mean +/− S.E.M. from four independent experiments. Significance versus MPP^+-induced cell death is indicated by **p*<0.05; ***p*<0.01; ****p*<0.001. Significance versus MPP^+ and JZL184 treated cells is indicated by #*p*<0.05yy

model of Parkinson's disease [11]. For a better understanding of the neuroprotective effect of JZL184, an in vitro assay based on SH-SY5Y was set up. These human neuroblastoma cells were chosen for this study because they have been widely used to investigate neuronal cell damage and death and they serve as an in vitro model for Parkinson's disease [17]. They produce dopamine [24], express dopamine receptors [25], and are able to introduce dopamine and MPP^+ through the norepinephrine transporter [26]. Furthermore, a fully functional endocannabinoid system is present in this cell line that markedly expresses enzymes involved in endocannabinoid production and more modest amounts of CB_1 and CB_2 receptors [18].

Using this cell line, we were able to detect the neuroprotective effect of JZL184 against MPP^+-induced neurotoxicity. Although there are several reports in the literature inducing neurotoxicity in SH-SY5Y cells with MPP^+, the doses used are very different [20, 21]. Based on dose–response curves, a 5 mM concentration was considered the optimal because it induced cell damage between 20 and 40 %, which is in agreement with previous studies carried out in SH-SY5Y [17]. JZL184 treatment improved SH-SY5Y cells viability indicating that the effect of the drug could be extended also to in vitro models. The concentration used to obtain a biological effect is larger (100 nM) than the IC_{50} reported for JZL184 (8 nM) using murine brain membranes [22], probably due to the human origin of SH-SY5Y cells and the use of an in vivo assay. The absence of changes in cell viability when cells are incubated with URB597 suggests that MAGL, but not FAAH inhibition, is necessary for neuroprotection. There are several reports in the literature that demonstrate the specificity of MAGL inhibition with JZL184 in vivo and in vitro [22, 11, 27]; it would be interesting to determine whether there is a correlation between the 2-AG levels and the extent of neuroprotection.

The neuroprotective effect of JZL184 has been demonstrated in animal models of Parkinson's disease [28, 11] and in methamphetamine-induced toxicity in mice [29] both affecting the dopaminergic system. However, no neuroprotective effect has been reported in an animal model of malonate-induced neurotoxicity and in M-213 cells, which have a phenotype similar to striatal neurons [30, 31]. This could be due to a specific neuroprotective effect of MAGL inhibition for the dopaminergic system or, in the case of the cell cultures, to the low concentration of JZL184 used (10 nM).

There is controversy regarding the expression of CB_2 receptors in SH-SY5Y cells [32, 18]. We addressed receptor expression in our SH-SY5Y cells by real-time PCR and surely, the CB_2 receptors are present although at modest levels, thus agreeing with data reported by Pasquariello et al. [18]. There are several reports that describe increased CB_2 receptor expression in the brain under pathological conditions, mainly in glial cells [16, 33, 34]. Although the expression of CB_2 receptors in neurons has been neglected for a long time, there is growing evidence of their presence in neurons and their modulation under different conditions, such as neuronal damage or drug abuse [35, 36]. Recently, the presence of CB_2 receptors in dopaminergic neurons from the human *substantia nigra pars compacta* has been described [14]. Using inverse agonists of cannabinoid receptors, we determined the involvement of CB_2 receptors in the neuroprotective effect of JZL184. Addition of 1 μM AM630 (K_i=31.2 nM) prevented the neuroprotection-induced by JZL184. The micro molar dose of AM630 necessary to reverse the effect of JZL184 could be related to the potency of the CB_2 receptor antagonist to block a signaling pathway involved in cell survival, although a coexistence of CB_2 receptor-dependent-independent mechanisms cannot be ruled out. Selective agonists and antagonists for a particular receptor induce unique, ligand-specific receptor conformation that frequently results in differential activation of signaling pathways. This differential activation may result in changes in intrinsic activity and/or potency at one signaling pathway versus another, but not in

the affinity for the receptor [37]. The confirmation of the results with the addition of JWH133 at a concentration of 8 nM, which is close to its K_i value (3.4 nM), further supports the involvement of CB_2 receptors in this neuroprotective effect.

Our results also show that the highest dose of rimonabant has a tendency to exacerbate the neuroprotective effect of JZL184. This could be due to the effect of rimonabant in mitochondrial CB_1 receptors that regulate energy metabolism in neurons [38]. CB_2 receptors are involved in neuroprotection under different pathological conditions [39–42, 29] including an animal model of Parkinson's disease [16]. In general, the protective role of CB_2 receptor activation is mostly attributed to its ability to reduce deleterious microglial activation [43, 44, 39, 40], although CB_2-mediated regulation of astroglial phenotype could also support the neuroprotective effect [45]. The presence of CB_2 receptors in dopaminergic neurons [14] in human brain together with the results of this study suggests a direct effect of JZL184 in dopaminergic cells in the absence of glia.

Both 2-AG and AEA are ligands of CB_2 receptors. However, AEA is much less effective at activating this receptor and can functionally antagonize the stimulatory effects of 2-AG at CB_2 receptors [46]. Here, we describe a distinct neuroprotective effect between MAGL and FAAH inhibition that could be mediated by the distinct type of interaction between 2-AG and AEA with CB_2 receptors. These results emphasize the different physiological role played by the two endocannabinoids in vivo.

Altogether, the present study demonstrates that the neuroprotective effect of MAGL inhibition by JZL184 described in animal models of Parkinson's disease can be extended to in vitro models such as SH-SY5Y cells treated with MPP^+. This tool allowed us to study the mechanisms of neuroprotection mediated by MAGL inhibition. We provide evidence for the direct effect of JZL184 in dopaminergic-like cells and for the involvement of CB_2 receptors in the improvement of cell survival.

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Conflict of Interest JA Sánchez-Arias is a co-founder of Oxoprobics Bioscience.

References

1. Mechoulam R, Parker LA (2013) The endocannabinoid system and the brain. Annu Rev Psychol 64:21–47. doi:10.1146/annurev-psych-113011-143739
2. Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S et al (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochem Pharmacol 50(1):83–90
3. Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A et al (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 258(5090):1946–1949
4. Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346(6284):561–564. doi:10.1038/346561a0
5. Munro S, Thomas KL, Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. Nature 365(6441):61–65. doi:10.1038/365061a0
6. Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, Kathuria S, Piomelli D (2002) Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci U S A 99(16):10819–10824. doi:10.1073/pnas.152334899
7. Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB (1996) Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. Nature 384(6604):83–87. doi:10.1038/384083a0
8. Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, Rice KC (1990) Cannabinoid receptor localization in brain. Proc Natl Acad Sci U S A 87(5):1932–1936
9. Ahn K, Johnson DS, Mileni M, Beidler D, Long JZ, McKinney MK, Weerapana E, Sadagopan N et al (2009) Discovery and characterization of a highly selective FAAH inhibitor that reduces inflammatory pain. Chem Biol 16(4):411–420. doi:10.1016/j.chembiol.2009.02.013
10. Petrosino S, Di Marzo V (2010) FAAH and MAGL inhibitors: therapeutic opportunities from regulating endocannabinoid levels. Curr Opin Investig Drugs 11(1):51–62
11. Fernandez-Suarez D, Celorrio M, Riezu-Boj JI, Ugarte A, Pacheco R, Gonzalez H, Oyarzabal J, Hillard CJ et al (2014) The monoacylglycerol lipase inhibitor JZL184 is neuroprotective and alters glial cell phenotype in the chronic MPTP mouse model. Neurobiol Aging. doi:10.1016/j.neurobiolaging.2014.05.021
12. Cabral GA, Raborn ES, Griffin L, Dennis J, Marciano-Cabral F (2008) CB2 receptors in the brain: role in central immune function. Br J Pharmacol 153(2):240–251. doi:10.1038/sj.bjp.0707584
13. Onaivi ES (2006) Neuropsychobiological evidence for the functional presence and expression of cannabinoid CB2 receptors in the brain. Neuropsychobiology 54(4):231–246. doi:10.1159/000100778
14. Garcia MC, Cinquina V, Palomo-Garo C, Rabano A, Fernandez-Ruiz J (2015) Identification of CB(2) receptors in human nigral neurons that degenerate in Parkinson's disease. Neurosci Lett 587:1–4. doi:10.1016/j.neulet.2014.12.003
15. Lastres-Becker I, Molina-Holgado F, Ramos JA, Mechoulam R, Fernandez-Ruiz J (2005) Cannabinoids provide neuroprotection against 6-hydroxydopamine toxicity in vivo and in vitro: relevance to Parkinson's disease. Neurobiol Dis 19(1–2):96–107. doi:10.1016/j.nbd.2004.11.009
16. Price DA, Martinez AA, Seillier A, Koek W, Acosta Y, Fernandez E, Strong R, Lutz B et al (2009) WIN55,212-2, a cannabinoid receptor agonist, protects against nigrostriatal cell loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. Eur J Neurosci 29(11):2177–2186. doi:10.1111/j.1460-9568.2009.06764.x
17. Fall CP, Bennett JP Jr (1999) Characterization and time course of MPP^+-induced apoptosis in human SH-SY5Y neuroblastoma cells. J Neurosci Res 55(5):620–628. doi:10.1002/(SICI)1097-4547(19990301)55:5<620::AID-JNR9>3.0.CO;2-S
18. Pasquariello N, Catanzaro G, Marzano V, Amadio D, Barcaroli D, Oddi S, Federici G, Urbani A et al (2009) Characterization of the endocannabinoid system in human neuronal cells and proteomic analysis of anandamide-induced apoptosis. J Biol Chem 284(43):29413–29426. doi:10.1074/jbc.M109.044412

19. Hynes J, Floyd S, Soini AE, O'Connor R, Papkovsky DB (2003) Fluorescence-based cell viability screening assays using water-soluble oxygen probes. J Biomol Screen 8(3):264–272. doi:10.1177/1087057103008003004
20. Yang MC, Chen KP, Lung FW (2014) Generalized estimating equation model and long-term exposure effect of antipsychotics on SH-SY5Y cells against oxidative stressors. Eur J Pharmacol. doi:10.1016/j.ejphar.2014.06.007
21. De Simoni S, Linard D, Hermans E, Knoops B, Goemaere J (2013) Mitochondrial peroxiredoxin-5 as potential modulator of mitochondria-ER crosstalk in MPP^+-induced cell death. J Neurochem 125(3):473–485. doi:10.1111/jnc.12117
22. Long JZ, Li W, Booker L, Burston JJ, Kinsey SG, Schlosburg JE, Pavon FJ, Serrano AM et al (2009) Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. Nat Chem Biol 5(1):37–44. doi:10.1038/nchembio.129
23. Mor M, Rivara S, Lodola A, Plazzi PV, Tarzia G, Duranti A, Tontini A, Piersanti G et al (2004) Cyclohexylcarbamic acid 3′- or 4′-substituted biphenyl-3-yl esters as fatty acid amide hydrolase inhibitors: synthesis, quantitative structure-activity relationships, and molecular modeling studies. J Med Chem 47(21):4998–5008. doi:10.1021/jm031140x
24. Biedler JL, Roffler-Tarlov S, Schachner M, Freedman LS (1978) Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. Cancer Res 38(11 Pt 1):3751–3757
25. Farooqui SM (1994) Induction of adenylate cyclase sensitive dopamine D2-receptors in retinoic acid induced differentiated human neuroblastoma SHSY-5Y cells. Life Sci 55(24):1887–1893
26. Buck KJ, Amara SG (1994) Chimeric dopamine-norepinephrine transporters delineate structural domains influencing selectivity for catecholamines and 1-methyl-4-phenylpyridinium. Proc Natl Acad Sci U S A 91(26):12584–12588
27. Kallendrusch S, Hobusch C, Ehrlich A, Nowicki M, Ziebell S, Bechmann I, Geisslinger G, Koch M et al (2012) Intrinsic up-regulation of 2-AG favors an area specific neuronal survival in different in vitro models of neuronal damage. PLoS ONE 7(12):e51208. doi:10.1371/journal.pone.0051208
28. Nomura DK, Morrison BE, Blankman JL, Long JZ, Kinsey SG, Marcondes MC, Ward AM, Hahn YK et al (2011) Endocannabinoid hydrolysis generates brain prostaglandins that promote neuroinflammation. Science 334(6057):809–813. doi:10.1126/science.1209200
29. Nader J, Rapino C, Gennequin B, Chavant F, Francheteau M, Makriyannis A, Duranti A, Maccarrone M et al (2014) Prior stimulation of the endocannabinoid system prevents methamphetamine-induced dopaminergic neurotoxicity in the striatum through activation of CB receptors. Neuropharmacology. doi:10.1016/j.neuropharm.2014.03.014
30. Giordano M, Takashima H, Herranz A, Poltorak M, Geller HM, Marone M, Freed WJ (1993) Immortalized GABAergic cell lines derived from rat striatum using a temperature-sensitive allele of the SV40 large T antigen. Exp Neurol 124(2):395–400
31. Valdeolivas S, Pazos MR, Bisogno T, Piscitelli F, Iannotti FA, Allara M, Sagredo O, Di Marzo V et al (2013) The inhibition of 2-arachidonoyl-glycerol (2-AG) biosynthesis, rather than enhancing striatal damage, protects striatal neurons from malonate-induced death: a potential role of cyclooxygenase-2-dependent metabolism of 2-AG. Cell Death Dis 4:e862. doi:10.1038/cddis.2013.387
32. Klegeris A, Bissonnette CJ, McGeer PL (2003) Reduction of human monocytic cell neurotoxicity and cytokine secretion by ligands of the cannabinoid-type CB2 receptor. Br J Pharmacol 139(4):775–786. doi:10.1038/sj.bjp.0705304
33. Garcia C, Palomo-Garo C, Garcia-Arencibia M, Ramos J, Pertwee R, Fernandez-Ruiz J (2011) Symptom-relieving and neuroprotective effects of the phytocannabinoid Delta(9)-THCV in animal models of Parkinson's disease. Br J Pharmacol 163(7):1495–1506. doi:10.1111/j.1476-5381.2011.01278.x
34. Gomez-Galvez Y, Palomo-Garo C, Fernandez-Ruiz J, Garcia C (2015) Potential of the cannabinoid CB receptor as a pharmacological target against inflammation in Parkinson's disease. Prog Neuropsychopharmacol Biol Psychiatry. doi:10.1016/j.pnpbp.2015.03.017
35. Wotherspoon G, Fox A, McIntyre P, Colley S, Bevan S, Winter J (2005) Peripheral nerve injury induces cannabinoid receptor 2 protein expression in rat sensory neurons. Neuroscience 135(1):235–245. doi:10.1016/j.neuroscience.2005.06.009
36. Onaivi ES, Ishiguro H, Gong JP, Patel S, Meozzi PA, Myers L, Perchuk A, Mora Z et al (2008) Brain neuronal CB2 cannabinoid receptors in drug abuse and depression: from mice to human subjects. PLoS ONE 3(2):e1640. doi:10.1371/journal.pone.0001640
37. Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL et al (2007) Functional selectivity and classical concepts of quantitative pharmacology. J Pharmacol Exp Ther 320(1):1–13. doi:10.1124/jpet.106.104463
38. Benard G, Massa F, Puente N, Lourenco J, Bellocchio L, Soria-Gomez E, Matias I, Delamarre A et al (2012) Mitochondrial CB(1) receptors regulate neuronal energy metabolism. Nat Neurosci 15(4):558–564. doi:10.1038/nn.3053
39. Palazuelos J, Davoust N, Julien B, Hatterer E, Aguado T, Mechoulam R, Benito C, Romero J et al (2008) The CB(2) cannabinoid receptor controls myeloid progenitor trafficking: involvement in the pathogenesis of an animal model of multiple sclerosis. J Biol Chem 283(19):13320–13329. doi:10.1074/jbc.M707960200
40. Palazuelos J, Aguado T, Pazos MR, Julien B, Carrasco C, Resel E, Sagredo O, Benito C et al (2009) Microglial CB2 cannabinoid receptors are neuroprotective in Huntington's disease excitotoxicity. Brain 132(Pt 11):3152–3164. doi:10.1093/brain/awp239
41. Castillo A, Tolon MR, Fernandez-Ruiz J, Romero J, Martinez-Orgado J (2010) The neuroprotective effect of cannabidiol in an in vitro model of newborn hypoxic-ischemic brain damage in mice is mediated by CB(2) and adenosine receptors. Neurobiol Dis 37(2):434–440. doi:10.1016/j.nbd.2009.10.023
42. Murikinati S, Juttler E, Keinert T, Ridder DA, Muhammad S, Waibler Z, Ledent C, Zimmer A et al (2010) Activation of cannabinoid 2 receptors protects against cerebral ischemia by inhibiting neutrophil recruitment. FASEB J 24(3):788–798. doi:10.1096/fj.09-141275
43. Ramirez BG, Blazquez C, Gomez del Pulgar T, Guzman M, de Ceballos ML (2005) Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. J Neurosci 25(8):1904–1913. doi:10.1523/JNEUROSCI.4540-04.2005
44. Maresz K, Carrier EJ, Ponomarev ED, Hillard CJ, Dittel BN (2005) Modulation of the cannabinoid CB2 receptor in microglial cells in response to inflammatory stimuli. J Neurochem 95(2):437–445. doi:10.1111/j.1471-4159.2005.03380.x
45. Garcia-Ovejero D, Arevalo-Martin A, Petrosino S, Docagne F, Hagen C, Bisogno T, Watanabe M, Guaza C et al (2009) The endocannabinoid system is modulated in response to spinal cord injury in rats. Neurobiol Dis 33(1):57–71. doi:10.1016/j.nbd.2008.09.015
46. Gonsiorek W, Lunn C, Fan X, Narula S, Lundell D, Hipkin RW (2000) Endocannabinoid 2-arachidonyl glycerol is a full agonist through human type 2 cannabinoid receptor: antagonism by anandamide. Mol Pharmacol 57(5):1045–1050