UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA

TESIS DOCTORAL

CB2, PPAR-γ and GPR55 as pharmacological targets for an anti-inflammatory and neuroprotective treatment of Parkinson's disease

CB2, PPAR-γ y GPR55 como dianas farmacológicas para un tratamiento anti-inflamatorio y neuroprotector en la enfermedad de Parkinson

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Sonia Burgaz García-Oteyza

Director

José Javier Fernández Ruiz

Madrid

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PROGRAMA DE DOCTORADO

Bioquímica, Biología Molecular y Biomedicina

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SUMMARY/RESUMEN

CB2, PPAR-γ and GPR55 as pharmacological targets for an anti-inflammatory and neuroprotective treatment of Parkinson's disease

Parkinson's disease (PD) is a chronic neurodegenerative disorder which courses with hypokinetic symptoms due to the selective death of the dopaminergic neurons of the substantia nigra pars compacta (SNpc) and the consequent dopaminergic denervation of the striatum. This disease is also a proteinopathy, as one of its features is the presence of aggregates of misfolded proteins, mainly α-synuclein, known as Lewy bodies (LB). The etiology of PD remains unknown, but several environmental and genetic risk factors have been described, together with aging as the main one. The main symptoms of PD are bradykinesia, rigidity, and resting tremor, usually determinant for the diagnosis, which appear when more than 50% of the dopaminergic neurons are already dead. However, there are non-motor symptoms such as sleep disturbances, mood symptoms, gastrointestinal problems, and olfactory dysfunction, which may be prodromal and could be useful for an early diagnosis. The pathological events that contribute to the neuronal death include not only protein aggregation, but also neuroinflammation, mitochondrial dysfunction, oxidative stress, and excitotoxicity. These mechanisms are known to be interconnected, worsening the dopaminergic neurodegeneration which disturbs the basal ganglia circuitry, preventing a correct motor functioning. The most frequent treatment is the replacement of dopamine levels, mainly with levodopa (L-DOPA), which recovers the mobility of the patients in the short term but causes irreversible dyskinesias after several years. This makes urgent the need of a disease-modifying treatment, and given the multifactorial pathophysiology of PD, we propose the use of pleiotropic molecules which can be effective at several levels, such as cannabinoids.

The endocannabinoid system (ECS) is one of the most abundant modulators of the nervous system, with important functions in neurotransmission modulation and interaction with other signalling systems. Among the effects of cannabinoids, the ones with more therapeutic interest are those addressed to preserve, rescue, repair, and/or replace neurons and glial cells against multiple insults that may potentially damage them, which seems specially promising for chronic neurodegenerative pathological conditions, such as PD. For instance, different cannabinoids acting on different cannabinoid targets have demonstrated to have neuroprotective properties that could slow the disease progression, unlike any of the treatments which are being used at the moment, in several preclinical models of PD. However, there are still new targets, new compounds, and new combinations of them that could produce advantages over the current evidence, and that need to be further investigated. Likewise, there are also new PD models that could contribute to a better insight into PD pathology and its pharmacological management.

Based on this background, the global aim of this Doctoral Thesis is to determine the efficacy of the different cannabinoid compounds, combining their antioxidant, anti-inflammatory and neuroprotective properties in *in vitro* an *in vivo* models of PD that represent the main neuropathological events (oxidative stress, inflammation, aggregation of α-synuclein and neuronal death). According to the receptor targeted by these treatments, this global aim can be divided in three specific aims, which will be represented as three different chapters:

Chapter 1. Preclinical evaluation of compounds targeting PPAR-γ: VCE-003.2 and other CBG derivatives

Given the efficacy shown by an intraperitoneal treatment with the CBG derivative VCE-003.2 in experimental models of PD (previous results to this Thesis), we have characterised an oral formulation of this derivative. We first studied the effects of the oral treatment with VCE-003.2 in the LPS model and in the 6-OHDA model, in which this compound had a neuroprotective and anti-inflammatory effect, together with improvements in the motor symptoms caused by the lesions. Only in the 6-OHDA mice, the dopamine levels in the striatum were restored by the treatment with VCE-003.2, indicating a functional recovery of the dopaminergic neurons. In this same study, we also tested the efficacy of two CBG derivatives in comparison with VCE-003.2. Their effects were similar or lower than those of VCE-003.2, and we confirmed *in vitro* that they were able to bind to the classical binding site of PPAR-γ, suggesting that the binding to the alternative site exerted by VCE-003.2 is specific of this cannabinoid and may be crucial for its particular effects. We also evaluated VCE-003.2 efficacy using a model of PD based on local overexpression of mutant (A53T) α-synuclein (α-SYN). We found a symptomatic relief in the VCE-003.2 treated group, but both α-SYN groups had lost all the dopaminergic neurons of the SN, due to an excessive dose of the virus. RNA sequencing of the striatum revealed important changes in the expression of immune response, inflammatory and lysosomal genes in the α-SYN-lesioned mice, as well as some transcriptomic changes after VCE-003.2 treatment, such as the promotion of damage associated microglia, the activation of the unfolded protein response or the inhibition of E2F1-mediated apoptosis, suggesting potential mechanisms of action. The results of this chapter are included in:

Burgaz, S., García, C., Gómez-Cañas, M., Muñoz, E., & Fernández-Ruiz, J. (2019). Development of An Oral Treatment with the PPAR-γ-Acting Cannabinoid VCE-003.2 Against the Inflammation-Driven Neuronal Deterioration in Experimental Parkinson's Disease. *Molecules (Basel, Switzerland)*, *24*(15), 2702.

Burgaz, S., García, C., Gómez-Cañas, M., Navarrete, C., García-Martín, A., Rolland, A., Del Río, C., Casarejos, M. J., Muñoz, E., Gonzalo-Consuegra, C., Muñoz, E., & Fernández-Ruiz, J. (2021). Neuroprotection with the cannabigerol quinone derivative VCE-003.2 and its analogs CBGA-Q and CBGA-Q-Salt in Parkinson's disease using 6-hydroxydopamine-lesioned mice. *Molecular and Cellular Neurosciences*, *110*, 103583.

Burgaz, S., [...] & Fernández-Ruiz, J. Oral VCE-003.2 as a disease-modifying treatment in AAV-α-synuclein model of Parkinson's disease. (Data not published yet).

Chapter 2. Preclinical evaluation of compounds targeting CB2: the CBD derivative VCE-004.8, and the phytocannabinoid THCV, alone and in combination with CBD

In this chapter we can differentiate two different compounds, which have in common its affinity for CB2 receptor, and a multitarget profile in the ECS. Firstly, we studied the CBD derivative, VCE-004.8, which is a CB2 and PPAR-γ activator. In the 6-OHDA model, we observed a behavioural and neuroprotective effect, accompanied by a reduction in neuroinflammation in the SN. We confirmed that both PPAR-γ and CB2 receptors contribute to the effect of VCE-004.8, although there was a major contribution of PPAR-γ receptors *in vitro*. Next, we evaluated a combined treatment of two phytocannabinoids, THCV and CBD. THCV, which is a CB2 agonist,

CB1 antagonist at low doses, and partial agonist of GPR55, had already shown potential in PD models, and CBD had also been effective in PD models, but both could still be improved. As expected, THCV was active in both LPS and 6-OHDA models, to similar o greater levels than CBD, especially in the LPS model, probably due to the relevance of CB2 in neuroinflammation. However, the combination of both compounds did not improve their individual effects. In the 6-OHDA model, indeed, the combined treatment did not show neuroprotective effect and did not improve the motor alterations. We observed *in vitro* that lower concentrations of both compounds, specially of THCV could improve the effects of the combined treatment. The effects of the treatment were reversed by the addition of antagonists aimed at blocking PPAR-γ and CB2 receptors, reinforcing the crosstalk in their pathways which needs to be studied. The results of this chapter are included in:

Burgaz, S., García, C., Gómez-Cañas, M., Rolland, A., Muñoz, E., & Fernández-Ruiz, J. (2021). Neuroprotection with the Cannabidiol Quinone Derivative VCE-004.8 (EHP-101) against 6-Hydroxydopamine in Cell and Murine Models of Parkinson's Disease. *Molecules (Basel, Switzerland)*, *26*(11), 3245.

Burgaz, S., [...] & Fernández-Ruiz, J. Investigating a combined strategy with THCV and CBD as a neuroprotective therapy in experimental models of Parkinson's disease. (Data not published yet).

Chapter 3. Preclinical evaluation of compounds targeting GPR55: VCE-006.1

In this last chapter, we characterized the potential of a synthetic cannabinoid, VCE-006.1, a GPR55 activator, again in our classic models of PD, LPS and 6-OHDA, as well as in two transgenic models of amyotrophic lateral sclerosis (ALS), in a complementary study of our lab. We observed that, VCE-006.1 was not active against neuroinflammation, but it was active against neuronal death, as in the 6-OHDA model significantly increased the survival of dopaminergic neurons of the SN, and in the ALS models, there was an increase in the motor neurons of the spinal cord after the treatment, although this did not correlate with a better neurological state, unlike in the 6-OHDA model. Thus, the efficacy of this compound may improve when combined with other cannabinoids with anti-inflammatory profile. The results of this chapter are included in:

Burgaz, S., García, C., Gonzalo-Consuegra, C., Gómez-Almería, M., Ruiz-Pino, F., Unciti, J. D., Gómez-Cañas, M., Alcalde, J., Morales, P., Jagerovic, N., Rodríguez-Cueto, C., de Lago, E., Muñoz, E., & Fernández-Ruiz, J. (2021). Preclinical Investigation in Neuroprotective Effects of the GPR55 Ligand VCE-006.1 in Experimental Models of Parkinson's Disease and Amyotrophic Lateral Sclerosis. *Molecules* (Basel, Switzerland), 26(24), 7643.

Taken all together, our results confirm that the treatment with cannabinoids is anti-inflammatory and neuroprotective in different preclinical models of PD. As we hypothesised, the activation of cannabinoid targets (PPAR-γ, CB2 and GPR55) is effective as a pharmacological strategy for PD, although targeting only one of them may be insufficient as a disease-modifying treatment. Thus, we propose the use of multitarget strategies based on cannabinoid compounds (in combination or with multitarget activity) in preclinical models and later in the clinical field. Hence, further research must be done to unveil the different mechanisms involved in the effects of cannabinoids against the multiple neuropathological events occurring in PD.

CB2, PPAR-γ y GPR55 como dianas farmacológicas para un tratamiento anti-inflamatorio y neuroprotector en la enfermedad de Parkinson

La enfermedad de Parkinson (EP) es una enfermedad neurodegenerativa crónica que cursa con pérdida de movilidad por la muerte selectiva de las neuronas dopaminérgicas de la sustancia nigra *pars compacta* (SNpc) y la consiguiente denervación dopaminérgica del cuerpo estriado. Esta enfermedad es también una proteinopatía, ya que presenta agregados de proteínas mal plegadas, principalmente α-sinucleína, conocidos como cuerpos de Lewy (LB). La etiología de la EP sigue siendo desconocida, pero se han descrito varios factores de riesgo ambientales y genéticos, además del envejecimiento. Los principales síntomas de la EP son la bradiquinesia, la rigidez y el temblor en reposo, que son determinantes para el diagnóstico, y aparecen cuando más del 50% de las neuronas dopaminérgicas ya están muertas. Sin embargo, hay síntomas no motores, como alteraciones del sueño, trastornos del ánimo, problemas gastrointestinales y disfunción olfativa, que pueden ser prodrómicas y útiles para un diagnóstico precoz. Los eventos patológicos que contribuyen a la muerte neuronal incluyen la agregación proteica, pero también la neuroinflamación, la disfunción mitocondrial, el estrés oxidativo y la excitotoxicidad. Estos mecanismos están interconectados, agravando la neurodegeneración dopaminérgica que altera el circuito de los ganglios basales, impidiendo un correcto funcionamiento motor. El tratamiento más frecuente es la reposición de los niveles de dopamina con levodopa, que recupera la movilidad de los pacientes a corto plazo, pero provoca disquinesias irreversibles tras varios años. Esto hace necesario un tratamiento modificador de la enfermedad, y dada la fisiopatología multifactorial de la EP, se propone el uso de moléculas pleiotrópicas, como los cannabinoides.

El sistema endocannabinoide (SEC) es uno de los moduladores más abundantes del sistema nervioso, con importantes funciones en la modulación de la neurotransmisión y la interacción con otros sistemas de señalización. Entre los efectos de los cannabinoides, los de mayor interés terapéutico son los dirigidos a preservar, rescatar, reparar y/o reemplazar neuronas y células gliales frente a múltiples insultos que pueden dañarlas potencialmente, lo que parece especialmente prometedor para condiciones patológicas neurodegenerativas crónicas, como la EP. Por ejemplo, diferentes cannabinoides que actúan sobre diferentes dianas cannabinoides han demostrado tener propiedades neuroprotectoras que podrían frenar la progresión de la enfermedad, a diferencia de cualquiera de los tratamientos que se están utilizando actualmente, en varios modelos preclínicos de EP. Sin embargo, todavía existen nuevas dianas, nuevos compuestos y nuevas combinaciones de estos que podrían producir ventajas respecto a las actuales, y que necesitan ser investigadas más a fondo. Asimismo, también hay nuevos modelos de EP que podrían contribuir a la mejor comprensión de la patología y su manejo farmacológico.

En base a estos antecedentes, el objetivo global de esta Tesis Doctoral es determinar la eficacia de diferentes compuestos cannabinoides, combinando sus propiedades antioxidantes, anti-inflamatorias y neuroprotectoras en modelos *in vitro* e *in vivo* de EP que representen los principales eventos neuropatológicos (estrés oxidativo, inflamación, agregación de α-sinucleína y muerte neuronal). Según el receptor diana del tratamiento, este objetivo global puede dividirse en tres objetivos específicos, representados en tres capítulos diferentes:

Capítulo 1. Evaluación preclínica de compuestos activadores de PPAR-γ: VCE-003.2 y otros derivados del CBG

Dada la eficacia mostrada por un tratamiento intraperitoneal con el derivado del CBG VCE-003.2 en modelos experimentales de EP (resultados previos a esta Tesis), se ha caracterizado una formulación oral de este derivado. Primero se estudiaron los efectos del tratamiento oral con VCE-003.2 en el modelo LPS y en el modelo 6-OHDA, en los que este compuesto tuvo un efecto neuroprotector y anti-inflamatorio, junto con mejoras en los síntomas motores causados por las lesiones. Sólo en los ratones con 6-OHDA, los niveles de dopamina en el estriado se restauraron con el tratamiento con VCE-003.2, lo que indica una recuperación funcional de las neuronas dopaminérgicas. En este mismo estudio, también probamos la eficacia de dos derivados del CBG en comparación con el VCE-003.2. Sus efectos fueron similares o inferiores a los del VCE-003.2, y confirmamos *in vitro* que eran capaces de unirse al sitio de unión clásico de PPAR-γ, lo que sugiere que la unión al sitio alternativo ejercida por el VCE-003.2 es específica de este cannabinoide y puede ser crucial para sus efectos particulares. También evaluamos la eficacia del VCE-003.2 utilizando un modelo de EP basado en la sobreexpresión local de la α-sinucleína mutante (A53T) (α-SYN). Encontramos un alivio sintomático en el grupo tratado con VCE-003.2, pero ambos grupos α-SYN habían perdido todas las neuronas dopaminérgicas de la SN, debido a una dosis excesiva del virus. La secuenciación del ARN del estriado reveló importantes cambios en la expresión de genes de respuesta inmune y lisosomales en los ratones con α-SYN, así como algunos cambios transcriptómicos tras el tratamiento con VCE-003.2, como la promoción de la microglía asociada al daño, la activación de la respuesta a las proteínas no plegadas o la inhibición de la apoptosis mediada por E2F1, lo que sugiere potenciales mecanismos de acción. Los resultados de este capítulo se incluyen en:

Burgaz, S., García, C., Gómez-Cañas, M., Muñoz, E., & Fernández-Ruiz, J. (2019). Development of An Oral Treatment with the PPAR-γ-Acting Cannabinoid VCE-003.2 Against the Inflammation-Driven Neuronal Deterioration in Experimental Parkinson's Disease. *Molecules (Basel, Switzerland)*, *24*(15), 2702.

Burgaz, S., García, C., Gómez-Cañas, M., Navarrete, C., García-Martín, A., Rolland, A., Del Río, C., Casarejos, M. J., Muñoz, E., Gonzalo-Consuegra, C., Muñoz, E., & Fernández-Ruiz, J. (2021). Neuroprotection with the cannabigerol quinone derivative VCE-003.2 and its analogs CBGA-Q and CBGA-Q-Salt in Parkinson's disease using 6-hydroxydopamine-lesioned mice. *Molecular and cellular neurosciences*, *110*, 103583.

Burgaz, S., [...] & Fernández-Ruiz, J. Oral VCE-003.2 as a disease-modifying treatment in AAV-α-synuclein model of Parkinson's disease. (Data not published yet).

Capítulo 2. Evaluación preclínica de compuestos activadores de CB2: el derivado del CBD VCE-004.8, y el fitocannabinoide THCV, sólo y en combinación con CBD

En este capítulo se estudiaron dos compuestos, ambos con afinidad por el receptor CB2 y un perfil multidiana en el SEC. Primero estudiamos el derivado del CBD, VCE-004.8, que es un activador de CB2 y PPAR-γ. En el modelo 6-OHDA observamos un efecto conductual y neuroprotector, acompañado de una reducción de la neuroinflamación en la SN. Confirmamos que tanto los receptores PPAR-γ como los CB2 contribuyen al efecto de VCE-004.8, aunque hubo una mayor contribución de los receptores PPAR-γ *in vitro*. A continuación, evaluamos un

tratamiento combinado de dos fitocannabinoides, el THCV y el CBD. El THCV, que es un agonista de CB2, antagonista de CB1 en dosis bajas, y agonista parcial de GPR55, ya había mostrado potencial en modelos de EP, al igual que el CBD, pero lo efectos de ambos podían ser mejorados. El THCV fue activo tanto en el modelo LPS como en el 6-OHDA, a niveles similares o mayores que el CBD, especialmente en el modelo LPS, probablemente debido a la relevancia del CB2 en la neuroinflamación. Sin embargo, la combinación de ambos compuestos no mejoró sus efectos individuales. En el modelo de 6-OHDA, de hecho, el tratamiento combinado ni siquiera fue neuroprotector en la SN ni mejoró las alteraciones motoras. En un estudio *in vitro* observamos que concentraciones más bajas de ambos compuestos, especialmente de THCV, podían mejorar los efectos del tratamiento combinado, cuyos efectos fueron parcialmente revertidos por la adición de antagonistas de PPAR-γ y CB2. Los resultados de este capítulo se incluyen en:

Burgaz, S., García, C., Gómez-Cañas, M., Rolland, A., Muñoz, E., & Fernández-Ruiz, J. (2021). Neuroprotection with the Cannabidiol Quinone Derivative VCE-004.8 (EHP-101) against 6-Hydroxydopamine in Cell and Murine Models of Parkinson's Disease. *Molecules (Basel, Switzerland)*, *26*(11), 3245.

Burgaz, S., [...] & Fernández-Ruiz, J. Investigating a combined strategy with THCV and CBD as a neuroprotective therapy in experimental models of Parkinson's disease. (Data not published yet).

Capítulo 3. Evaluación preclínica de compuestos activadores de GPR55: VCE-006.1

En este último capítulo caracterizamos el potencial de un cannabinoide sintético, VCE-006.1, un activador de GPR55, de nuevo en los modelos clásicos de EP, de LPS y 6-OHDA, así como en dos modelos transgénicos de esclerosis lateral amiotrófica (ELA), en un estudio complementario de nuestro laboratorio. Observamos que el VCE-006.1 no era activo contra la neuroinflamación, pero sí contra la muerte neuronal, ya que en el modelo de 6-OHDA aumentó significativamente la supervivencia de las neuronas dopaminérgicas de la SN, y en los modelos de ELA hubo un aumento de las neuronas motoras de la médula espinal tras el tratamiento, aunque esto no se correlacionó con un mejor estado neurológico, a diferencia del modelo de 6-OHDA. Por tanto, la eficacia de este compuesto podría mejorar en combinación con otros cannabinoides con perfil anti-inflamatorio. Los resultados de este capítulo se incluyen en:

Burgaz, S., García, C., Gonzalo-Consuegra, C., Gómez-Almería, M., Ruiz-Pino, F., Unciti, J. D., Gómez-Cañas, M., Alcalde, J., Morales, P., Jagerovic, N., Rodríguez-Cueto, C., de Lago, E., Muñoz, E., & Fernández-Ruiz, J. (2021). Preclinical Investigation in Neuroprotective Effects of the GPR55 Ligand VCE-006.1 in Experimental Models of Parkinson's Disease and Amyotrophic Lateral Sclerosis. *Molecules* (Basel, Switzerland), 26(24), 7643.

En conjunto, nuestros resultados confirman que el tratamiento con cannabinoides es anti-inflamatorio y neuroprotector en diferentes modelos preclínicos de EP. Como hipotetizamos, la activación de dianas cannabinoides (PPAR-γ, CB2 y GPR55) es eficaz como estrategia farmacológica para la EP, aunque dirigirse solo a una de ellas puede ser insuficiente para modificar la enfermedad. Por tanto, proponemos el uso de estrategias multidiana basadas en compuestos cannabinoides (combinados o con actividad multidiana) en modelos preclínicos y después en la clínica. Además, se debe seguir investigando para desvelar los distintos mecanismos implicados en los efectos de los cannabinoides contra los múltiples eventos neuropatológicos que ocurren en la EP.

ABBREVIATIONS

2-AG	2-araquidonoilglicerol
3-OMD	3-*O*-methyl-DOPA
3-MT	3-methoxytyramine
6-OHDA	6-hydroxydopamine
AA	Arachidonic acid
AADC	Aromatic L-amino acid decarboxylase
AAV	Adeno-associated virus
ABDH	α/β- domain hydrolase
Abn-CBD	Abnormal cannabidiol
AC	Adenylate ciclase
ACEA	Arachidonoyl-2-chloroethylamide
AD	Alzheimer's disease
AEA	Anandamide
AIMs	Abnormal involuntary movements
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
AMT	Anandamide membrane transporter
AP-1	Activator protein 1
Arg1	Arginase 1
ATF2	Activating transcripction factor 2
BBB	Blood brain barrier
BP	Biological process
BSA	Bovin serum albumin
cAMP	Cyclic adenosine monophosphate
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CBD	Cannabidiol
CBG	Cannabigerol
CD68	Cluster of differentiation 68
CNS	Central Nervous System
COMT	Catechol-O-methyltransferase
COX-2	Ciclooxygenase type 2
CPM	Counts per million
CREB	cAMP response element-binding
CRG	Centre for Genomic Regulation
CRT	Cylinder rearing test
CSF	Cerebrospinal fluid
DA	Dopamine
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DAM	Damaged associated microglia
DAT	Dopamine transporter
DBS	Deep brain stimulation
DEGs	Differentially expressed genes
DJ-1	Parkinson's disease protein 7
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DOPAC	3,4-dihydroxy-phenylacetic acid
EBST	Elevated body swing test

eCBs	Endocannabinoids
ECS	Endocannabinoid system
EHP	Emerald Health Pharmaceuticals
EMT	Endocannabinoid membrane transporter
ERK1/2	Extracellular signal-regulated kinase 1/2
ESCs	Embryonic stem cells
FAAH	Fatty acid amide hydrolase
FABPs	Fatty acid binding proteins
FBS	Fetal bovine serum
FDR	False discovery rate
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GASP-1	G-protein coupled receptor associated sorting protein 1
GBA1	Glucosylceramidase β1
GCase	Glucocerebrosidase
GDNF	Glial cell derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GIRK	G-protein activated internal rectifying K^+
GO	Gene ontology
GPCR	G-protein coupled receptor
GPe	External globus pallidus
GPi	Internal globus pallidus
GRK	G-protein receptor kinase
GSEA	Gene set enrichment analysis
GWAS	Genome-wide association studies
hiPSC	Human induced pluripotent stem cell
HD	Huntington's disease
IL-1β	Interleukin 1β
iNOS	Inducible nitric oxide synthase
IP3	inositol 1,4,5- triphosphate
iPSCs	Induced pluripotent stem cell
KPBS	Potassium phospate-buffered saline
L-DOPA	Levodopa
LAMP-1	Lysosomal-associated membrane protein 1
LB	Lewy bodies
LID	Levodopa-induced dyskinesias
LPI	Lysophosphatidylinositol
LPS	Lypopolisaccharide
LRRK2	Leucine-rich repeat kinase 2
MAGL	Monoacylglycerol lipase
MAO-B	Monoamine oxydase B
MAPK	Mitogen-activated protein kinase
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic resonance imaging
MSNs	Medium spiny neurons
mtCB1	Mitochondrial cannabinoid receptor type 1
mTORC1	Mammalian target of rapamycin complex 1
NADPH	Nicotinamide adenine dinucleotide phosphate

NAPE	*N*-arachidonoyl phosphatidylethanolamine
NAPE-PLD	*N*-arachidonoyl phosphatidylethanolamine phospholipase D
NFAT	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	*N*-methyl-D-aspartate
Nrf2	Nuclear factor erythroid 2–related factor 2
NSAIDs	Non-steroidal anti-inflammatory drugs
NSCs	Neural stem cells
OEA	*N*-oleoylethanolamide
PBS	Phospate-buffered saline
PCA	Principal component analysis
PCR	Polimerase chain reaction
PD	Parkinson's disease
PEA	*N*-palmitoylethanolamide
PET	Possitron emission tomography
PGC-1	Peroxisome proliferator-activated receptor gamma coactivator 1
PGRN	Progranulin
PINK-1	Serine/threonine-protein kinase 1
PI3K	Phosphoinositide-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PNS	Peripheral Nervous System
PPAR	Peroxisome-proliferator activated receptor
PPRE	Peroxisome-proliferator activated receptor response elements
REM	Rapid eye movement
RhoA	Ras homolog family member A
RIN	RNA integrity number
RNAseq	RNA sequencing analysis
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SN	Substantia nigra
SNCA	Synuclein α gene
SNpc	Substantia nigra *pars compacta*
SNpr	Substantia nigra *pars reticulata*
STN	Subthalamic nucleus
TH	Tyrosine hydroxilase
THC	Δ^9-tetrahydrocannabinol
THCV	Δ^9-tetrahydrocannabivarin
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor α
TPM	Transcripts per million
TRP	Transient receptor potential channel
UPR	Unfolded protein response
VCE	Vivacell Biotechnology España
VMAT2	Vesicular monoamine transporter 2
WT	Wild type

INTRODUCTION

1. PARKINSON'S DISEASE

Parkinson's disease (PD) is a chronic neurodegenerative disease, also classified as the most prevalent basal ganglia disorder, described by Dr. James Parkinson in 1817 as "the shaking palsy" (Parkinson, 2002). The hypokinetic symptoms of PD are caused by the selective death of the dopaminergic neurons of the substantia nigra *pars compacta* (SNpc), and the consequent denervation and depletion of dopamine (DA) in the striatum. The other main neuropathological event is the presence of protein inclusions, known as the Lewy bodies (LB), formed of misfolded proteins, mainly of α-synuclein. Although the contribution of different risk factors has been described, including aging, genetic and environmental factors, the final cause of these alterations remains unknown. The first treatment for PD was based on DA replacement, which ameliorates the motor impairments the first years, but worsens them on the long term, causing irreversible dyskinesia. Other therapies have been tried or are currently being studied, but a disease-modifying treatment must still be found (Poewe et al., 2017).

1.1. Epidemiology and etiology

PD represents the second most prevalent degenerative disorder after Alzheimer's disease (AD), and the first basal ganglia disease. PD is an age-related disorder, not common in the population under 50 years, but with an increasing incidence every decade from the 60 to 90 years (Figure 1). The mean prevalence is around 0,3% of the total population, but this number increases sharply with age, being around 1% of the population above 60 years of age and more that 3% in those over 80 years of age (Pringsheim et al., 2014). Without any treatment, the mortality associated with PD is three times higher than in the rest of the population at the same age (D'Amelio et al., 2006). Moreover, the increase in life expectancy together with the improvements in health care have led to an increased prevalence of PD, which will have doubled by 2030 (Dorsey et al., 2007).

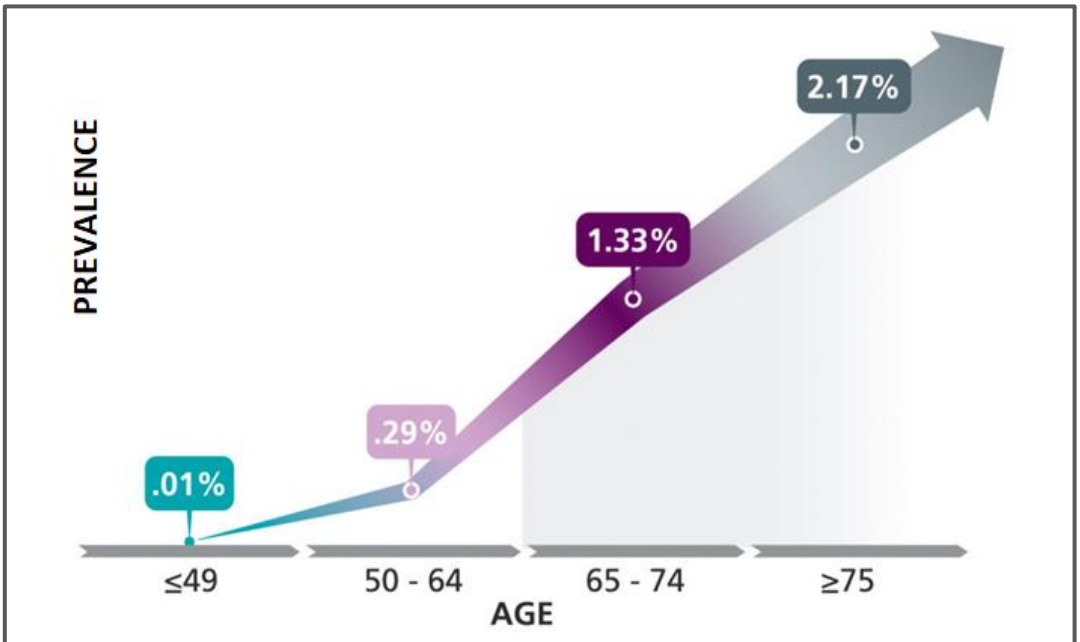

Figure 1. Prevalence of PD increases with age. (*Hamilton and Yang et al., 2019*).

In most populations the epidemiological numbers imply twice as many men than women, which could be due to the protective effect of estrogens, but this remains to be further invetigated (Van Den Eeden et al., 2003). Among the factors implicated in these differences, some sex-specific effects may be due to a differential exposure to environmental risk factors as well as the contribution of hormones such as testosterone. In the same way, there are prevalence differences between some populations, mainly geographically defined populations, which could be partially due to environmental factors (Lix et al., 2010). However, there are also genetic factors shared in these populations that could contribute to the epidemiological gaps between them.

Still today, the cause of PD remains unknown in most cases, but age is known to be the main non modifiable risk factor (Lee and Gilbert, 2016). Although this has been known for a long time, neuromelanin accumulation has been proposed recently as an age-related feature that contributes to PD pathology (Hirsch et al., 1989). Non-human animals do not express neuromelanin, whose synthesis in humans is mediated by tyrosinase and that is known to accumulate throughout life. Neuromelanin was considered just an intracellular pigment with little influence in the pathogenesis of PD, but useful as a marker of disease progression as it disappeared with neuronal death (depigmentation). Now, it has been suggested that when the production of neuromelanin granules reaches a threshold level in the dopaminergic neurons of the SNpc, it can cause neuronal dysfunction in the first place, and then trigger neuronal death at a more advanced stage (Carballo-Carbajal et al., 2019).

Moreover, it has been proposed that, in addition to aging, the sporadic cases are a result of the interaction between environmental modifiable factors and susceptibility genes. Large genome-wide association studies (GWAS) have confirmed that some of the genes associated with familiar PD are also affected in sporadic PD (Figure 2), with already 90 loci significantly associated with the pathology (Nalls et al., 2019). Even though heritable forms of PD, this is, the cases in which the cause has been identified, only represent around 5 to 15%, they provide information about their molecular pathways which could help to unravel the neuropathology of PD, such as SNCA gene in relation with α-synuclein accumulation in LB (Chu and Kordower, 2007). Familiar cases imply a genetic origin and an earlier development of the disease. Most of the familiar cases are monogenetic, due to mutations in identified mendelian genes, such as *LRRK2*, *SNCA*, *parkin*, *PINK-1*, *DJ-1*, etc. However, different alterations in the same gene can have different consequences, so most of them can be both causative and risk factors, depending on their variability (Kim and Alcalay, 2017). Recently, mutations in the *GBA1* gene have been identified as the main genetic risk factor for PD. *GBA1* is the β-glucosylcermidase gene 1, which codes for glucocerebrosidase (GCase), a lysosomal enzyme whose dysfunction has been related to Gaudier's disease and more recently to α-synuclein aggregation and the risk of developing PD (Do et al., 2019). In fact, the *GBA1*-associated PD presents with the same clinical manifestations as idiopathic PD, except for a more aggressive course and an increased risk of developing dementia. In addition, idiopathic PD patients are deficient in GCase, even without *GBA1* mutations, in correlation with higher α-synuclein levels (Blandini et al., 2019). Lastly, *GAK* and *HLA-DRB5* are recent examples of low-

risk, high-frequency gene variants identified by GWAS, as well as some genes, such as *LRRK2*, which are found in several or all of the categories (Figure 2).

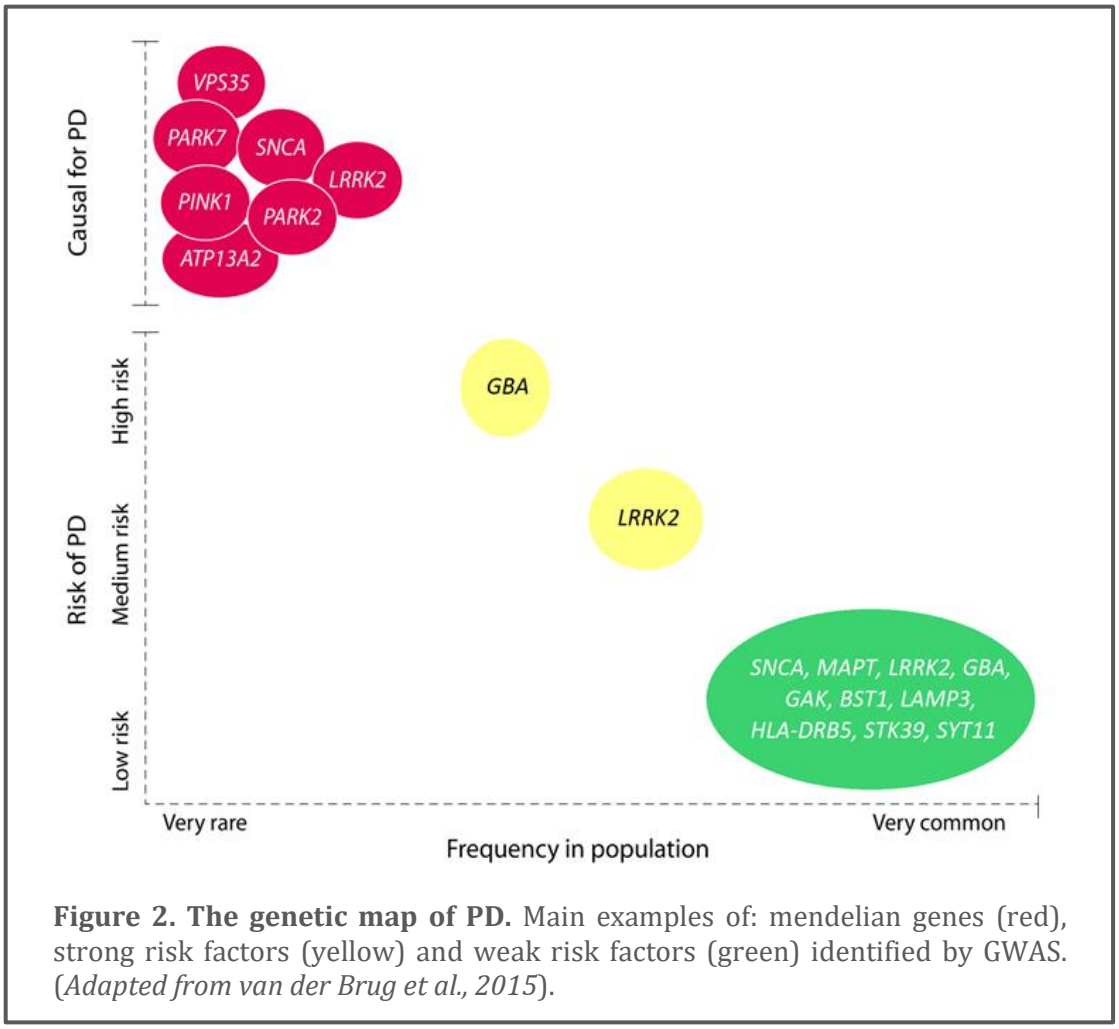

Figure 2. The genetic map of PD. Main examples of: mendelian genes (red), strong risk factors (yellow) and weak risk factors (green) identified by GWAS. (*Adapted from van der Brug et al., 2015*).

Regarding the environmental factors that have been related with PD, there is an increased number of sporadic cases in individuals exposed to toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), present in opioid preparations, and pesticides (such as paraquat or rotenone), all of them inhibitors of mitochondrial complex I (Langston et al., 1983). Other risk factors are the exposure to dairy products and to methamphetamine, as well as people who suffer from melanoma or traumatic brain injury. Conversely, there are other environmental factors that can be considered as protective because the incidence of PD is lower among consumers of caffeine, tea, tobacco, non-steroidal anti-inflammatory drugs (NSAIDs) and ibuprofen (Ascherio and Schwarzschild, 2016).

1.2. Symptoms

The clinical manifestation of PD is known as parkinsonism, a syndrome characterized by hypomobility symptoms which include bradykinesia, rigidity, resting tremor, postural instability, and incapability or slowness to start a voluntary movement (Dickson et al., 2009). Even though PD is the most common cause of parkinsonism, there are other forms of this syndrome. For example, secondary parkinsonism, which implies damage in the basal ganglia,

can be a result of an ischemic, neoplastic, or infective injury, or a kind of parkinsonism induced by drug consumption. Essential tremor and atypical parkinsonism have also been described as parkinsonian syndromes, which can be distinguished from PD by the symmetry of the symptoms or the absence of response to L-DOPA (Balestrino and Schapira, 2020).

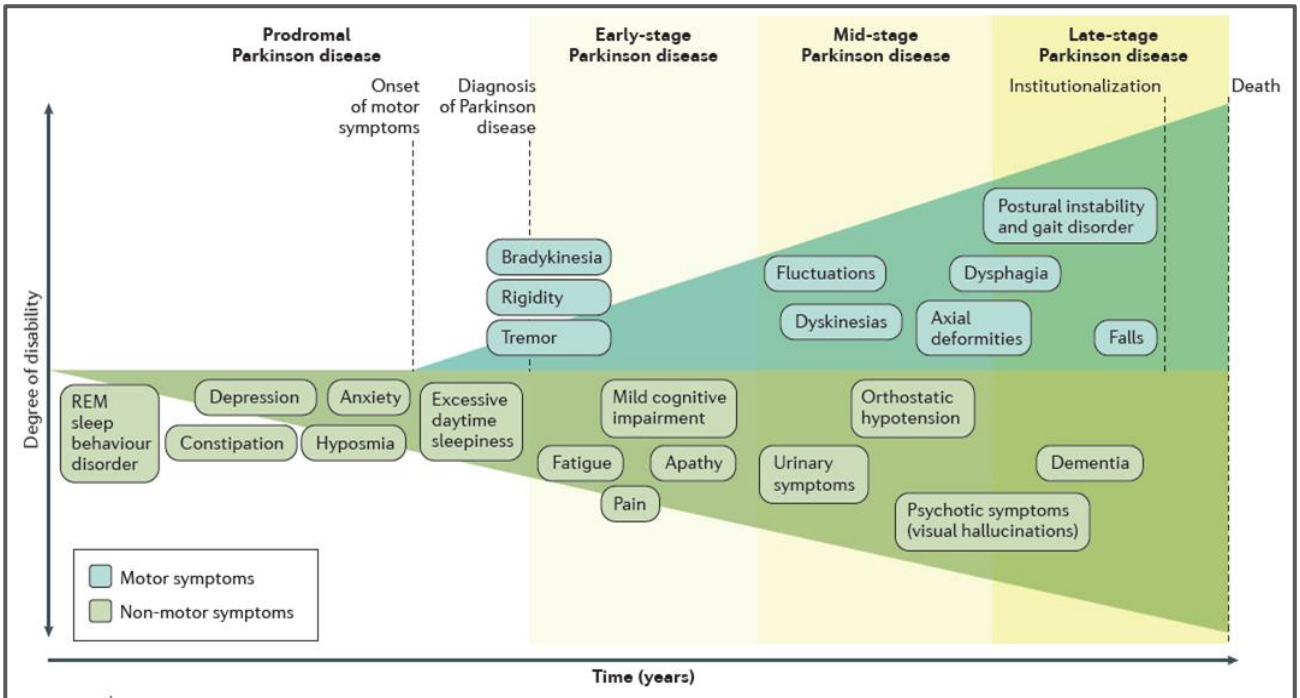

Figure 3. Clinical symptoms associated with PD progression. (*From Poewe et al., 2017*).

Regarding PD symptoms, the three pillars would be bradykinesia, rigidity, and resting tremor. Postural instability is also frequent, but not considered the fourth main feature anymore, as well as gait and balance disturbances. The motor symptoms are the ones used for the clinical diagnosis, but when the neuronal death has not been detected yet we can talk about prodromal PD (Figure 3). There are also specific non-motor symptoms, such as olfactory dysfunction, accompanied by gastrointestinal problems, sleep disturbances, rapid eye movement (REM) and neuropsychiatric symptoms, which can last years or even decades and which can appear years before the motor symptoms in many cases. There can also be some minor issues in the ability to walk (Poewe and Mahlknecht, 2009). The motor symptoms do not appear until around 50% of the dopaminergic neurons of the SNpc are already dead, and the striatum results to be significantly denervated. At this point (early-stage PD), patients are usually diagnosed based on their difficulties to start voluntary movements, with slowness and decreased amplitude (bradykinesia), together with resting tremor (3-6 Hz) or rigidity, almost always in an asymmetric way (Kalia and Lang, 2015). This diagnostic delay is due to compensatory mechanisms in the dopaminergic pathways that keep the circuitry working until levels of DA are very low. For this reason, the identification of the prodromal phase and its features would be fundamental for an early diagnosis, opening a window for early treatments, even before the massive neuronal death has occurred (Heinzel et al., 2019). After the diagnosis (mid-stage PD), there is an increasing severity of the cardinal motor features,

while the non-motor symptoms become increasingly prevalent and obvious over the course of the illness, although they can be present to a variable degree throughout all stages of PD (Figure 3). They occur as an extension of the degenerative process, as the cognitive symptoms appear, due to cell loss in the cortex. There is dementia, cognitive decline, and psychosis in more advanced stages, and a dysfunction of cholinergic, noradrenergic, and serotonergic transmission (Sveinsbjorndottir, 2016), which may sometimes result by the chronic therapy with L-DOPA. Finally, patients develop L-DOPA-induced motor complications (dyskinesia) and the evolution of poorly L-DOPA-responsive motor disabilities, such as postural instability, gait problems and dysphagia (late-stage PD), until death (Figure 3).

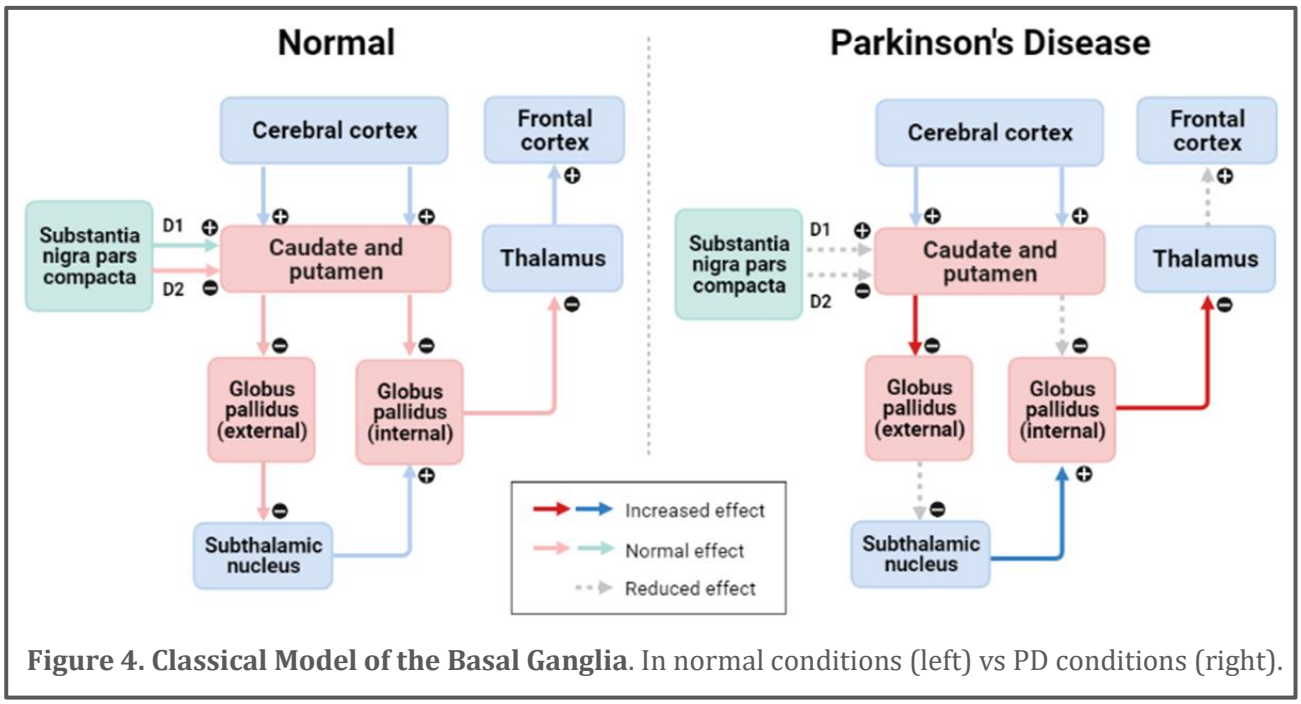

Figure 4. Classical Model of the Basal Ganglia. In normal conditions (left) vs PD conditions (right).

PD is classified as a movement disease because the SNpc is part of the basal ganglia, a group of brain areas involved in the control of movement through different connections between them and the central cortex (Alexander et al., 1990). There are different models that try to explain how movement is controlled by this circuitry, such as the parallel model or the centre-surround model, being the most accepted one the classical model. According to this model, there are two pathways that control movement, which start from the striatum, controlled in turn by the DA of the SNpc. The most abundant cells in the striatum are the GABAergic medium spiny neurons (MSNs). These interneurons can be divided in two subtypes: the ones that express D1 receptors, which are coupled to Gs proteins, and project directly to the internal globus pallidus (GPi)/substantia nigra *pars reticulata* (SNpr), that conform the direct pathway; and the ones that express D2 receptors, which are coupled to Gi proteins and project to the external globus pallidus (GPe) , then the subthalamic nucleus (STN), and finally the GPi/SNpr, that conform the indirect pathway. Some recent studies have identified some new elements in this circuitry, which conform the hyperdirect pathway, but its function remains to be identified (Nambu et al., 2002).

In a physiological situation, with normal DA levels coming from the SNpc, the effect on both pathways will be opposite, activating the direct pathway (D1) and inhibiting the indirect pathway (D2). The result of this effect modulates the output of GPi/SNpr neurons which inhibit the thalamus, then resulting in higher or lower activity of the thalamocortical neurons (Figure 4). In the parkinsonian condition, the loss of SNpc results in a decreased dopaminergic transmission in the motor region of the striatum. This causes hypoactivity of the direct pathway and hyperactivity of the indirect pathway. This results in an excessive GPi/SNpr output which implies a chronic and progressive increase in GABAergic inhibition of thalamocortical projections, leading to the suppression of movement and giving rise to the parkinsonian symptoms (McGregor et al., 2019).

1.3. Mechanisms/pathophysiology

The main neuropathological event of PD is the death of the dopaminergic neurons in the SNpc, with the consequent denervation of the striatum (Figure 5). The loss of nigrostriatal neurons starts in the ventrolateral SNpc, and spreads through the midbrain as the disease progresses (Damier et al., 1999). The early degeneration has also been described in terms of loss of neuromelanin levels, which represents the decrease in the volume of the SNpc of PD patients and has been seen to correlate nigral function with motor, cognitive and behavioural function (Biondetti et al., 2020). The other main feature is the presence of intracellular aggregates of α-synuclein or LB, which makes this disease to be also considered as a proteinopathy. In addition, there are many other underlying pathological events, such as altered proteolysis, neuroinflammation, mitochondrial dysfunction, oxidative stress, and excitotoxicity. These events have been shown to contribute to the neurodegeneration (Figure 6), to be interconnected between each other and they appear related to proteins coded by several genes associated with PD according to GWAS (Nalls et al., 2019).

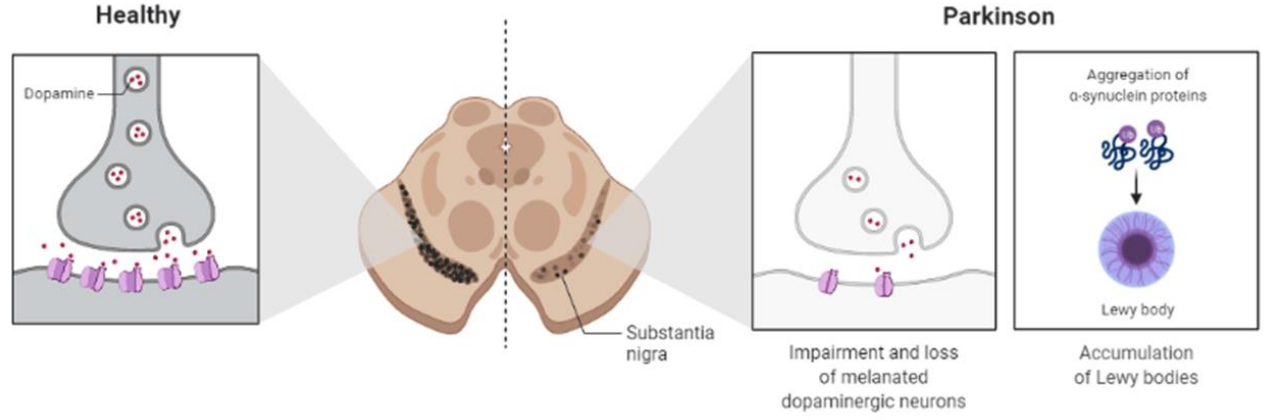

Figure 5. Progression of PD in the substantia nigra. In healthy (left) vs PD patients (right).

1.3.1. Proteostasis dysregulation/synucleinopathy

LB are intraneuronal protein aggregates present in PD patients, mainly formed by α-synuclein and other misfolded proteins, including neurofilaments and ubiquitin (Spillantini et al., 1998). The physiological function of α-synuclein has not been fully determined, but it is

known to work as a chaperone and to participate in synaptic vesicle dynamics and in mitochondrial function. The neurotoxic properties of α-synuclein come from its conversion from monomers to oligomers that progressively form protofibrils whose combination result in insoluble α-synuclein fibrils (Han et al., 2020). The accumulation of α-synuclein and its aggregation has also been related to the impairment of degradation systems, such as the ubiquitin-proteasome system and lysosomal autophagy, as well as to the presence of oxidative stress within the cell (Figure 6). Also, there are many PD causal or susceptibility genes which are related to (or participate) in the proteasomal function, such as *parkin* or *SNCA* itself, and that could contribute to the formation of α-synuclein aggregates.

An additional mechanism of aggregation that has been proposed is the prion-like propagation of α-synuclein, consistent with the PD pathology spreading to more brain areas as the disease progresses. It has been suggested that nearby neurons can uptake α-synuclein aggregates that have been transported intra-axonally, where they will trigger new endogenous α-synuclein aggregation (Angot et al., 2010). This transmission can occur through exosomes or nanotubules. There are different hypotheses regarding the onset of the aggregation. Classically, synucleinopathy has been proposed to start in the periphery of the Central Nervous System (CNS), specifically in the olfactory bulb, which would match with the early anosmia described for PD patients (Braak et al., 2003). However, other authors suggest that the mechanism of the gut-origin hypothesis looks more plausible, since it would also be supported by the gut disturbances on early PD stages (Scheperjans et al., 2018).

1.3.2. Neuroinflammation

Microglial cells are the main effectors of the immune system in the CNS and are responsible of carrying out support functions such as maintaining tissue homeostasis, pruning and remodelling of axon collaterals, phagocytosis of neurons in apoptosis, the remyelination of axons and to provide a response to injury or infection (Nayak et al., 2014). For instance, basal ganglia are within the CNS structures with greater presence of glial cells. As in other neurodegenerative diseases, it has been seen that in PD microglial cells are activated, there is astrogliosis, and there can be infiltration of peripheral immune system cells into the CNS, such as macrophages and monocytes (McGeer and McGeer, 2004). Neuroinflammation is one of the main contributors to the pathogenesis of PD, even though it does not seem to be the initial trigger of the disease (Hirsch and Hunot, 2009).

The proinflammatory phenotype of microglial cells is known as the M1 activation state, which seems to contribute to neuroinflammation, as the levels of proinflammatory cytokines associated to M1 microglia are increased both in serum and cerebrospinal fluid (CSF) of PD patients and correlate with more disability and worse prognosis of the disease (Scalzo et al., 2010). In addition, the M1 type cells have been found in post-mortem samples of PD midbrain in high levels, and in the vicinity of the LB, correlating with the high levels of α-synuclein aggregates (Moehle and West, 2015). Nevertheless, microglia can also have a phagocytic phenotype, the M2 activation state, in charge of removing residual components such as myelin and cell debris. As a result, a neuroprotective role has also been proposed for microglia in the clearance of α-synuclein that has been released from neurons, which would be then engulfed

and degraded by autophagy. Moreover, it has been described that when the "synucleinphagy" process gets disrupted in mice expressing human α-synuclein there is neuronal degeneration in the midbrain accompanied by α-synuclein deposition (Choi et al., 2020). However, there are still other reasons to consider neuroinflammation as a pathogenic factor of PD, as subjects chronically treated with NSAIDs due to peripheral inflammatory conditions have lower risk of developing PD and experimental PD can be reached through exposure to LPS.

1.3.3. Mitochondrial dysfunction

The mitochondrial respiration is an essential function of every cell, and its alteration implies dysfunctions in such cells. The relationship between PD pathogenesis and mitochondrial dysfunction first came from studies about accidental infusions of MPTP, a toxin that inhibits complex I of the mitochondria (Langston et al., 1983). The inhibition of complex I by this toxin has been described to provoke parkinsonism in humans, as well as in animal models, where the injection of mitochondrial toxins (MPTP, paraquat, rotenone or 6-hydroxidopamine (6-OHDA)) replicates the main events of PD pathology. Rotenone and paraquat are pesticides, and MPTP is a subproduct of opioid drugs, which can enter through intranasal way. Also, it is known that there is a deficiency of complex I expression in samples of PD patients (Schapira et al., 1990).

Additionally, several mutated genes related in GWAS to the familiar forms of PD, such as *parkin*, *DJ-1*, *PINK*, and *LRRK2*, also play important roles in different mitochondrial processes, suggesting a crucial role of the mitochondria in the pathogenesis of PD (Bose and Beal, 2016). Some of these genes and alterations also imply lysosomal dysfunction and an impaired mitophagy, this is, the elimination of damaged mitochondria. Both in PD patients and animal models, the dysfunction of mitophagy has been related with increased degeneration and neurotoxicity, suggesting that this mechanism is a critical component of PD pathogenesis (Liu et al., 2019). For instance, the peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) family, which acts as a master regulator of mitochondrial biogenesis, has been found to be dysregulated in PD and thus proposed as a therapeutic target (Piccinin et al., 2021). Axonal degeneration has also been proposed as an important consequence of mitochondrial dysfunction, probably due to energy deficiency, which could be one of the first neurodegenerative events, taking place years before the neuronal cell bodies in the SNpc start to die (Kordower et al., 2013).

Furthermore, and in concordance with the central role of the mitochondria in any cell, these alterations have been related with other neuropathogenic mechanisms, such as oxidative stress and inflammation, but mainly with the aggregation of α-synuclein (Figure 6). For instance, oxidative stress is one of the main consequences of mitochondrial dysfunction, described in the next section. Both lysosomal function and microglia take part in the clearance of the LB, so when any of these mechanisms may be compromised, they would exacerbate each other's contribution to neurodegeneration in PD patients (Devi et al., 2008).

1.3.4. Oxidative stress

Oxidative stress is the consequence of the imbalance between the levels of reactive oxygen species (ROS) produced and the antioxidant capacity of the biological system to detoxify the reactive intermediates, in order to avoid an oxidative state that could contribute to cellular damage. ROS production occurs mainly through the increase in the expression and activity of NADPH oxidase enzymes and the inducible nitric oxide synthase (iNOS). The brain consumes around 20% of the oxygen of the body, and part of it is converted to ROS, both in neurons and glia, mainly in the electron transport chain, as ROS are necessary for signalling (Dias et al., 2013). For example, dopamine metabolism produces ROS, and thus potentiates the oxidative stress to which the dopaminergic neurons are exposed.

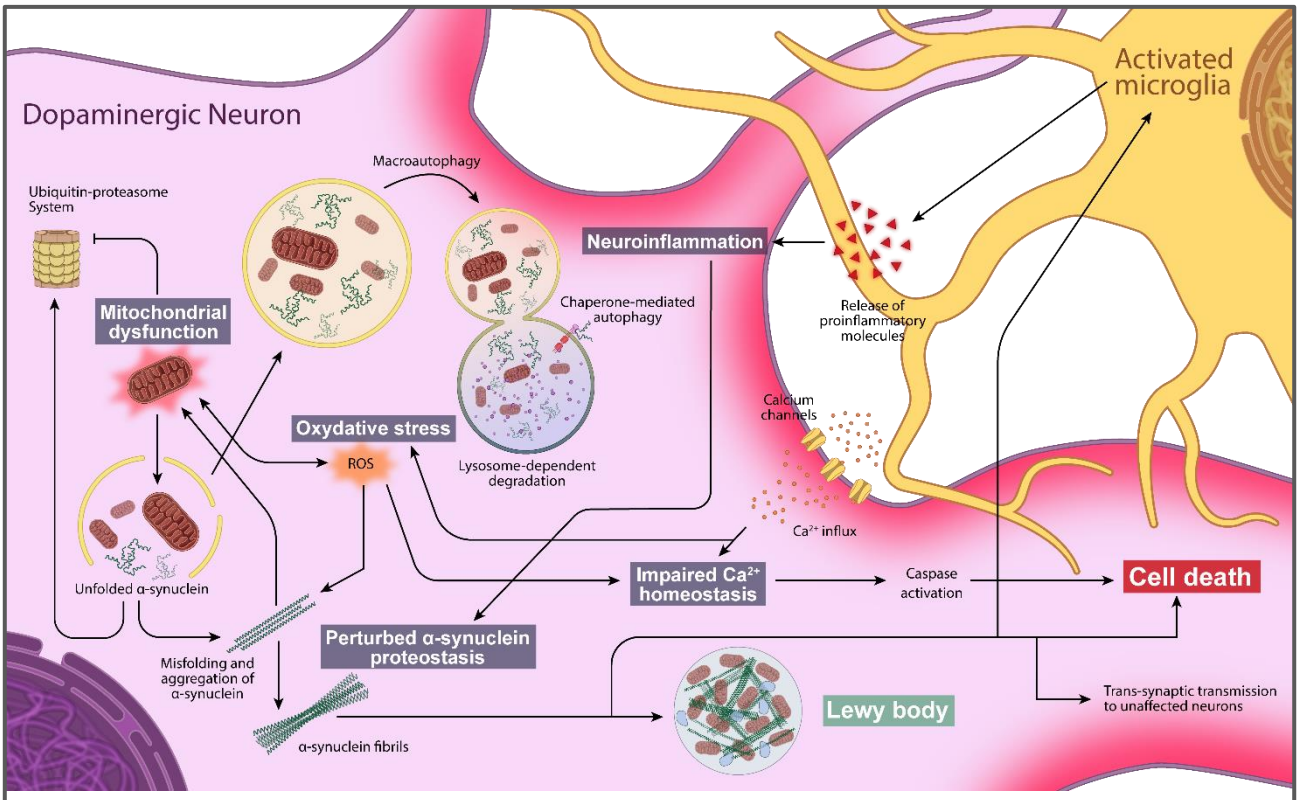

Figure 6. Molecular mechanisms involved in PD. Schematic diagram depicting interactions between major molecular pathways that are implicated in the pathogenesis of PD.

The role of oxidative stress in PD has been widely studied, and it has been proposed that selective death of nigral dopaminergic neurons answers to a higher vulnerability of these neurons to metabolic and oxidative stress, for different reasons. On the one hand, nigrostriatal projections have very long and unmyelinated axons with a large number of synapses, which require high energy consumption, together with activity involving calcium oscillations and calcium extrusions, which also consume energy (Pissadaki and Bolam, 2013). On the other hand, elevated levels of cytosolic DA and the generation of its metabolites (which are ROS, as we mentioned above) have been seen to be neurotoxic, and the reduction of such levels implied neuroprotection to the dopaminergic neurons of the SNpc (Mosharov et al., 2009). Again, these events are intimately linked to other neuropathogenic events of PD, such as

mitochondrial and lysosomal dysfunction, which are also a source of ROS (Figure 6), and with some genes which are related to familiar form of PD, such as *DJ-1*, and that are associated with increased cellular oxidative stress (Blesa et al., 2015).

1.3.5. Excitotoxicity

Excitotoxicity is one of the common pathological events that contribute to cell death in neurodegenerative diseases and is caused by the excess of extracellular glutamate in the synaptic space. Since glial cells are the main responsible of re-uptaking glutamate, an impairment of the reabsorption function in such cells would lead to excitotoxicity, thus contributing to neurodegeneration (Iovino et al., 2020). The same happens as a consequence of an elevated glutamate tone. In this respect, as a part of the basal ganglia circuitry, there are some glutamatergic connections, such as cortical inputs in both SN and striatum, which could contribute to the pathogenesis of PD if their glutamate homeostasis suffered any alteration. For instance, the hyperactivity of STN increases with the death of dopaminergic neurons in the SNpc, leading to abnormally high levels of glutamate. In addition to the excitotoxicity generated, the hyperactivity of the STN has been related with specific clinical manifestations, such as the parkinsonian tremor (Steigerwald et al., 2008).

1.4. Treatment

Most of the diagnostic criteria currently used are based on clinical symptoms, which eliminates the possibility of early detection of the disease and therefore of a window of action that necessarily implies greater efficacy of disease-modifying drugs. Thus, there is a clear need for the identification and detection of early biomarkers of the disease, such as the measurement of α-synuclein and neurofilaments in cerebrospinal fluid and blood (Parnetti et al., 2019), the use of imaging procedures or the prodromal clinical signs (anosmia or depression). Recently, purified cell populations, such as peripheral monocytes, have been suggested as novel biomarkers, as they show an altered expression of several genes, which are also altered in microglia and SN of PD patients (Navarro et al., 2021).

Meanwhile, PD management has classically focused on the motor symptoms of the disease. Considering that PD is characterized by the deficiency of dopamine, the first and main treatment strategy is dopamine replacement (LeWitt and Fahn, 2016). First, L-DOPA, a dopamine precursor molecule capable of crossing the blood-brain barrier (BBB), unlike dopamine by itself, was used to restore dopamine tone in the basal ganglia. Once in the striatum, L-DOPA is captured by the surviving dopaminergic cells and converted to dopamine by the aromatic L-amino acid decarboxylase enzyme (AADC), not only in the dopaminergic neurons but also in serotoninergic neurons, which have the same or similar decarboxylases. In this way, it is possible to improve motor-type symptoms, although the development of the disease will not be slowed down. Furthermore, an important drawback of this treatment is the long-term appearance of dyskinesias (5 to 10 years after treatment onset), caused by the discontinuous drug delivery due to the short half-life of L-DOPA. Dyskinesias are motor fluctuations, abnormal involuntary movements (AIMs) that cannot be controlled, due to the

pulsatile stimulation of the striatum, and that require the reduction of L-DOPA doses to avoid the worsening of these side effects (Heumann et al., 2014).

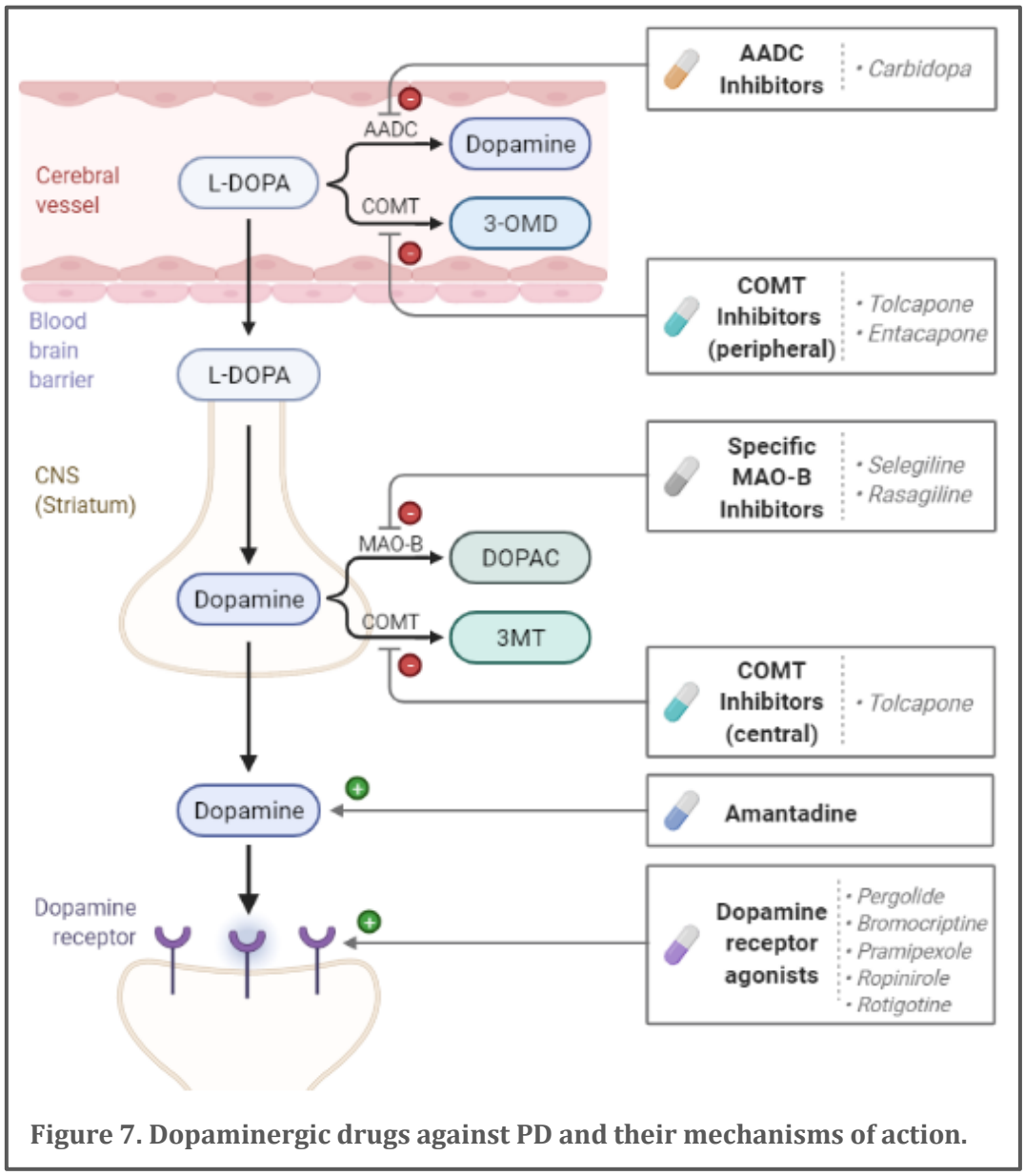

Figure 7. Dopaminergic drugs against PD and their mechanisms of action.

To enhance the biodisponibility and half-life of L-DOPA through the prevention of its metabolism, L-DOPA started to be used in combination with inhibitors of its degradation enzymes (Figure 7). To avoid its peripheral degradation, there are AADC inhibitors, such as carbidopa (benzeraside), as well as inhibitors of the catechol-O-methyltransferase (COMT), such as tolcapone, to prevent its metabolism to 3-*O*-methyl-DOPA (3-OMD). Likewise, there is a central degradation of DA, mediated by COMT, which produces 3-methoxytyramine (3-MT), and by monoamine oxidase-B (MAO-B), which produces 3,4-dihydroxy-phenylacetic acid (DOPAC), so again inhibitors of these enzymes, such as selegiline, need to be used and can also help to control motor fluctuations (Figure 7). For the same reason, dopamine agonists that have a longer half-life (and therefore more constant levels) as well as formulations of continuous delivery have started to be used, thus avoiding the pulsatile stimulation of striatal dopamine receptors and the motor complications derived from it (Poewe and Antonini,

2015). However, these novel formulations still have some complaints with respect to the chronic administration of L-DOPA, since they have less overall effect and could also have other side effects derived from the activation of dopaminergic receptors in the reward system and other CNS structures (Amstrong and Okun, 2020). Nevertheless, these drawbacks could be useful for patients who fail to respond to L-DOPA treatment.

Complementarily, non-dopaminergic therapies have also been developed, since not all symptoms are caused by alterations in dopamine levels (such as parkinsonian tremor) and in particular to compensate for the prodyskinetic effects of the dopaminergic treatment. In this context, the treatment that has been shown to be most effective against L-DOPA-induced dyskinesias has been amantadine, an NMDA receptor antagonist. There are also other promising treatments under development, especially targeting serotonin, adenosine, or acetylcholine receptors (Connolly and Lang, 2014), as well as some cannabinoid-based treatments, which are effective reducing levodopa-induced dyskinesias (LID; Espadas et al., 2020).

Another important approach is deep brain stimulation (DBS) of the STN (or less frequently of the GPi), which is effective for the motor symptoms and for the L-DOPA-induced dyskinesias. However, there must be a previous response to L-DOPA treatment, otherwise the DBS is unlikely to be effective (Bronstein et al., 2011). The DBS consists of a high frequency stimulation of the STN that aims to compensate for the motor fluctuations derived from the pharmacological treatment with L-DOPA, especially in the off state. This therapy has demonstrated to improve quality of life in PD patients, and to change the disease course by postponing L-DOPA-induced complications, allowing reduction of medication (Deuschl and Agid, 2013). A similar strategy under study is the use of focused ultrasounds, which has the advantage of not needing surgery (Martínez-Fernández et al., 2021).

As a movement disorder, part of the PD treatment also includes physical therapy or rehabilitation. The use of exercise-based treatment is under study, to try to improve and unify the different approaches. The evidence has been found in studies that use different types of exercises, from strength to high-intensity exercises, or that include specific parameters, such as gait speed or balance control, and that could be promising as a complementary treatment for PD (Keus et al., 2009).

Next, another historical, but still under study treatment for PD, is cell therapy. The rationale for such strategy is directed to replace the loss of dopaminergic neurons with transplanted DA-producing cells of different origins. It began with chromaffin cells obtained from pigs in the past century, followed by fetal nigral cell transplantation, aiming for dopaminergic synapse formation and the consequent dopamine supplementation. Although it was effective in some patients for reducing motor symptoms and dyskinesias, the functional recovery was not always sufficient (Olanow et al., 2003), and the occurrence of problems was frequent. Intrastriatal transplantation of the autologous carotid body was also tested, and none of the patients developed dyskinesia after the surgery. Some patients showed clinical amelioration, which could be due to the secretion of glial cell derived neurotrophic factor (GDNF) by the transplanted cells (Patel and Gill, 2007). Currently, different types of stem cells

are being tested such as embryonic stem cells (ESCs), neuronal stem cells (NSCs) and induced pluripotent stem cells (iPSCs), with promising results for slowing the disease progression (Yasuhara et al., 2017). These studies try to demonstrate that the benefit of cell therapy is not only to manage to replace the damaged tissue, but also to have a proneurotrophic scenario for the host environment, activating mechanisms that could lead to neuroprotection, neurogenesis, and anti-inflammation.

Finally, a promising treatment to be considered is gene therapy, as both non-disease and disease modifying transgenes have been tested for PD gene therapy in animal and human studies, with promising results (Axelsen and Woldbye, 2018). More recently, many of these studies are centred in the use of adeno-associated virus (AAV) coding for *GBA1* (Sucunza et al., 2021). The GCase enhancement has been demonstrated to prevent the dopaminergic neurodegeneration in the SNpc of rodents and macaques by reducing α-synuclein burden. Moreover, there is a new type of AAV that can penetrate the BBB (AAV9-PHP), thus it can be administered systemically, increasing the efficacy of this disease-modifying therapy (Sucunza et al., 2021).

1.5. Parkinson's disease models

There are different experimental tools for studying PD, such as patient samples or cell cultures, but the most widely used are animal models, which seek to best represent human pathology. Currently, there is not a fully valid model, as the precise pathogenetic mechanism in humans remains unknown. However, there are different types of models, which continue being improved with the advances in scientific knowledge and techniques, most of them developed in rodents or non-human primates (Chia et al., 2020). Neurotoxin-based models have been primarily used to recapitulate symptoms of the idiopathic PD form, while genetic models are based on human mutations related to the familiar forms of PD. There are also double hit models, which combine two insults, and other models mainly based on generating α-synucleinopathy (Kin et al., 2019).

1.5.1. Genetic models

This group includes animal models obtained by gene manipulation. In PD, there are many genes which are used to obtain genetic models, most of them because they have been identified as causal genes in different forms of familiar PD, such as *α-synuclein*, *parkin*, *DJ-1*, *PINK1* and *LRRK2*. The issue with these models is that in the sporadic PD forms, which are the most common ones, alterations can be found in several risk genes (not causal genes), so the monogenic models only represent a minor proportion of the different PD forms (Jagmag et al., 2016).

However, these models can be used to study some specific features related with the manipulated gene. The knockouts of *parkin*, *DJ-1* and *PINK1* (separately and altogether) may be useful to understand compensation mechanisms and early alterations of the mitochondrial function. Similarly, the transgenic models of *LRRK2* can serve to study early pathology of the dopaminergic system, although they never reach a substantial neuronal death, probably due to the partial penetrance of its mutations in humans (Dawson et al., 2010).

Finally, numerous models which overexpress α-synuclein (both wild type (WT) and with mutations) have been developed, considering the central role of α-synuclein in sporadic PD, strongly supported by the presence of LB in the pathology, as well as the complete penetrance of the mutations of the *SNCA* gene. However, the phenotype outcome strongly depends on the promoter used for transgene expression, and none of these models managed to reproduce progressive loss of dopaminergic neurons and motor alterations compatible with PD clinical signs (Chesselet, 2008).

1.5.2. Neurotoxin models

This group includes models obtained by any kind of toxin insult. They are usually based on neurotoxins which elicit the nigrostriatal degeneration observed in patients who suffer from PD, for example pesticides (Zeng et al., 2018). Shortly, this kind of models search for dopaminergic death, but they usually lack a physiopathological or etiological correlation, as in general they do not achieve to reproduce α-synuclein aggregation. Generally, they are generated by the intracerebral injection of the neurotoxin, in a specific area, either striatum, medial forebrain bundle, or SNpc, and they are usually maintained from two weeks to few months, thus being considered chronic models which allow the screening of potential chronic treatments. Moreover, the lesion can be made unilateral, so the non-lesioned hemisphere may serve as a control for certain analysis. Some of the most used neurotoxin models of PD are:

- Reserpine model: based on the injection of reserpine, which blocks the vesicular monoamine transporter 2 (VMAT2), thus causing a complete monoamine depletion, including DA. This blockade causes PD-like motor impairment as it induces akinesia, catalepsy, rigidity, and oral tremor. This model has been mainly used for screening of potential symptomatic drugs, as its phenotype is completely reversed by L-DOPA treatment. However, regarding the molecular features, the reserpine model lacks construct validity, as there is neither neurodegeneration nor protein aggregation (Leão et al., 2015).
- MPTP model: based on the injection of MPTP, which can cross the BBB to be converted into 1-methyl-4-phenylpyridinium (MPP+). MPP+ is an inhibitor of mitochondrial complex I and causes a great loss of dopaminergic neurons in the SN and their terminals in the striatum. An advantage of this model is that the susceptibility to this toxin increases with age, just as happens on PD. This model does not show the typical behaviour of PD patients, except for minor motor complications. Although the neuronal death is specific of the nigrostriatal neurons, it cannot be considered a long-term model, as the neurodegeneration occurs too rapidly and aggressively to appreciate the efficacy of chronic treatments (Jackson-Lewis and Przedborski, 2007).
- Rotenone model: based on the injection of rotenone, a pesticide that crosses the BBB and blocks the mitochondrial electron transport chain through inhibition of complex I. This toxin also blocks mitosis and inhibits cell proliferation, which could contribute to neurodegeneration. The molecular features of the rotenone model could be representative of PD pathogenesis, as it causes slow and specific nigrostriatal cell death, but the high

mortality rates provoked by this pesticide makes it too acute to follow the disease progression (Fleming et al., 2004).

- 6-OHDA model: based on the injection of 6-OHDA, a neurotoxin captured by the dopamine transporter (DAT) in the axons which causes retrograde neuronal degeneration of the SN, through the inhibition of complex I and IV of the mitochondria. This toxin also promotes auto-oxidation, as it accumulates in the cytosol and induces the formation of hydrogen peroxide and other ROS (Simola et al., 2007). As a result, there is a downregulation of the dopamine synthesis, which can be compensated with an increased dopamine production in the non-lesioned striatum, where the 6-OHDA is injected. To avoid the same effect on other monoaminergic neurons, which would mean an unspecific lesion, it is necessary to also administer desipramine, to block the noradrenergic transporters (Alvarez-Fischer et al., 2008). 6-OHDA also activates apoptosis through caspase-3 and leads to neuroinflammation in the SNpc as a consequence of neuronal death. The 6-OHDA model can reproduce the motor impairments of the disease, especially in the limbs contralateral to the lesioned hemisphere. The main drawback of this model would be the absence of LB formation (Alvarez-Fischer et al., 2008).
- LPS model: the lipopolysaccharide (LPS) model has been validated due to the key role of neuroinflammation in both the etiology and the progression of PD. The model is based on the intrastriatal injection of LPS (Hunter et al., 2009), which targets the Toll-like receptor 4 (TLR4) in microglial cells, although it can also be obtained by intranigral injection (Machado et al., 2011). The activation of TLR4 triggers a primary and chronic inflammatory response. After the primary neuroinflammation, there can be other secondary alterations caused by the malfunctioning of the glial cells, such as oxidative stress and mitochondrial dysfunction, which will progressively lead to neuronal death in the SNpc. The specific dopaminergic neurodegeneration is accompanied by striatal dopamine depletion and the consequent progressive motor impairment.

1.5.3. Proteinopathy models

The lack of a complete parkinsonian phenotype in the classic models, both genetic and based on neurotoxins, has promoted the search of other models, based on the presence of pathological events correlated with α-synuclein aggregation that led to the specific dopaminergic neurodegeneration. Although α-synuclein can propagate when injected systemically (Kuan et al., 2021) or in the enteric nervous system (Manfredsson et sl., 2018), we will centre on its propagation when injected in the CNS of rodents:

- Prion-like models: the injection of brain homogenates with α-synuclein (preformed fibrils) has shown to provoke a prion-like propagation in the striatum of WT mice (Luk et al., 2012). Between three and six months later, a slow dopaminergic degeneration and motor complications appear in these mice, modelling a parkinson-like phenotype.
- rAAV-mediated models: the injection of viral vectors coding for the A53T mutated α-synuclein in the SNpc of mice lead to the formation of α-synuclein aggregates (Castro-Sánchez et al., 2018). As a result, there is dopamine depletion in the striatum consequent

with the neuronal death observed in SNpc, accompanied by microgliosis and alterations in their motor performance in motor tests.

- Neuromelanin aggregation model: the overexpression of tyrosinase, the first-step enzyme which synthesizes neuromelanin, in the SN of rats led to intracellular neuromelanin accumulation followed by formation of α-synuclein aggregates, hypokinetic symptoms, and dopaminergic degeneration of the nigrostriatal neurons (Carballo-Carbajal et al., 2019), supporting the new role attributed to neuromelanin beyond its classic biomarker assignment as the underlying pathological mechanism of aging.

1.5.4. In vitro *models*

Although *in vitro* models are less representative than a complete biological system, they are a useful tool for the study of molecular mechanisms of specific features of PD in a more controlled environment, as well as for a first-step compound screening. The range of models is wide, so we will mention some of them, including the ones used in this Doctoral Thesis:

- Human induced pluripotent stem cell (hiPSC)-based models: different studies have given proof-of-principle that it is feasible to produce hiPSC-derived dopaminergic neurons from patients with sporadic PD, although most hiPSC-based PD models have been developed from patients carrying genetic mutations. These models present some pathological features which have been described in post-mortem PD brains or in relevant animal models, such as α-synuclein accumulation, mitochondrial dysfunction, or oxidative stress (Kouroupi et al., 2020).
- SH-SY5Y cells: SH-SY5Y is a human neuroblastoma cell line widely used as PD model. This cell line can undergo differentiation to different types of neurons, but even without differentiation they express DAT and the DA synthesis enzymes (Xie et al., 2010). To mimic PD pathology in these cells, different strategies have been used, mainly based on (i) neurotoxin exposures, for example to MPTP, 6-OHDA and rotenone, which are used to generate *in vivo* models (Xicoy et al., 2017), mainly reproducing the mitochondrial dysfunction, oxidative stress, and cell death; and (ii) genetic approaches, which can be either knockdown or forced overexpression of genes associated with familiar PD. For example, one model comes from the overexpression of α-synuclein (WT or with a PD associated mutation, such as A53T) to provoke lysosomal dysfunction and autophagy impairment in the SH-SY5Y neurons (Nascimento et al., 2020).
- LPS-stimulated BV2 cells: the BV2 microglial cell line when stimulated with LPS provides a model of neuroinflammation, one of the pathological events of PD. As neuroinflammation is a common feature in other pathologies this model is not exclusively of PD but is very useful for the study of anti-inflammatory compounds, especially if compared with an *in vivo* model (Yan et al., 2019). This approach can also be useful for neuroprotection studies, as other cell lines (neurons) can be exposed to the conditioned media generated after the stimulation with LPS of the BV2 cells, to study the effects of an inflammatory environment.

2. CANNABINOIDS

The term “cannabinoids” was initially used to name the terpenophenolic compounds of the *Cannabis sativa* plant, but further research allowed this term to also include the endogenous lipids that target specific elements of the endocannabinoid system (ECS). From that moment, the cannabinoid compounds can be classified as plant-derived or natural (phytocannabinoids), endogenous signalling lipids (endocannabinoids) and also the synthetic cannabinoid derivatives obtained in the laboratory (Andre et al., 2016). Though *Cannabis sativa* has been used for thousands of years, for both medical and recreational purposes, the scientific research on this field just started to develop some decades ago. The first phytocannabinoid to be described was the Δ^9-tetrahydrocannabinol (THC), the main psychoactive compound of the plant (Gaoni and Mechoulam, 1964). By now, more than 140 phytocannabinoids have been described. They are characterized by a highly lipophilic profile, which influences their biodisponibility and mechanism of action, and they have been related to a large number of physiological functions and pathological conditions. Consequently, during the last decades, the research on cannabinoids and their therapeutic potential has gained great interest and many other cannabinoids and their different targets on the human body have progressively been described (Cristino et al., 2020).

2.1. Endocannabinoid system

The endocannabinoid signalling system is one of the most abundant cell and tissue modulators of the nervous system, with important functions such as synapsis modulation in neurons and interaction with other neural cells and communicating systems. Moreover, the ECS can modulate the function of proteins and nuclear factors involved in cell proliferation, differentiation, and survival (Bisogno, 2008). Likewise, it has been shown that cannabinoids play an important role in neuroprotection, nociceptive control, memory, neurogenesis, regulation of appetite, metabolism, emotional processes, thermoregulation, modulation of the immune response, and endocrine regulation systems (Di Iorio et al., 2013). Some of the effects with more interest for therapeutic research are those addressed to preserve, rescue, repair, and/or replace neurons and glial cells against multiple insults that may potentially damage these cells. This potential seems specially promising for both acute and chronic neurodegenerative pathological conditions (Fernández-Ruiz, 2009).

The main elements forming the ECS are the endogenous ligands or endocannabinoids (eCBs), the enzymes involved in their synthesis and degradation, and the different cellular receptors available for eCBs, in which cannabinoid receptors type 1 and 2 (CB1 and CB2, respectively), are the most relevant. The two main endogenous ligands of the ECS are anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Figure 8). They are small signalling molecules, derived from lipids, which are synthesised on demand. The presence of specific lipid precursors and the increase in the levels of intracellular calcium trigger the synthesis of eCBs (Joshi and Onaivi, 2019).

AEA is a partial agonist of the CB1 receptor and has lower effect on the CB2 receptor, but it has an agonist effect on other cannabinoid related receptors, such as the transient receptor potential vanilloid type 1 (TRPV1). AEA is synthesized from *N*-arachidonoyl

phosphatidylethanolamine (NAPE) by the enzyme NAPE-phospholipase D (NAPE-PLD), both in the postsynaptic terminals (Figure 9) and in glial processes (Di Marzo et al., 1994). Also in the postsynapsis, AEA hydrolysis to arachidonic acid (AA) and ethanolamide is carried out by fatty acid amide hydrolase (FAAH) (Figure 9). This enzyme also participates in the degradation of other acylethanolamines, such as *N*-palmitoylethanolamine (PEA) and *N*-oleoylethanolamine (OEA), which are other minoritarian endocannabinoids (Ahn et al., 2008).

2-AG is a full agonist of CB1 and CB2 receptors and more abundant than AEA. It is mainly synthesized from diacylglycerol (DAG) by the isoform α of diacylglycerol-lipase (DAGLα), expressed in microglia, astrocytes, and postsynaptic neurons (Tanimura et al., 2010). Once the 2-AG has exerted its function, it is metabolized to AA and glycerol (Figure 9), mainly by monoacylglycerol lipase (MAGL), which is located in the axon terminals of the presynaptic neurons (Dinh et al., 2002). However, 2-AG can also be degraded by other enzymes, such as FAAH and the α/β-domain hydrolase 6 (ABDH6) and 12 (ABDH12). ABDH6 is mostly localized in the postsynaptic terminal (contrarily to MAGL), while ABDH12 is expressed mainly in microglial cells (Navia-Paldanius et al., 2012).

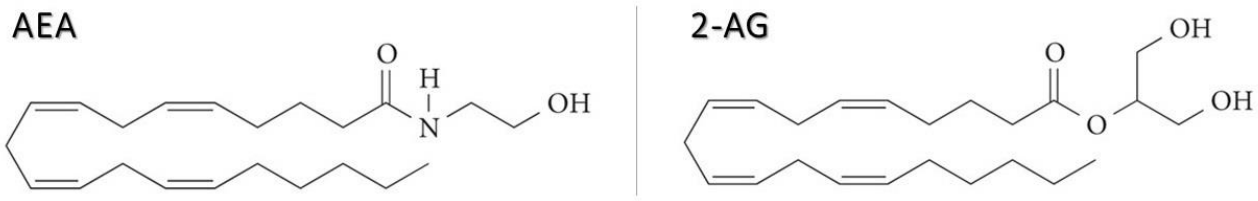

Figure 8. Chemical structure of the main endocannabinoids.

While their biosynthesis and metabolism have been clearly described, the transport mechanisms of the eCBs across the cell membrane still need to be elucidated and determine if there is a putative endocannabinoid membrane transporter (EMT), as some pharmacological (inhibition) studies indicate (Nicolussi and Gertsch, 2015). Alternatively, other less specific mechanisms have been suggested, such as passive diffusion through the membrane and binding to caveole-rich membrane domains, as well as their transport through the cytosol by fatty acid binding proteins (FABPs). However, there would still be a lack of specificity that needs to be further discussed (Nicolussi and Gertsch, 2015).

2.2. Cannabinoid receptors

The cannabinoid receptors, CB1 and CB2, are part of class A of the superfamily of G-protein coupled receptors (GPCRs), located in the plasmatic membrane with 7 transmembrane domains, an extracellular N-terminal domain, and an intracellular C-terminal domain, and generally associated with Gi/o heterotrimeric proteins. Their protein sequences share a 44% of homology (Munro et al., 1993) and are highly conserved in the other animal organisms, both vertebrates and invertebrates (Elphick, 2012).

CB1 receptor is the most abundant metabotropic receptor of the brain, but it is also present in the peripheral nervous system (PNS) (Zou and Kumar, 2018). Its expression is

particularly high in the basal ganglia, hippocampus, and cerebellum (Herkenham et al., 1990). This receptor is mainly localized in the presynaptic membrane of the axonal terminals, predominantly in both GABAergic and glutamatergic neurons, but also in other phenotypes as well as postsynaptically and in glial cells and neuronal progenitors (Howlett, 2002). From a subcellular perspective there is also an important expression of CB1 in the mitochondrial membrane, mitochondrial CB1 receptor (mtCB1), which has a separate regulation involved in the modulation of mitochondrial respiration (Hebert-Chatelain et al., 2014).

The primary function of the CB1 receptor is to modulate the presynaptic neurotransmitter release (Figure 9), which makes it crucial for essential neuronal plasticity processes, such as depolarization-induced suppression of inhibition (DSI) and of excitation (DSE). Also, when it happens on the long term, CB1 is involved in long-term potentiation (LTP) and long-term depression (LTD), mainly by retrograde signalling (Castillo et al., 2012). The activation of CB1 receptor implies the activation of $G_{i/o}$ proteins, which inhibit the activity of the adenylate cyclase (AC), thus reducing the levels of cAMP (cyclic adenosine monophosphate) (Bonhaus et al., 1998). This leads to a reduction in the activation of protein kinase A (PKA) and of the intracellular calcium levels, by closing N and P/Q calcium channels, and to the opening of G-protein activated internal rectifying K^+ (GIRK) channels, causing a more negative resting membrane potential (Figure 10). Right after, CB1 is phosphorylated by GRK (G-protein receptor kinase) to recruit β-arrestins, resulting in the receptor internalization (Walsh and Andersen, 2020). The activation of CB1 also triggers other signalling pathways, such as ERK1/2 and MAPK pathway as well as PI3K/Akt/mTORC1 pathway. Under some conditions, CB1 is also able to bind to G_s and G_q proteins, ending in an increased calcium release from the endoplasmic reticulum via phospholipase C (PLC) and 1,4,5-triphosphate (IP3) and therefore in the activation of other downstream signalling pathways (Zou and Kumar, 2018). Being CB1 expression and signalling pathways so diverse, it seems important to bring out the fact that the efficacy of the G-protein is not intrinsic to the receptor, as it has been demonstrated that there are variations according to the cellular type and the subcellular localization of CB1 (Jimenez-Blasco et al., 2020).

CB2 receptor is mainly expressed in immune tissues and cells (Galiègue et al., 1995) among other peripheral tissues, and thus it has always been considered the "peripheral" cannabinoid receptor. However, in the CNS its expression has been well described in microglial cells (Núñez at al., 2004) and astrocytes (Fernández-Trapero et al., 2017), especially in a neuroinflammatory context. Likewise, after years of controversy, CB2 expression has been demonstrated in a subset of neurons at detectable and functionally relevant levels (Atwood and Mackie, 2010). Despite its lower expression in comparison with CB1, it is remarkable that CB2 plays an important role in different neurological processes, such as nociception, neuroinflammation and neurogenesis (Palazuelos et al., 2012). As for CB1, the signalling pathway triggered after CB2 activation is mediated mainly by $G_{i/o}$ proteins (Figure 10). It is also important to mention the existence of heteromers formed between CB1 and CB2, which are characterized by a bidirectional cross-antagonism phenomenon, as CB1 antagonists can prevent the activation of CB2 by its agonists and vice versa (Callén et al., 2012).

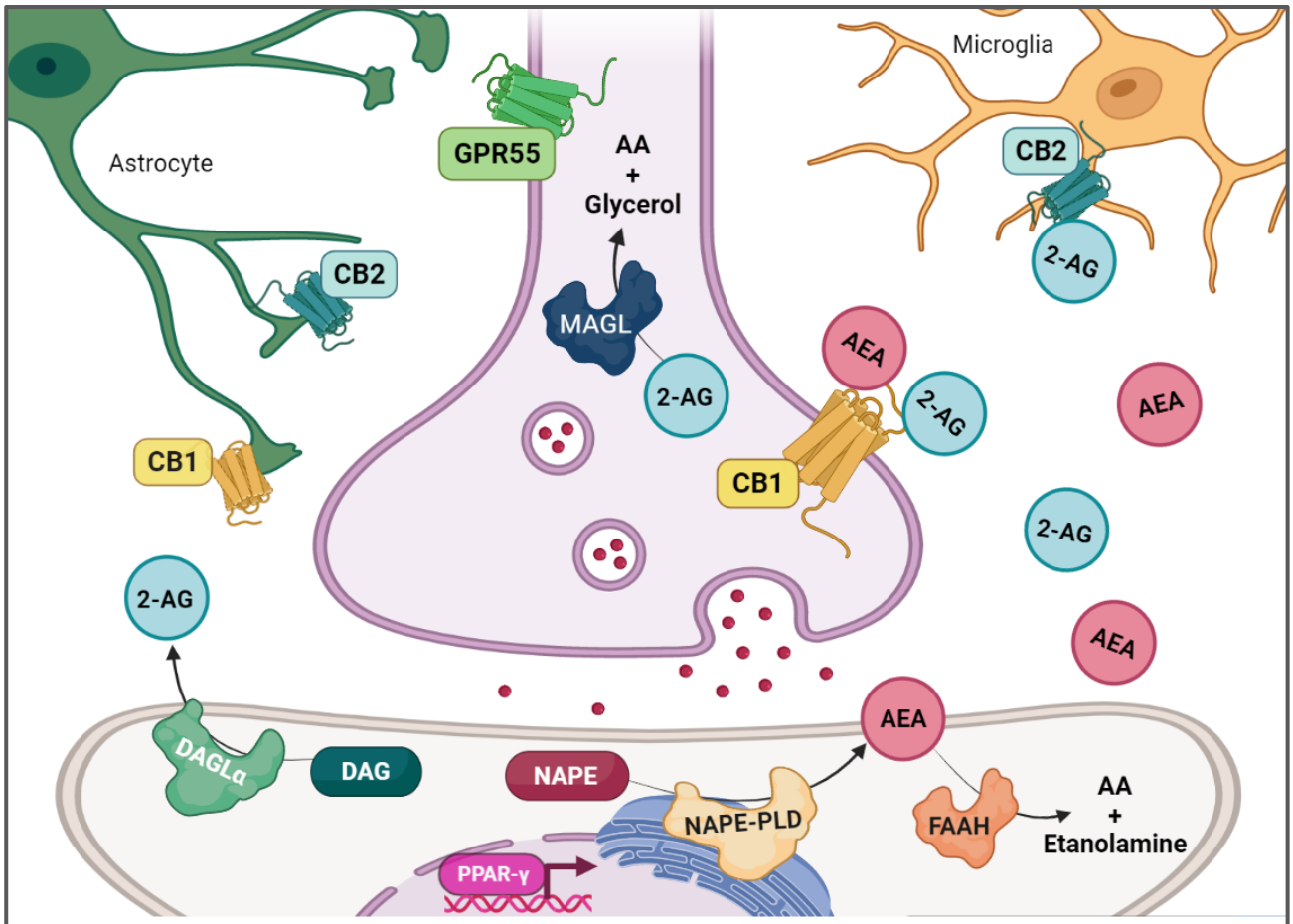

Figure 9. Schematic distribution of some of the ECS elements in the synapse cleft and pre- and post-synaptic compartments

2.2.1. Other receptors activated by cannabinoids

Apart from CB1 and CB2, there are other "non-cannabinoid" receptors that are targeted by different cannabinoid ligands. There is a great variety of this non-cannabinoid targets, as they include ionotropic receptors, such as TRPV1 (Pertwee et al., 2010); metabotropic receptors, such as the orphan receptors GPR55, GPR18 and GPR119 (Irving et al., 2017); and nuclear receptors, such as the peroxisome-proliferator activated receptors (PPARs) (O'Sullivan, 2016).

TRPV1, also known as the capsaicin receptor and the vanilloid receptor 1, is expressed in the somatosensory and viscerosensory nociceptive systems, involved in the perception of nociceptive inputs and peripheric inflammation signals. This receptor is present both in the PNS and CNS, and it has been considered as the ionotropic receptor of the ECS (Frias and Merighi, 2016). When capsaicin activates TRPV1 receptors in neurons, this channel opens and the entrance of calcium leads to membrane depolarization, activation of sodium channel and finally the generation of an action potential. Similarly, TRPV1 can be activated by AEA and other synthetic derivatives of this endocannabinoid ligand, though with less efficacy than its canonical ligands, which could be related with the analgesic functions that have been largely described for cannabinoids (Pertwee et al., 2010).

GPR55 expression has been reported in diverse CNS-derived cells, regulating several physiological and pathophysiological processes (Henstridge et al., 2011), and in both neurons and glia, remarking a potential role in neuroimmunological regulation for GPR55 (Pietr et al., 2009). Despite the several cannabinoid ligands which are able to activate GPR55, this receptor lacks the classical cannabinoid binding pocket, present in both CB1 and CB2 (Petitet et al., 2006). The binding site in the active conformation of the GPR55 receptor is vertical, deep, and with many hydrophilic residues, contrarily to the one of CB1 and CB2, which is highly hydrophobic (Kotsikorou et al., 2011). Also contrarily to CB1 and CB2, which are coupled to G-proteins of the $G_{i/0}$ subfamily, GPR55 has been shown to utilize other G-proteins, such as $G_{\alpha 13}$, $G_{\alpha q/11}$, $G_{\alpha q}/G_{\alpha 12}$ and $G_{\alpha 12/13}$ (Henstridge et al., 2011; Waldeck-Weiermair et al., 2008), for signal transduction, being especially related to inflammation (Yang et al., 2016). The signalling pathway activated by GPR55 depends on the ligand and type of cell studied, being the most relevant ones the activation of RhoA and activation of PLC (Figure 10), which triggers intracellular calcium release, both leading to the activation of MAPK pathways (phosphorylation of ERK1/2) (Henstridge et al., 2011). This signal transduction is associated with temporal changes in membrane-bound DAG, actin filament formation and plasma membrane topology, which is remodelled due to the recruitment of protein kinase C (PKC; Kapur et al., 2009). The activation of GPR55 in most cases affects the activity or formation of the same intracellular molecules, regardless of the G_{α} subunit implicated in signal initiation, ending in the activation of the MAPK cascades and transcription factors, such as NFAT, NF-κB, CREB and ATF2 (Shore and Reggio, 2015).

Both CB1 and CB2 can form heteromers with GPR55, which in general seem to attenuate GPR55 signalling pathways (Kargl et al., 2012; Balenga et al., 2014). In relation to the trafficking of this receptor, it has been seen that GPR55 is downregulated after a lingering agonist stimulation, via targeted degradation from the GPCR associated sorting protein-1 (GASP-1). Indeed, when GASP-1 is absent, GPR55 avoids intracellular degradation and is recycled back to the cell surface, suggesting an essential role of this protein in GPR55 availability (Kargl et al., 2012). Another signalling route for the internalization GPR55 goes through its interaction with β-arrestin (Kapur et al., 2009).

Regarding its pharmacology, lysophosphatidylinositol (LPI) has been confirmed as the main non-cannabinoid endogenous ligand of GPR55, acting as a potent and direct agonist (Kotsikorou et al., 2011). The main endocannabinoid ligands, 2-AG and AEA, are GPR55-activating compounds, as well as the cannabinoid molecules THC, cannabidiol (CBD) and Abn-CBD (abnormal cannabidiol), which have been also described as antagonist and inverse agonist (Shore and Reggio, 2015). Furthermore, it has been found that some cannabinoid ligands may inhibit or enhance the LPI-induced activation of GPR55 (Anavi-Goffer et al., 2012), supporting the existence of both an orthosteric and an allosteric binding site on GPR55. This follows from some cannabinoid ligands acting as agonists when applied alone but inhibiting non-competitively LPI-induced activation when applied in tandem. Thus, these findings propose the existence of bitopic ligands, which can work both as agonists and antagonists of GPR55 (Henstridge, 2012). This would be the case of AM251 and rimonabant,

cannabinoid ligands, both arylpyrazoles, which have been reported to have different effects on GPR55 depending on the assay.

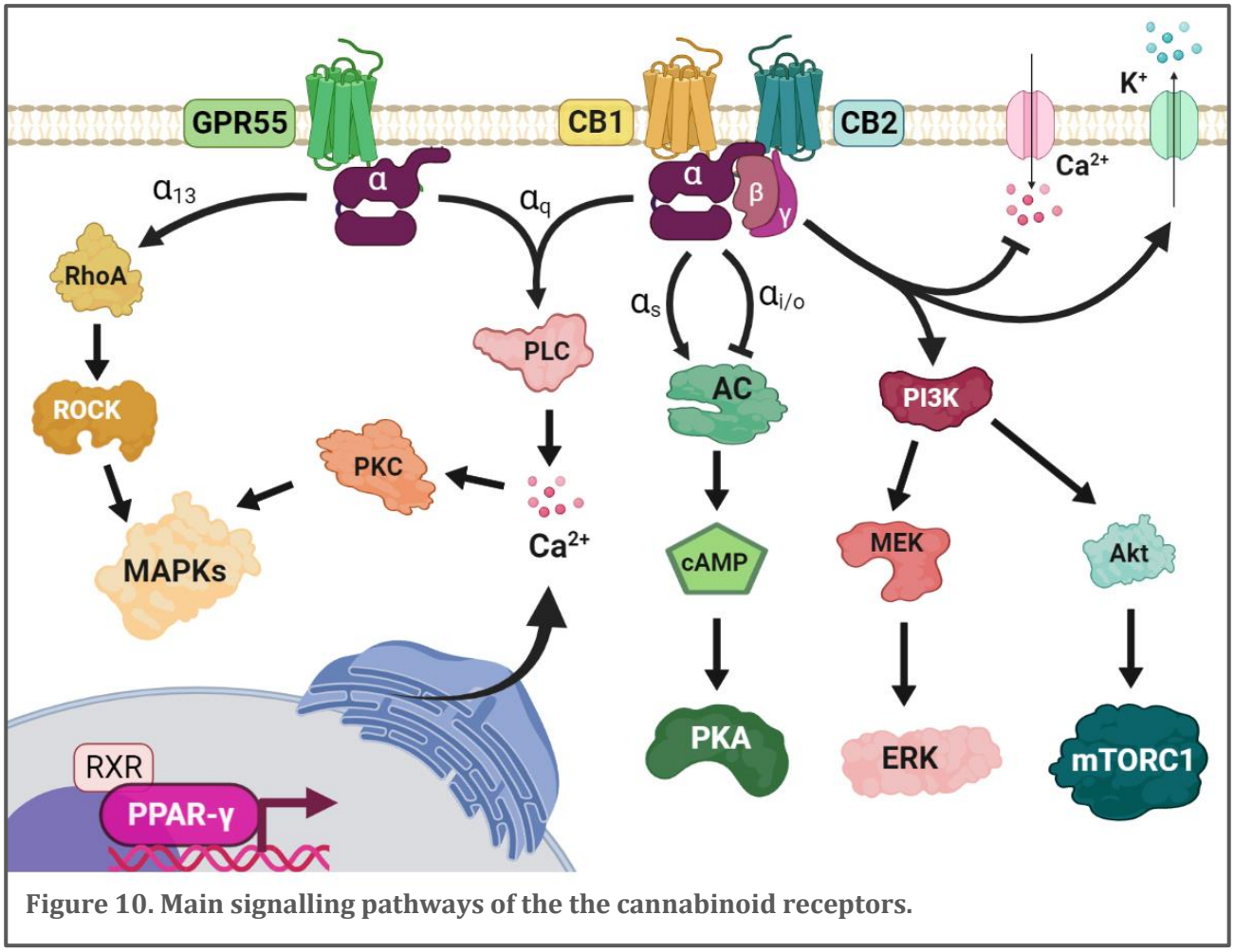

Figure 10. Main signalling pathways of the the cannabinoid receptors.

PPARs are a family of receptors expressed ubiquitously throughout the body and formed by three isoforms: α, β/δ and γ. These receptors have a DNA-binding region, highly conserved between the isoforms, and a ligand-binding region. The binding of a ligand induces the heteromerization of PPAR with the retinoid X receptor (RXR) which allows its translocation to the nucleus and the binding to PPAR response elements (PPRE) in the DNA (Figure 10). This leads to the stabilization of the complex by different coactivators (replacing corepressors) and the triggering of transcriptional answers (Kota et al., 2005). The target genes of this signalling are mostly associated with glucose and lipid metabolism, energetic homeostasis, cell differentiation and inflammation (Mirza et al., 2019).

The γ subtype of the PPAR family (PPAR-γ) is a nuclear receptor for hormones, fatty acids, eicosanoid, etc., that regulates mainly glucose and lipid metabolism, with special relevance in adipose tissue, where this receptor modulates adipogenesis. In the brain, PPAR-γ is expressed in all regions and mainly in neurons (Figure 9), while its expression in microglia and astrocytes is lower, and increases in response to brain damage, such as a neuroinflammatory state, thus depending on the functional state of the receptor (Warden et al., 2016). As a result, PPAR-γ receptors have been related to diverse brain functions (Cai et al., 2018), most of them

with potential therapeutic value, such as inflammation and neurodegeneration (Chiang et al., 2015), as well as in neurogenic processes, such as neuronal progenitor proliferation and neuronal differentiation (Quintanilla et al., 2014).

Eicosanoids and prostaglandin J2 are the main endogenous ligands for PPAR-γ, but there are a great variety of physiological substances that can activate this receptor, such as long-chain ω-3 fatty acids and endocannabinoids. PPAR-γ is also activated by synthetic ligands, including thiazolidinediones (such as rosiglitazone and pioglitazone), and also by some natural compounds such as phytocannabinoids, though in general they are considered weak agonists, because they are less potent than many other PPAR ligands (O'Sullivan et al., 2016; Morales et al., 2017). However, recent research has proposed a crosstalk between CB2 and PPAR-γ signalling between some cannabinoid compounds (Youssef et al., 2019), which could be interesting for a pharmacological approach aiming for an anti-inflammatory effect.

2.3. Cannabinoid ligands

2.3.1. Phytocannabinoids

Cannabis sativa is the source of this subtype of cannabinoid compounds, as "phytocannabinoids" refers to the molecules extracted from this plant which generally have a terpenophenolic structure (Figure 11).

THC is the most abundant component of the plant and is responsible for the psychotropic effects of marijuana as well as for the so-called cannabinoid-induced tetrad: hypolocomotion, hypothermia, catalepsy and antinociception (Pertwee, 2008). THC is a classic cannabinoid, as it binds both CB1 and CB2 receptors, with preference for CB1, which also mediates most of the THC activities (Zimmer et al., 1999).

CBD is the second main phytocannabinoid, the major non-psychotropic one, and it is an atypical cannabinoid, this is, it does not elicit the cannabinoid-induced tetrad, as it has low (or negligible) affinity for CB1 and CB2 receptors (McPartland et al., 2007), though it has been recently suggested to act as an allosteric modulator of both CB1 (Laprairie et al., 2015) and CB2 (Martinez-Pinilla et al., 2017). Most of its effect are mediated by its interaction with non-cannabinoid receptors, such as serotonin receptors 5-HT1A (Espejo-Porras et al., 2013) and PPAR-γ receptors (Esposito et al., 2011); in an indirect way, the adenosine receptors 2A (Mecha et al., 2013); as well as many other channels and enzymes.

Among many other biological effects, CBD is involved in immunomodulation, angiogenic processes, and modulation of neuronal and cardiovascular function, which makes it useful for different pathological conditions, such as neurological diseases and cancer. CBD has also demonstrated to exert important antioxidant properties, as it modulates intracellular calcium and reduces glutamate release. The efficacy of CBD as an anticonvulsant drug has been demonstrated in different type of epilepsy syndromes, even as a treatment for refractory epilepsies, such as those present in Dravet syndrome, both in animal models (Kaplan et al., 2017) and clinical trials (Devinsky et al., 2017), currently known under the brand name Epidiolex®. Another important feature of CBD is to act as an entourage molecule, modulating

the collateral effects of THC, which increases its own potential as a therapeutic agent (Pisanti et al., 2017) and could be related to its potential allosteric activity at the CB1 receptor. Indeed, the combination of both compounds in a 1:1 proportion, known as Sativex®, has already been approved to treat multiple sclerosis spasticity (Patti et al., 2016), as well as for the treatment of pain in cancer patients (Johnson et al., 2013).

CBG (cannabigerol) is another important but less studied phytocannabinoid, which is also devoid psychotropic effects and has no activity at neither CB1 nor CB2 receptors. It has been seen that CBG is the most potent agonist of the PPAR-γ receptors among the phytocannabinoids, showing antioxidant, anti-inflammatory, and neuroprotective effects (Granja et al., 2012). However, as many other cannabinoids, it has multiple targets, as it can also act as an agonist of TRPV1, TRPA1 and α2 adrenergic receptors, and as an antagonist of TRPM8 and 5-HT1A receptors (Cascio et al., 2010; Borrelli et al., 2014).

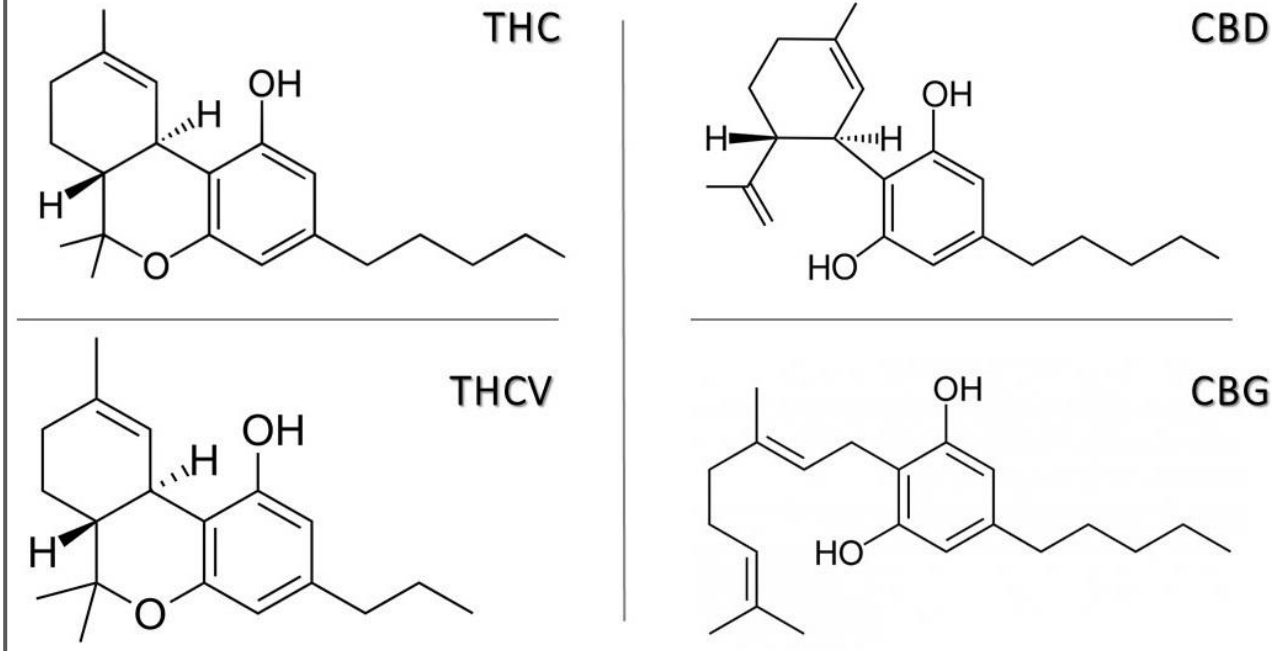

Figure 11. Chemical structures of the main phytocannabinoids.

THCV (Δ^9-tetrahydrocannabivarin) is the *n*-propyl analogue of THC, which also shares analogue effects with THC, as it can produce cataleptic behaviour and antinociception through the activation of CB1 receptor, but with less potency than THC (Pertwee, 2008). However, it has also been shown that THCV administered *in vivo* at lower doses (under 5 mg/Kg) can act as a CB1 antagonist, as it can block the CB1-mediated effects of endocannabinoids or other CB1 agonists (Thomas et al., 2005). Regarding CB2 receptors, THCV was initially proposed as an antagonist *in vitro*, but when administered *in vivo* it behaves as a CB2 partial agonist, displaying anti-inflammatory and neuroprotective responses to similar extent as other full CB2 agonists, such as HU-308 (García et al., 2011). Other studies have also reported THCV to

be an activator or a partial agonist of GPR55, different TRP channels and 5-HT1A receptors (Morales et al., 2017).

2.3.2. Synthetic cannabinoids

Since the study of cannabinoids began, a variety of new chemical structures of these compounds has been generated in numerous laboratories and pharmaceutical companies, which has allowed new forms of classification of cannabinoids, as well as to synthesize analogues or derivatives of both endocannabinoids and phytocannabinoids. Specifically, 'synthetic cannabinoid' refers to any compound that targets the ECS or that comes from another cannabinoid, but that has been chemically synthesized in a laboratory.

The **agonist ligands** can be divided in four groups, according to their chemical structure:

- Classical cannabinoids, which include all the dibenzopyran derivatives. They are mainly represented by THC and its synthetic analogue HU-210, with higher affinity and efficacy for CB1/CB2 receptors because of its dimetylheptyl chain (Ottani and Giuliani, 2001). Another important synthetic cannabinoid of this group is the CB2-selective agonist, JWH-133 (Huffman, 2005).
- Nonclassical cannabinoids, which include bicyclic and tricyclic analogues of THC that lack a pyran ring, such as CP-55940, which is also a non-selective agonist of both CB1 and CB2 receptors, but with a higher affinity and potency (Pertwee et al., 2010). This group also includes a potent and highly selective CB2-agonist, the synthetic cannabinoid HU-308 (Hanus et al., 1999) which also binds some off-target receptors, such as TRPV1 and DAT (Soethoudt et al., 2017).
- Eicosanoids, having the structure of the endocannabinoids AEA and 2-AG. This group includes compounds like R-(+)-methanandamide (more stable than AEA) and arachidonoyl-2-chloroethylamide (ACEA), which are both CB1-selective agonists (Pertwee et al., 2010).
- Aminoalkylindoles, which are the most different ones in structure, as they are derived from pravadoline, a NSAID-like compound. The main representant of this group is the full CB1/CB2 agonist R-(+)-WIN55212, as well as the CB2-selective agonists JWH-015 and AM-1241 (Howlett et al., 2002).

Most of the CB1/CB2 agonists mentioned contain chiral centres and have a greater pharmacological activity than their stereoisomers in cannabinoid receptor binding assays. The exception is the endocannabinoids, both AEA and 2-AG, which lack a chiral centre. Another aspect to be considered is that, although some of the cannabinoid agonists mentioned are selective of the CB1 and/or CB2 receptors, others can also have off-target receptors outside the ECS (Pertwee, 2010).

The **antagonist ligands** are usually selective, with much greater affinity for one of the cannabinoid receptors, unlike the agonists, which can be non-selective in many cases. Also attending to the chemical structure, one of the main types of antagonists are the diarylpyrazoles, being the most known ones SR141716A (rimonabant), a CB1-selective antagonist, and SR114528, a CB2-selective antagonist (Shire et al., 1999). Another important

chemical series is 6-ioprovadalina (AM-630) which is a CB2-selective antagonist, though it has other non-cannabinoid targets, such as TRPA1 (Howlett et al., 2002). As well as blocking CB1 or CB2 receptors competitively, these compounds can behave as inverse agonists, as they by themselves produce inverse cannabimimetic effects (Pertwee, 2010). Neutral antagonists are scarce, all of them synthetic cannabinoids derived from rimonabant or phytocannabinoids, except for the phytocannabinoid THCV, which is a neutral CB1-antagonist at low doses.

3. CANNABINOIDS IN PARKINSON'S DISEASE

There is extensive evidence about the important relationship between the ECS and PD that supports the use of a cannabinoid-based therapy to combat the pathological events underlying this disease, mainly based on four facts: (a) the different elements of the ECS are highly expressed in the basal ganglia structures and their expression and regulation appear affected by the disease; (b) the role of the endocannabinoid system in the basal ganglia implies the modulation of neurotransmission and thus is fundamental both in healthy and pathological conditions (Aymerich et al., 2018); (c) the basal ganglia structures control the general motor function, and the modulation of the ECS in these areas is associated with important motor responses, even in pathological conditions, as PD; (d) the increasing preclinical evidence of effective pharmacological treatments with cannabinoids in different parkinsonian models (Fernández-Ruiz et al., 2015).

Endocannabinoids are highly expressed in the basal ganglia and their levels increase during PD, both in humans and animal models (Figure 12). In CSF samples of PD patients AEA levels were two-fold higher than in controls, with no correlation between AEA levels and disease progression. However, this overexpression was not present in patients under treatment with L-DOPA or dopaminergic agonists (Pisani et al., 2011). In the striatum of MPTP-treated monkeys both AEA and 2-AG levels were upregulated, and both were reversed to the control levels after levodopa treatment without correlation with the development of levodopa-induced dyskinesias (van der Stelt et al., 2005). These results are consistent with rodent neurotoxin models of PD, as in the reserpine model in rats, where the hypokinetic symptoms were accompanied by high levels of 2-AG in the globus pallidus and were reverted after dopaminergic replacement (Di Marzo et al., 2000). Also, in 6-OHDA-lesioned rats, the levels of AEA in the striatum become elevated as a consequence of a decreased activity of the anandamide membrane transporter (AMT) and the degradation enzyme FAAH, which leads to the overactivation of spontaneous glutamatergic activity (Gubellini et al., 2002). Again, these abnormal levels were completely reverted after the chronic treatment with L-DOPA and the inhibition of FAAH restored normal glutamatergic activity in 6-OHDA-lesioned rats, suggesting that the increased AEA levels imply a compensatory mechanism for the abnormal glutamatergic input (Maccarone et al., 2003). Similarly, the chronic administration of JZL184, a MAGL inhibitor, reduced the motor impairment and preserved the dopaminergic neurons of the SN of MPTP-treated mice (Fernández-Suarez et al., 2014).

CB1 receptor is expressed presynaptically in the GABAergic striatal projections that conform the direct and the indirect pathway, that is, co-expressed with D1 and D2 receptors respectively, in the MSNs. CB1 can also be found in the nuclei that receive these projections:

GPe, GPi, and SNpr. In the striatum, CB1 also appears in the interneurons expressing parvalbumin. Regarding glutamatergic neurons, CB1 is located in the presynapsis of the cortical afferents that target the basal ganglia and in the nigral projections to the subthalamic nucleus. Contrarily, nigrostriatal dopaminergic neurons do not express CB1 receptors, though they can display cannabinoid signalling as they do express TRPV1 receptors, targeted by many cannabinoids (Fernández-Ruiz et al., 2011).

As CB1 is highly expressed in the basal ganglia, its modulation can have different effects on the motor symptoms depending on the development stage of PD. In pre-symptomatic stages of the disease, when neuronal malfunctioning begins, CB1 receptor undergoes desensitization or downregulation, favouring the progression of the nigral degeneration due to an enhancement of the excitotoxicity and, to a lower extent, more oxidative stress, and increased inflammation. In intermediate or advanced symptomatic phases, when the bradykinesia has shown up and there is degeneration, including neuronal death, there is an overexpression of CB1 receptor. The biphasic dysregulation of CB1 has been described in genetic models lacking parkin and α-synuclein (García-Arencibia et al., 2009b), in neurotoxin models, such as 6-OHDA-treated rats (Walsh et al., 2010) and MPTP-treated marmosets, as well as in the basal ganglia of post-mortem samples of PD patients (Lastres-Becker et al., 2001), where the increase could be reversed by L-DOPA treatment (Figure 12). Using PET and MRI, the higher levels of expression of CB1 receptors have been also described *in vivo* in PD patients, with no correlation with levodopa-induced dyskinesia severity (Van Laere et al., 2012).

This CB1 upregulation implies motor inhibition, suggesting that the blockade of CB1 could have beneficial effects on the hypokinetic symptoms. Nevertheless, CB1 deficient mice show increased vulnerability to 6-OHDA lesions, suggesting a potential neuroprotective effect for this receptor too (Pérez-Rial et al., 2011). Other studies have shown that CB1-activating compounds are able to reduce the tremor, compensating the overactivity of the subthalamic nucleus (Sañudo-Peña et al., 1998). CB1 agonists can reduce the glutamate toxicity in the basal ganglia through the inhibition of neurotransmitter release, thus showing a neuroprotective profile, and have also shown anti-dyskinetic effects in some LID models (Morgese et al., 2007). However, they may not be so useful for PD on the long term, as the activation of CB1 receptors would worsen the hypokinetic signs (such as bradykinesia, immobility, postural instability) due to an increased GABAergic activity, despite the reduction of the tremor, and let alone the unwanted central effects triggered by CB1 in the CNS (García-Arencibia et al., 2009a). The efficacy of CB1 antagonists has been described mainly as a hypokinetic therapy, enhancing the dopaminergic agonists symptom relieving (Cao et al., 2007), as well as ameliorating the dyskinetic state after chronic L-DOPA treatment (Gutiérrez-Valdez et al., 2013)

CB2 receptor is mainly expressed in glial cells, both in astrocytes and microglia, but with low expression in healthy conditions. However, in response to different types of cytotoxic insults that imply inflammation-driven neurodegeneration, as those taking place in PD, CB2 receptor expression gets upregulated in astrocytes and activated microglia (Ashton and Glass, 2007). Although the presence of CB2 receptors in neurons has been controversial, recent

studies support their expression in some basal ganglia structures, such as the globus pallidus (Lanciego et al., 2011) as well as in the nigrostriatal tyrosine hydroxylase (TH) positive neurons of the SNpc, which are the dopaminergic neurons that degenerate during PD progression (García et al., 2015).

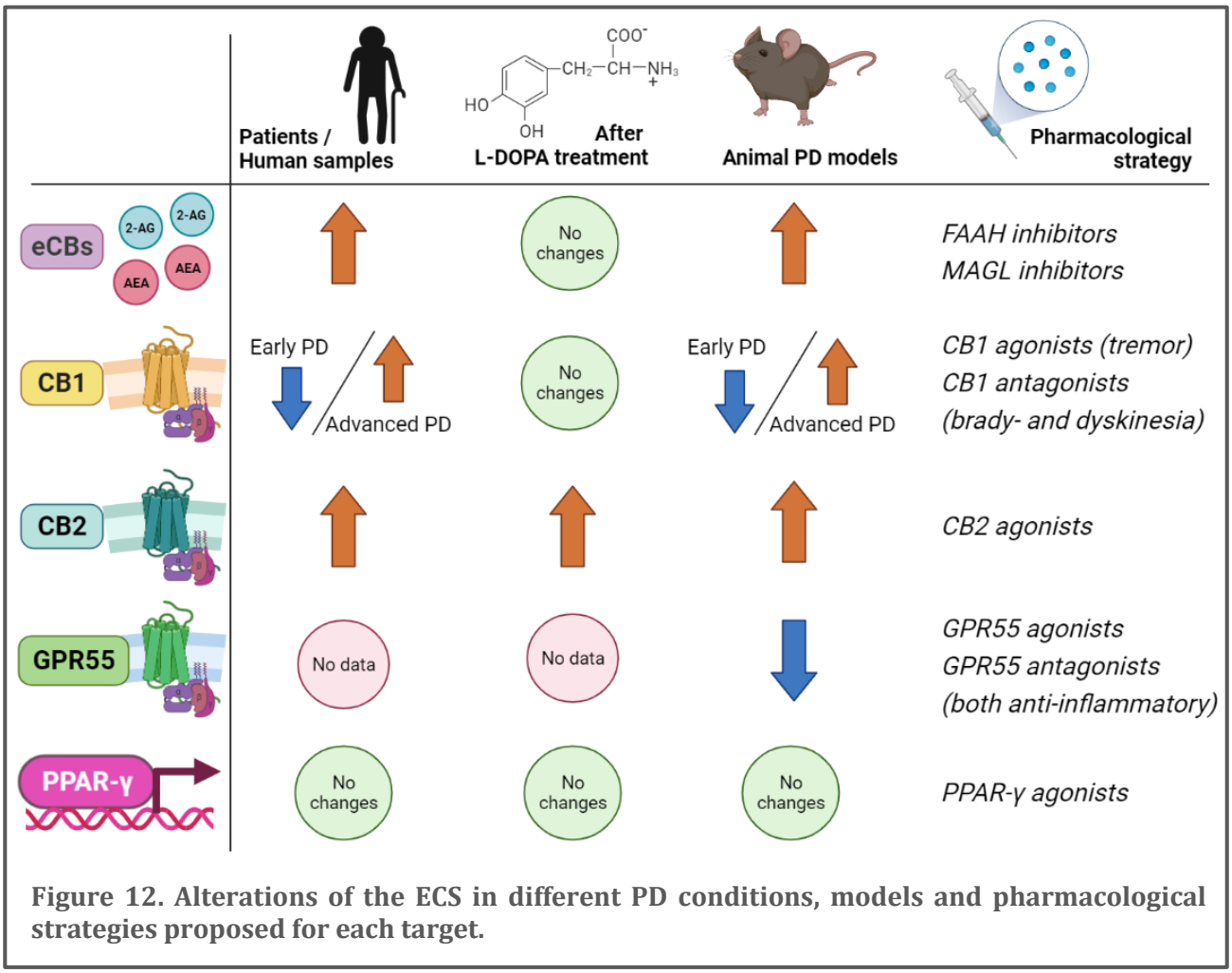

Figure 12. Alterations of the ECS in different PD conditions, models and pharmacological strategies proposed for each target.

In accordance with the neuroinflammation present in PD, there is an overexpression of CB2 receptors in the SN of PD patients (Figure 12). Its cellular substrates could be both microglial cells (Gómez-Gálvez et al., 2016) and astrocytes (Navarrete et al., 2018). CB2 upregulation has also been described in the SN and striatum of different PD models, such as MPTP-treated rats (Price et al., 2009), LPS-lesioned mice (García et al., 2011; Gómez-Gálvez et al., 2016) and rotenone-lesioned mice (Concannon et al., 2016). Furthermore, these studies show how the genetic deletion of CB2 receptors in LPS-lesioned mice implies more vulnerability to the insult, as well as the efficacy of different CB2-activating cannabinoids against local inflammation and gliosis. Activation of CB2 was also efficient in MPTP-lesioned mice, reducing neurotoxic and neuroinflammatory events and preserving dopaminergic neurons (Chung et al., 2016; Shi et al., 2017). However, in the 6-OHDA model, CB2 receptors are poorly upregulated and the genetic deficiency of CB2 in these mice had no effects on the vulnerability to the insult (García et al., 2011). Anyway, CB2 agonists were beneficial in 6-

OHDA-treated rats, though to a lesser extent, by partially reducing dopamine depletion in the striatum (García-Arencibia et al., 2007).

GPR55 receptor represents a novel target for the activity of certain cannabinoids, including that it could be a potential target in the treatment of neurodegeneration. In parkinsonian conditions, in the MPTP mice model, the expression of GPR55 is reduced both in the striatum and the SN (Celorrio et al., 2017) (Figure 12). However, the heteroreceptor complexes formed between GPR55 and either CB1 or CB2 showed increased expression levels in the striatum of MPTP-treated non-human primates, and not in the group of animals which also received chronic levodopa treatment (Martinez-Pinilla et al., 2020). Also, GPR55 knockout mice, subjected to behavioural assays, showed impaired movement coordination (Wu et al., 2013). However, GPR55 deficiency in these mice did not affect the concentrations of any component of the endocannabinoid system, neither synaptic transmission nor short and long-term synaptic plasticity, suggesting that GPR55 is not essential for the CNS development or learned behaviours, but plays an important role in motor coordination (Wu et al., 2013).

LPI-induced GPR55 activation has been observed to preserve dentate gyrus granule cells and to reduce the number of activated microglia after an excitotoxic lesion induced by NMDA (Kallendrusch et al., 2013). GPR55 has also been related with neuroinflammation, as its expression seems to be controlled by the microglial activation state, in a similar way to CB2, a fact found, for example, after the stimulation with LPS of the microglial cell line BV2, an *in vitro* model of neuroinflammation (Pietr at al., 2009). Furthermore, the pharmacological modulation of GPR55 has shown great potential in inflammatory environments, but with some contradictory results. The activation of GPR55 induces neuroprotection after an inflammatory insult (Hill et al., 2019), has anti-inflammatory effects in microglial cells stimulated with LPS (Minamihata et al., 2020) and prevents motor impairment in the MPTP model of PD protecting the dopaminergic cell bodies (Celorrio et al., 2017). However, other studies have seen the opposite, as the pharmacological treatment with GPR55 antagonists also shows anti-inflammatory effects in primary microglial cells stimulated with LPS (Saliba et al., 2018). These controversial effects of GPR55 modulation have also been described with respect to the motor functions in the 6-OHDA model in rats, where the intrastriatal administration of either LPI or of ML193 (a GPR55 antagonist) exerted the same beneficial effects over the motor impairment present in the parkinsonian rats (Fatemi et al., 2021).

PPAR-γ receptors are also targeted by different types of cannabinoids. Many studies have revealed the important role of PPAR-γ agonists in microglial activation as well as in monocytes and T-cells differentiation, in which they act as key regulators of immune responses through their ability to block the NFκB, AP-1 and Jak-Stat pathways in different CNS diseases (Carta et al., 2011). Regarding PD, the activation of PPAR-γ by non-cannabinoid ligands, such as thiazolidinediones, has shown to be effective in different PD experimental models and has recently entered in clinical investigation (Carta et al., 2015). For example, the treatment with pioglitazone of MPTP-treated mice, leads to a reduction in the loss of positive TH cells in the SN (Figure 12), although dopamine levels in the striatum were not totally recovered (Dehmer et al., 2004). Similarly, the chronic treatment with rosiglitazone prevented

the motor impairment and the dopaminergic neurodegeneration in the SN of MPTP-lesioned mice as well as inhibiting microglial and astroglial reactivity (Schintu et al., 2009). Also, rosiglitazone was able to alleviate levodopa-induced AIMs in 6-OHDA-lesioned rats, without decreasing striatal levodopa and dopamine bioavailability, as well as not affecting levodopa anti-parkinsonian activity (Martínez et al., 2015). However, thiazolidinediones present many clinical side effects such as weight gain, fluid retention, and osteoporosis (Rosen, 2010) which make difficult their clinical use, whereas small molecules as cannabinoids, also capable to act at PPARs, may provide a more secure anti-inflammatory and neuroprotective activity.

The phytocannabinoid CBG, through the activation of PPAR-γ, has demonstrated to be neuroprotective (Valdeolivas et al., 2015), and to have antioxidant (Hill et al., 2012) and anti-inflammatory properties (Granja et al., 2012). However, in an attempt to improve the pharmacokinetic profile and the potency of this compound, Emerald Health Pharmaceuticals (EHP; previously Vivacell Biotechnology España (VCE)) designed and synthesized a series of quinone derivatives of CBG (VCE-003 series), which are PPAR-γ activators but lack CB1/CB2 activity. The administration of these compounds has managed to alleviate neuroinflammation and provide neuroprotection in animal models of multiple sclerosis (Granja et al., 2012), experimental autoimmune encephalomyelitis (Carrillo-Salinas et al., 2014), and Huntington's disease (HD; Díaz-Alonso et al., 2016), disorders which share the above-mentioned pathological events with PD (Figure 13). Similarly, EHP has developed a series of quinone derivatives of CBD (VCE-004 series), which act as dual CB2/PPAR-γ agonists and that have shown great potential as therapeutic agents in systemic sclerosis (García-Martín et al., 2019), as well as anti-inflammatory and remyelination agent in multiple sclerosis (Navarrete et al., 2020). However, these compounds have also shown important peripheral effects, for example in obesity (Palomares et al., 2018) and scleroderma (García-Martin et al., 2018), which seems consistent with the abundant expression of CB2 receptors in non-nervous tissues.

Likewise, many cannabinoid compounds have cannabinoid-receptor independent antioxidant properties, due to the scavenger activity of their phenolic structure (Figure 13). The phytocannabinoids THC and CBD have shown neuroprotective potential in PD models which seem to be due to their antioxidant properties, as they can reduce ROS release and activate antioxidant pathways mediated by Nrf2 (Lastres-Becker et al., 2005; García-Arencibia et al., 2007). Also, synthetic cannabinoids may owe their neuroprotective effects to their antioxidant properties, such as the CB1/CB2 agonist CP55,940, which was effective against dopaminergic toxicity in a pesticide-based PD model (Jimenez del Río et al., 2008).

There are also many cannabinoid compounds which lack a unique target, this is, they act on a dual or multi-target manner. For example, the phytocannabinoid THCV works as a CB1 agonist at high doses and as an antagonist at low doses, which can reduce the dyskinesia produced by levodopa therapy (Pertwee et al., 2007), as well as being an antioxidant compound and a CB2 agonist, which has been seen to be neuroprotective for dopaminergic nigral neurons (García et al., 2011). It has also been described its activity on GPR55 receptor, which could contribute to its neuroprotective properties (Figure 13). In the same way, there are GPR55 ligands under development (Morales et al., 2016), which may also be useful as a pharmacological treatment for PD.

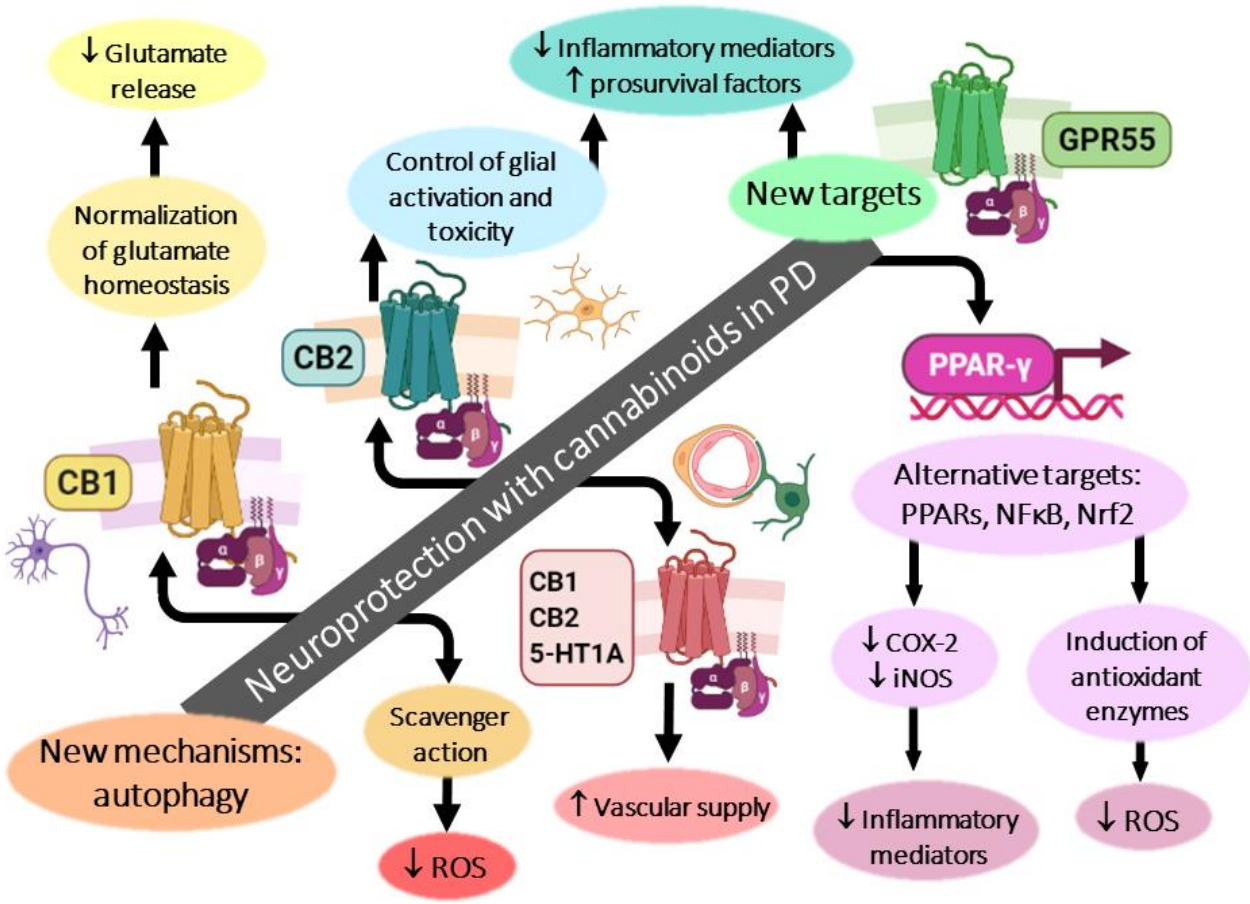

Figure 13. Molecular and cellular mechanisms proposed for the neuroprotective effects of compounds that act on the ECS. *(Adapted from Fernández-Ruiz et al., 2015).*

Finally, some of these multi-target compounds have been tried on clinical studies with PD patients, but there are still very few trials and with poor results, as none of them have shown neuroprotective properties, just subtle symptomatic amelioration, and partial relief of levodopa-induced dyskinesias (Stampanoni et al., 2017). However, the strategy followed so far on these clinical trials is not fully supported by the preclinical evidence. According to the results obtained in different PD models and mentioned above, in order to obtain an effective therapy against the main neuropathological events involved in PD, the compound or combination of compounds should be based on: 1) blocking CB1 to reduce the motor impairment; 2) activating CB2 to control the inflammatory events; 3) promoting the antioxidant effects produced by cannabinoid receptor-independent mechanisms; and 4) targeting the alternative cannabinoid receptors GPR55 and/or PPAR-γ which can contribute to the already mentioned mechanisms (Figure 13).

AIMS

As presented in the Introduction, PD is a highly prevalent disorder which implies important social and economic burdens, and which remains without a cure. Given the multifactorial pathophysiology of PD, the use of pleiotropic molecules which can be effective at several levels of the neurodegenerative process, such as cannabinoids, is needed. Besides, cannabinoids have demonstrated to have neuroprotective properties that could slow the disease progression unlike any of the treatments which are being used at the moment. In this context, different cannabinoid ligands acting on different cannabinoid targets have shown promising potential in preclinical models of PD. However, there are still new compounds or combinations of them that could report advantages over the current evidence that need to be further investigated, as well as new PD models that could contribute to a better insight into PD pathology and its pharmacological management.

Our **hypothesis** is that the administration of different cannabinoid compounds targeting CB2, PPAR-γ or GPR55 receptors will exert anti-inflammatory and neuroprotective effects in *in vitro* and *in vivo* models of PD. These cannabinoid compounds will activate different signalling pathways related to some of the neuropathological events of PD, through at least one of these receptors or the combination of the three of them. As a result, there will be a better protective effect against the death of nigrostriatal dopaminergic neurons and consequently an improvement of the motor impairments caused by the disease.

The **global aim** of this Doctoral Thesis is to progress in the development of a cannabinoid-based pharmacological therapy in PD through its preclinical evaluation in different models of PD. To determine the efficacy of the different compounds, we will focus on evaluating their antioxidant, anti-inflammatory and neuroprotective properties in *in vitro* an *in vivo* models of PD that represent these neuropathological events (mitochondrial dysfunction, oxidative stress, inflammation, aggregation of α-synuclein and neuronal death). According to the target receptor of the treatment, this global aim can be divided in three **specific aims**, which will be presented as three different chapters:

1. Preclinical evaluation of compounds targeting PPAR-γ: VCE-003.2 and other CBG derivatives.
2. Preclinical evaluation of compounds targeting CB2: the CBD derivative VCE-004.8, and the phytocannabinoid THCV, alone and in combination with CBD.
3. Preclinical evaluation of compounds targeting GPR55: VCE-006.1.

RESULTS

CHAPTER 1

Preclinical evaluation of compounds targeting PPAR-γ: VCE-003.2 and other CBG derivatives

In this chapter, we have developed a complete characterization of an oral formulation of the CBG derivative VCE-003.2, in different *in vitro* and *in vivo* models of PD, including the study of the molecular mechanisms involved in its anti-inflammatory and neuroprotective effects. In a previous study of our research group (García et al., 2018), VCE-003.2 showed potential as an anti-inflammatory and neuroprotective compound in two experimental models of PD, through the activation of PPAR-γ receptors.

Starting from there, our first aim was to increase the evidence over this compound to get closer to its clinical development, using an oral formulation of VCE-003.2, better for human use, in the inflammatory model based on LPS lesion. Although not in all the parameters analysed, VCE-003.2 presented a clearly beneficial effect in LPS-lesioned mice, as in the intraperitoneal administration. These include the reduction of the motor impairments, the recovery in the autophagic dysfunction and astrogliosis, and only a modest trend to reduce neuronal death and microgliosis in the SN.

Next, to reinforce the potential of VCE-003.2, we wanted to check its efficacy in a different model of PD, as this disease is generally characterized by different neuropathological events, needing to be investigated in different preclinical models that partially reproduce specific events of the disease. In the 6-OHDA model, related with mitochondrial damage and oxidative stress, we observed a greater neuronal death with more evident motor impairments, whereas the inflammatory parameters are secondary. The oral treatment with VCE-003.2 was able to markedly reduce the hemiparesis and the bradykinesia measured in the behavioural tests, together with a neuroprotective effect in the SN, which completely recovered the microgliosis and astrogliosis of the area. Moreover, we checked the DA/DOPAC levels in the striatum, and both were increased in the lesioned animals after the oral treatment with VCE-003.2, indicating not only a structural but also a functional recovery of the TH+ neurons of the SN. Complementarily to this study, we also tested the efficacy of two CBG derivatives in comparison with VCE-003.2. However, their effects were similar o lower than those of VCE-003.2, even though we confirmed that they were able to bind the classical binding site of PPAR-γ.

Finally, as one of the main shortcomings of these two models, LPS and 6-OHDA, is the lack of α-synuclein aggregates, we decided to evaluate VCE-003.2 efficacy using a model of PD based on local overexpression of mutant (A53T) α-synuclein administered using AAV-technology. We found a symptomatic relief in the treated group in the behavioural motor test, but both α-SYN groups had lost almost all the dopaminergic neurons, due to an excessive dose of the virus, with similar levels of microgliosis and astrogliosis independently from the VCE-003.2 treatment. RNA sequencing of the striatum revealed important changes in the expression of immune response and lysosomal genes and/or pathways in the α-SYN mice, and some of them were reverted after VCE-003.2 treatment (eg. E2F1 regulon), suggesting potential mechanisms of action.

Papers included in this chapter:

Burgaz, S., García, C., Gómez-Cañas, M., Muñoz, E., & Fernández-Ruiz, J. (2019). **Development of An Oral Treatment with the PPAR-γ-Acting Cannabinoid VCE-003.2 Against the Inflammation-Driven Neuronal Deterioration in Experimental Parkinson's Disease**. *Molecules (Basel, Switzerland)*, *24*(15), 2702.

Burgaz, S., García, C., Gómez-Cañas, M., Navarrete, C., García-Martín, A., Rolland, A., Del Río, C., Casarejos, M. J., Muñoz, E., Gonzalo-Consuegra, C., Muñoz, E., & Fernández-Ruiz, J. (2021). **Neuroprotection with the cannabigerol quinone derivative VCE-003.2 and its analogs CBGA-Q and CBGA-Q-Salt in Parkinson's disease using 6-hydroxydopamine-lesioned mice**. *Molecular and Cellular Neurosciences*, *110*, 103583.

Burgaz, S., [...] & Fernández-Ruiz, J. **Oral VCE-003.2 as a disease-modifying treatment in AAV-α-synuclein model of Parkinson's disease.** (Data not published yet).

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MDPI

Article

Development of An Oral Treatment with the PPAR-γ-Acting Cannabinoid VCE-003.2 Against the Inflammation-Driven Neuronal Deterioration in Experimental Parkinson's Disease

Sonia Burgaz [1,2,3], Concepción García [1,2,3], Maria Gómez-Cañas [1,2,3], Eduardo Muñoz [4,5,6,7] and Javier Fernández-Ruiz [1,2,3,*]

[1] Instituto Universitario de Investigación en Neuroquímica, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain
[2] Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), 28040 Madrid, Spain
[3] Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), 28040 Madrid, Spain
[4] Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), 14004 Córdoba, Spain
[5] Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, 14004 Córdoba, Spain
[6] Hospital Universitario Reina Sofía, 14004 Córdoba, Spain
[7] Vivacell Biotechnology España, 14004 Córdoba, Spain
* Correspondence: jjfr@med.ucm.es; Tel.: +34-913941450

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Abstract: In a recent study, we described the neuroprotective properties of VCE-003.2—an aminoquinone derivative of the non-psychotropic phytocannabinoid cannabigerol (CBG)—administered intraperitoneally (i.p.) in an inflammatory model of Parkinson's disease (PD). We also demonstrated that these properties derive from its activity on the peroxisome proliferator-activated receptor-γ, in particular at a regulatory site within this receptor type. In the present study, we wanted to further confirm this neuroprotective potential using an oral lipid formulation of VCE-003.2, developed to facilitate the clinical development of this phytocannabinoid derivative. To this end, we evaluated VCE-003.2, administered orally at two doses (10 and 20 mg/kg), to mice subjected to unilateral intrastriatal injections of lipopolysaccharide (LPS), a classic model of inflammation-driven neuronal deterioration that recapitulates characteristics of PD. The administration of VCE-003.2 to these mice showed, as expected, poor activity in the different motor tests (rotarod, computer-aided actimeter) used in experimental parkinsonism, in general due to the lack of evident changes in these behaviors by LPS lesion. However, VCE-003.2, at 20 mg/kg, was highly active in improving the changes detected in LPS-lesioned mice in the cylinder rearing test. In addition, the histopathological analysis of the basal ganglia revealed a trend towards recovery at 20 mg/kg VCE-003.2 in the loss of tyrosine hydroxylase-containing nigrostriatal neurons, as well as a complete reduction in the elevated LAMP-1 immunolabeling (reflecting autophagy impairment) caused by LPS lesion. These effects were not seen at 10 mg/kg. This was associated with a partial reduction in the intense glial reactivity provoked by LPS in the substantia nigra, in particular the astroglial reactivity labeled with glial fibrillary acidic protein. The analysis using qPCR in the striatum of proinflammatory mediators, such as tumor necrosis factor-α, interleukin-1β, inducible nitric oxide synthase, and cyclooxygenase-2, showed that the marked elevations provoked by the LPS lesion tended to be, in general, attenuated by VCE-003.2 treatment, with the greatest effects normally found with the highest dose of 20 mg/kg. In summary, our data confirm the neuroprotective potential of an oral formulation of VCE-003.2 against neuronal injury in an in vivo model of PD based on neuroinflammation, and this study opens the possibility to further the development of oral VCE-003.2 in the clinic.

Keywords: cannabinoids; VCE-003.2; oral formulation; PPAR-γ; γ receptors, inflammation, Parkinson's disease.

1. Introduction

Inflammation appears to be a key etiologic factor and pathogenic event in Parkinson's disease (PD), to the point that the risk of developing PD has been found to be lower in subjects chronically treated with nonsteroidal anti-inflammatory agents for other pathological conditions [1], whereas subjects bearing inflammatory cytokine gene polymorphisms might have increased risk [2]. This has promoted the investigation of strategies against neuroinflammation to limit neuronal deterioration in this disease [3–5]. Cannabinoids have been found to be highly active anti-inflammatory agents [6,7], an effect that has been normally linked to the activation of the cannabinoid receptor type-2 (CB_2), whose benefits have been already investigated in experimental models of PD [8–10]. The anti-inflammatory properties of cannabinoids have recently been reinforced with the observation that different phytocannabinoids and their derivatives, as well as the endocannabinoids anandamide and 2-arachidonoylglycerol, and related signaling lipids such as palmitoylethanolamide and oleylethanolamide, can also bind and activate specific receptor types of the peroxisome proliferator-activated receptor (PPAR) family [11,12]. These nuclear receptors play an important role in some cell and tissue functions and, in the case of PPAR-γ, they have long been involved in the control of neuroinflammatory responses [13]. This also includes the case of PD for which non-cannabinoid PPAR-γ activators such as glitazones have been found to be active in experimental PD models and have recently entered clinical investigation [14], but with the risk of important side effects. Therefore, the fact that some cannabinoids are able to activate PPAR-γ [12,15,16] enables these compounds to exert anti-inflammatory effects besides their CB_2 receptor-mediated anti-inflammatory activity and they may present a safer profile.

A series of quinone derivatives of the phytocannabinoid cannabigerol (CBG), which behave as PPAR-γ activators showing negligible affinity at the CB_1/CB_2 receptors unlike their phytocannabinoid template, have been recently designed, synthesized, and characterized [17–19]. One of them, the aminoquinone derivative of CBG, so-called VCE-003.2, was recently investigated in murine models of Huntington's disease [19] confirming its neuroprotectant profile exerted by activating PPAR-γ and its ability to cross the blood–brain barrier after systemic administration. In a more recent study [20], these anti-inflammatory and neuroprotectant properties of VCE-003.2 were investigated in lipopolysaccharide (LPS)-lesioned mice, the experimental model of PD which better reproduces inflammation as a pathogenic event in this disease [9,10]. Our data, in this in vivo PD model [20], reveal its efficacy via intraperitoneal (i.p.) administration against the inflammation-driven nigrostriatal neuronal deterioration—a fact further confirmed in in vitro studies using cultured BV2 cells stimulated with LPS as well as experiments with cultured M-213 exposed to BV2-dependent conditioned media [20]. Furthermore, the beneficial effects of VCE-003.2 were confirmed to be mediated by the activation of PPAR-γ, by binding to a functional alternative binding site within this receptor (with regulatory functions) that would be different to the canonical binding site used by glitazones [21]. We confirmed that VCE-003.2 binds to the PPAR-γ at this different site using

docking and transcriptional analyses [20]. In the present study, we wanted to further investigate this neuroprotective potential in PD using an oral lipid formulation of VCE-003.2 to facilitate the development of this phytocannabinoid derivative. Therefore, we evaluated VCE-003.2, administered orally at two doses (10 and 20 mg/kg), to mice subjected to unilateral intrastriatal injections of LPS, the same model of inflammation-driven nigrostriatal neuronal deterioration used in our previous study [20].

2. Results

The oral administration of the two doses of VCE-003.2 (10 and 20 mg/kg) to LPS-lesioned mice, as expected, resulted in poor activity in two motor tests, rotarod, and computer-aided actimeter, frequently used in our laboratory and other laboratories to detect behavioral abnormalities in experimental parkinsonism (see Reference [6] for review). However, it is important to note that such behavioral abnormalities have not been seen, in general, in LPS-lesioned mice possibly because the lesion was unilateral [9,10,20], and the present study was not an exception. Thus, the rotarod performance was not altered in LPS-lesioned mice compared to control mice (post-lesion: $F(3,49) = 0.508$, ns; before autopsy: $F(3,48) = 1.059$, ns; Figure 1). Similar results were obtained using the computer-aided actimeter with the ambulatory activity (post-lesion: $F(3,45) = 1.019$, ns; before autopsy: $F(3,49) = 0.265$, ns; Figure 1), resting time (post-lesion: $F(3,49) = 1.491$, ns; before autopsy: $F(3,49) = 0.216$, ns; Figure 1), and vertical activity (post-lesion: $F(3,47) = 0.553$, ns; before autopsy: $F(3,43) = 0.687$, ns; Figure 1), as well as with other parameters, e.g., slow and fast movements, mean and maximal velocity (data not shown).

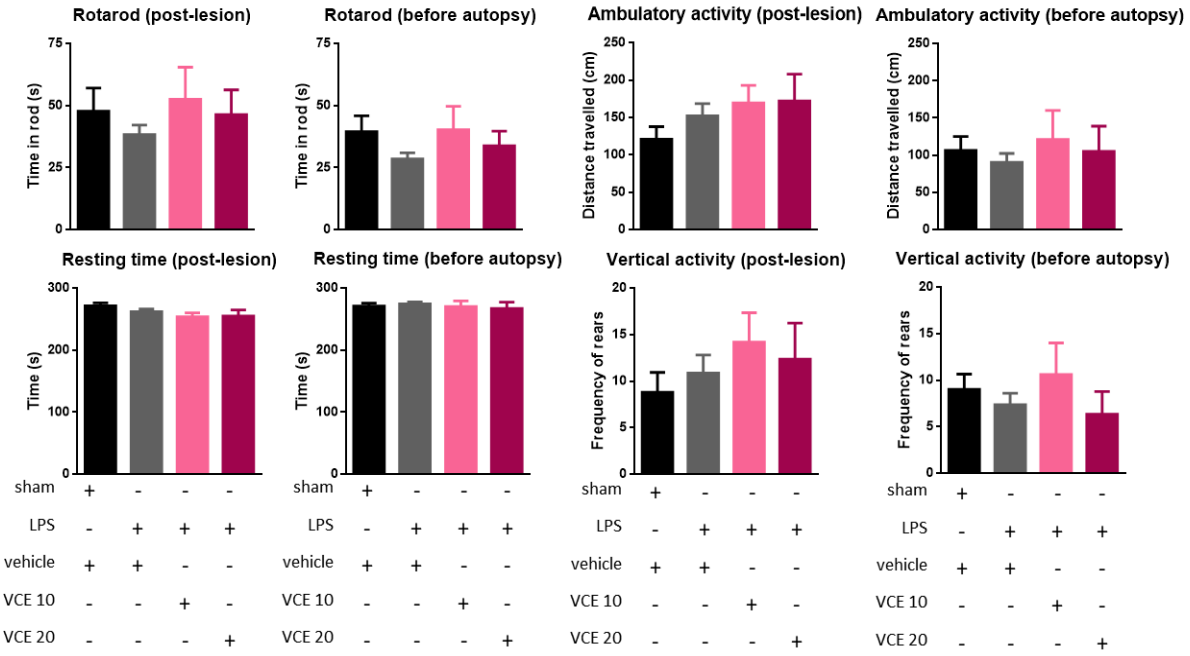

Figure 1. Rotarod performance, horizontal (ambulation) and vertical (rearing) activities, and resting time measured in a computer-aided actimeter in control and lipopolysaccharide (LPS)-lesioned mice orally treated for 28 days after lesion with vehicle (corn oil) or VCE-003.2 at the doses of 10 (VCE 10) or 20 (VCE 20) mg/kg. Data correspond to only one week after the onset of the treatment (post-lesion), or to only one day before the autopsy and after four weeks of treatment (before autopsy). Values are means ± SEM of more than six subjects per group. Data were assessed using the one-way analysis of variance followed by the Bonferroni test.

Under these conditions, the possible beneficial effects of VCE-003.2 at the two doses investigated here could not be demonstrated (Figure 1), despite certain trends that could be appreciated with the dose of 10 mg/kg of VCE-003.2 (but not with 20 mg/kg). The lack of statistical significance in the above F values, degrees of freedom, and probability levels supports that these apparent trends have no biological relevance in contrast to other trends that are discussed below.

However, in a separate experiment conducted at the highest 20 mg/kg dose of VCE-003.2 after the previous data revealed no changes at the behavioral level, we were able to prove that this CBG derivative was highly active in improving the changes detected in LPS-lesioned mice in another PD-related test, the cylinder rearing test, that may be a more relevant functional endpoint when the lesion is unilateral. Our data demonstrate that LPS lesion caused a preference for the ipsilateral paw in the cylinder rearing test, which was evident at both 1 week after the onset of the treatment ($F(2,30) = 4.542$, $p < 0.05$; Figure 2) and directly before autopsy after 4 weeks of treatment ($F(2,30) = 3.642$, $p < 0.05$; Figure 2), whereas the treatment with VCE-003.2 partially corrected this alteration, in particular at 1 week after the onset of the treatment (Figure 2).

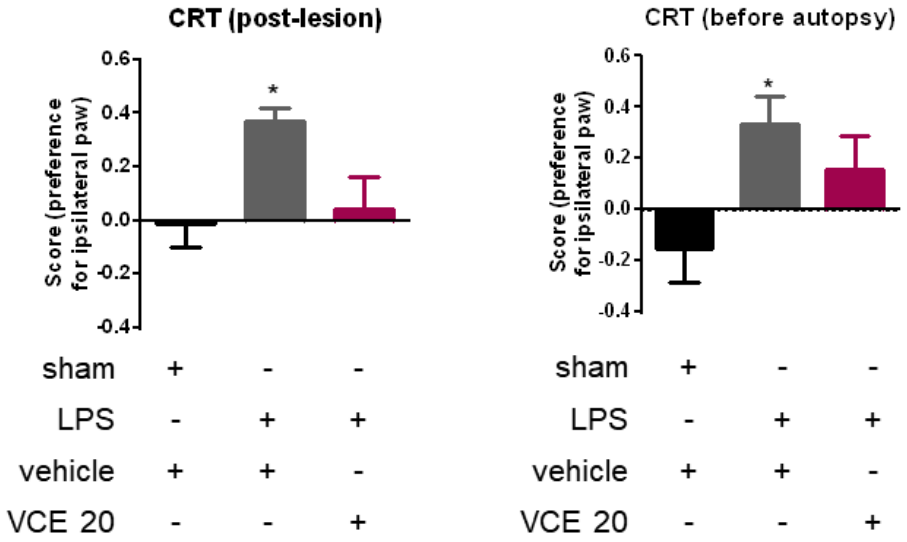

Figure 2. Preference for the ipsilateral paw measured in the cylinder rearing test in control and LPS-lesioned mice orally treated for 28 days after lesion with vehicle (corn oil) or VCE-003.2 at the dose of 20 mg/kg (VCE 20). Data corresponds to only one week after the onset of the treatment (post-lesion), or to only one day before the autopsy and after four weeks of treatment (before autopsy). Values are means ± SEM of more than six subjects per group. Data were assessed using the one-way analysis of variance followed by the Bonferroni test (*$p < 0.05$ versus vehicle-treated control (sham) mice).

The histopathological analysis of the basal ganglia revealed a trend towards a recovery by VCE-003.2, at 20 mg/kg but not at 10 mg/kg, in the loss of tyrosine hydroxylase (TH)-containing nigrostriatal neurons caused by the LPS lesion ($F(3,45) = 2.266$, $p = 0.095$; Figure 3). We also analyzed LAMP-1, a marker of autophagy, which was elevated, reflecting autophagy dysregulation in the experimental models of PD [22,23], including LPS-lesioned mice [24], and also in biological samples of patients [25]. Our data indicate a 2-fold elevation in LAMP-1 in LPS-lesioned mice and a complete recovery after the treatment with VCE-003.2, but only at the higher dose ($F(3,47) = 24.94$, $p < 0.0001$; Figure 4).

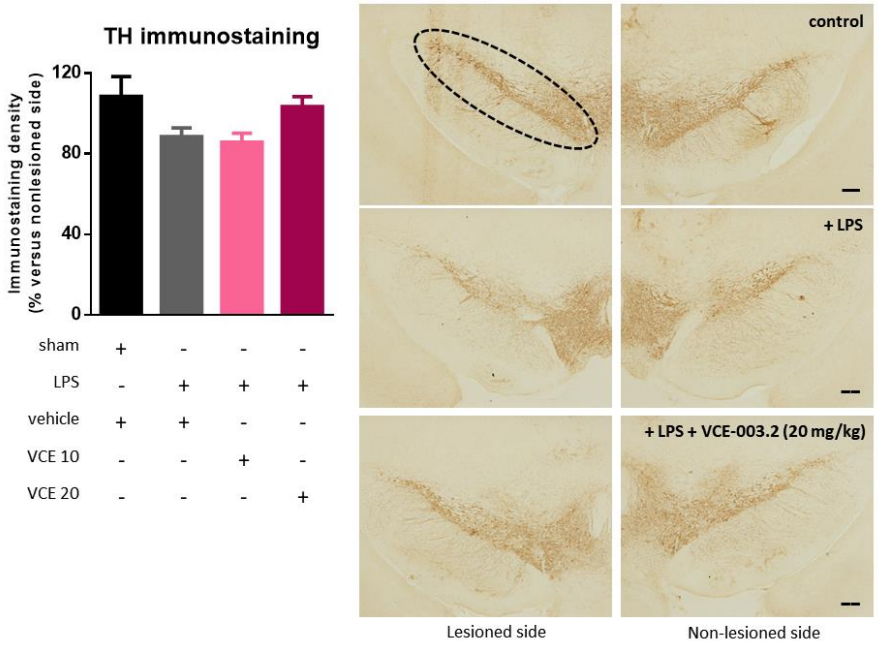

Figure 3. Intensity of the immunostaining for tyrosine hydroxylase (TH) measured in a selected area of the substantia nigra pars compacta of control and LPS-lesioned mice orally treated for 28 days after lesion with vehicle (corn oil) or VCE-003.2 at the doses of 10 (VCE 10) or 20 (VCE 20) mg/kg. Immunoreactivity values are measures in the lesioned side over the non-lesioned side and correspond to means ± SEM of more than six subjects per group. Data were assessed using the one-way analysis of variance followed by the Bonferroni test. Representative immunostaining images for sham and LPS-lesioned mice treated with vehicle or VCE-003.2 at 20 mg/kg, with indication of the approximate area quantified, are shown at right (scale bar = 200 μm).

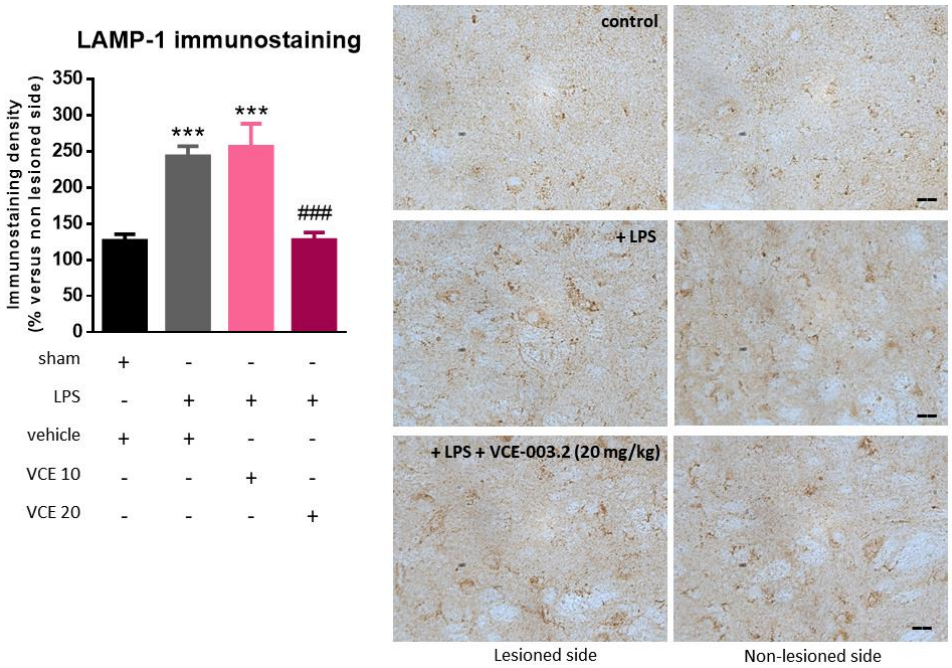

Figure 4. Intensity of the immunostaining for LAMP-1 measured in a selected area of the substantia nigra pars compacta of control and LPS-lesioned mice orally treated for 28 days after lesion with vehicle (corn oil) or VCE-003.2 at the doses of 10 (VCE 10) or 20 (VCE 20) mg/kg. Immunoreactivity values are measured in the lesioned side over the non-lesioned side and correspond to means ± SEM of more than six subjects per group. Data were assessed using the one-way analysis of variance followed by the Bonferroni test (***$p < 0.005$ versus vehicle-treated control (sham) mice; ###$p < 0.005$ versus vehicle-treated LPS-lesioned mice). Representative immunostaining images for sham and LPS-lesioned mice treated with vehicle or VCE-003.2 at 20 mg/kg are shown to the right (scale bar = 25 μm).

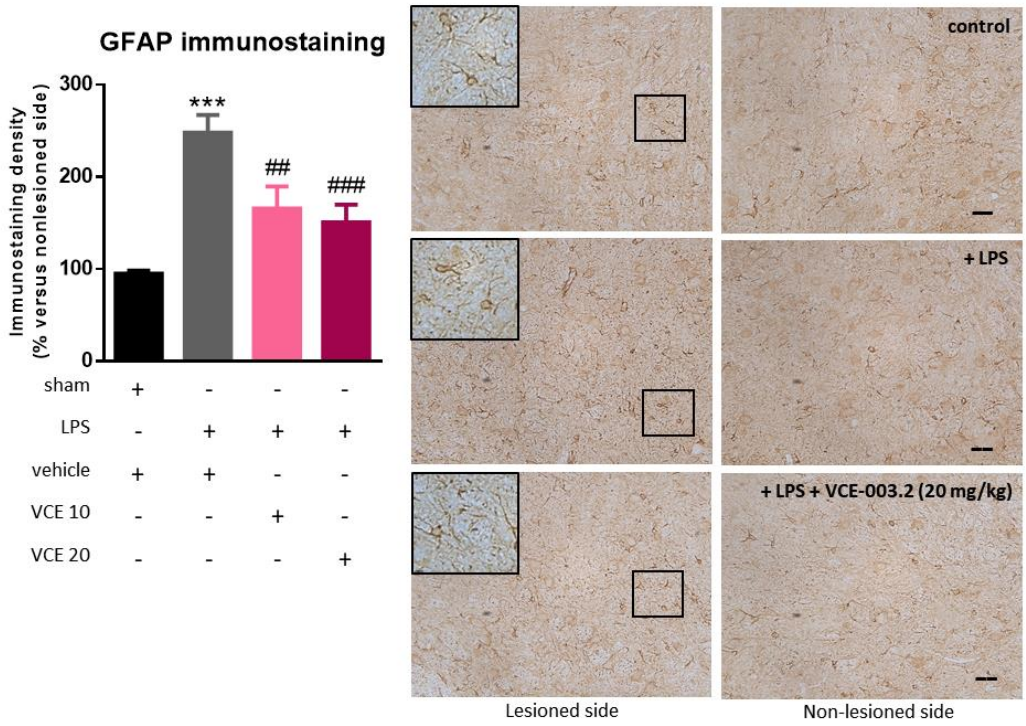

Figure 5. Intensity of the immunostaining for GFAP measured in a selected area of the substantia nigra pars compacta of control and LPS-lesioned mice orally treated for 28 days after lesion with vehicle (corn oil) or VCE-003.2 at the doses of 10 (VCE 10) or 20 (VCE 20) mg/kg. Immunoreactivity values are measured in the lesioned side over the non-lesioned side and correspond to means ± SEM of more than six subjects per group. Data were assessed using the one-way analysis of variance followed by the Bonferroni test (***$p < 0.005$ versus vehicle-treated control (sham) mice; ##$p < 0.01$, ###$p < 0.005$ versus vehicle-treated LPS-lesioned mice). Representative immunostaining images for sham and LPS-lesioned mice treated with vehicle or VCE-003.2 at 20 mg/kg are shown at right (scale bar = 50 μm), including a specific inlet showing the morphological characteristics of GFAP-labeled cells (2x magnified).

These changes were associated with a partial reduction triggered by VCE-003.2, at both doses, in the intense astroglial reactivity provoked by LPS in the substantia nigra, measured by GFAP immunostaining ($F(3,47) = 17.16$, $p < 0.0001$; Figure 5). In addition, treatment with VCE-003.2 reversed the ameboid characteristics of GFAP-labeled cells (increased cell volume and reduced length of processes), when activated by the LPS lesion, towards a more quiescent phenotype having lower cell volume and longer processes (see inlets in Figure 5). A modest trend towards a reduction was also observed for microglial reactivity labeled with CD68 ($F(3,46) = 16.60$, $p < 0.0001$; Figure 6), although the post-hoc test did not show statistically significant differences for the two doses of VCE-003.2 compared to vehicle-treated LPS-lesioned mice (Figures 5 and 6).

Lastly, the analysis by qPCR of proinflammatory mediators, such as tumor necrosis factor-α (TNF-α; $F(3,48) = 5.149$, $p < 0.005$) and interleukin-1β (IL-1β; $F(3,46) = 10.59$, $p < 0.0001$), in the striatum showed that the marked elevations provoked by the LPS lesion were, in general, attenuated by the treatment with VCE-003.2 with the greatest effects found with the dose of 20 mg/kg (except for IL-1β; Figure 7). Similar trends were found in relation with inducible nitric oxide synthase (iNOS; $F(3,49) = 0.839$, ns) and cyclooxygenase-2 (COX-2; $F(3,51) = 1.031$, ns) (Figure 7), although the differences did not reach statistical significance in most cases, partly due to the high variability in the lesioned groups, in particular in vehicle-treated LPS-lesioned mice.

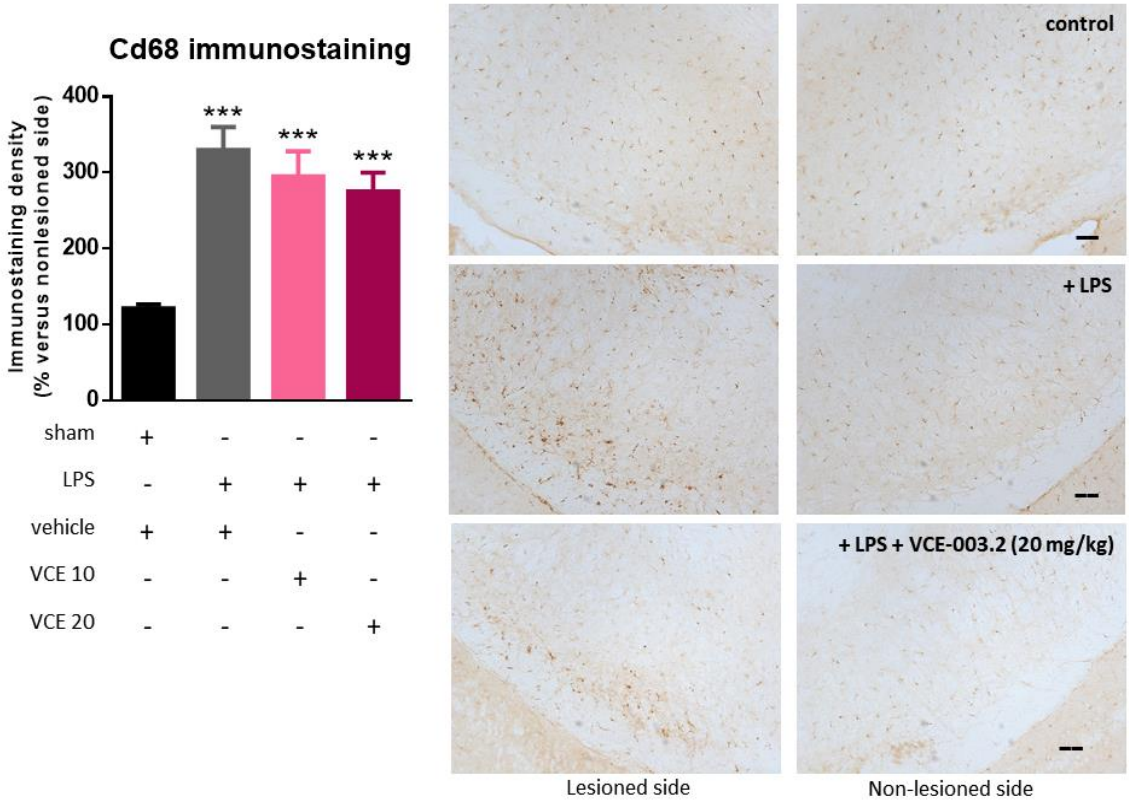

Figure 6. Intensity of the immunostaining for CD68 measured in a selected area of the substantia nigra *pars compacta of* control and LPS-lesioned mice orally treated for 28 days after lesion with vehicle (corn oil) or VCE-003.2 at the doses of 10 (VCE 10) or 20 (VCE 20) mg/kg. Immunoreactivity values are measured in the lesioned side over the non-lesioned side and correspond to means ± SEM of more than 6 subjects per group. Data were assessed using the one-way analysis of variance followed by the Bonferroni test (***$p < 0.005$ versus vehicle-treated control (sham) mice). Representative immunostaining images for sham and LPS-lesioned mice treated with vehicle or VCE-003.2 at 20 mg/kg are shown at right (scale bar = 200 μm).

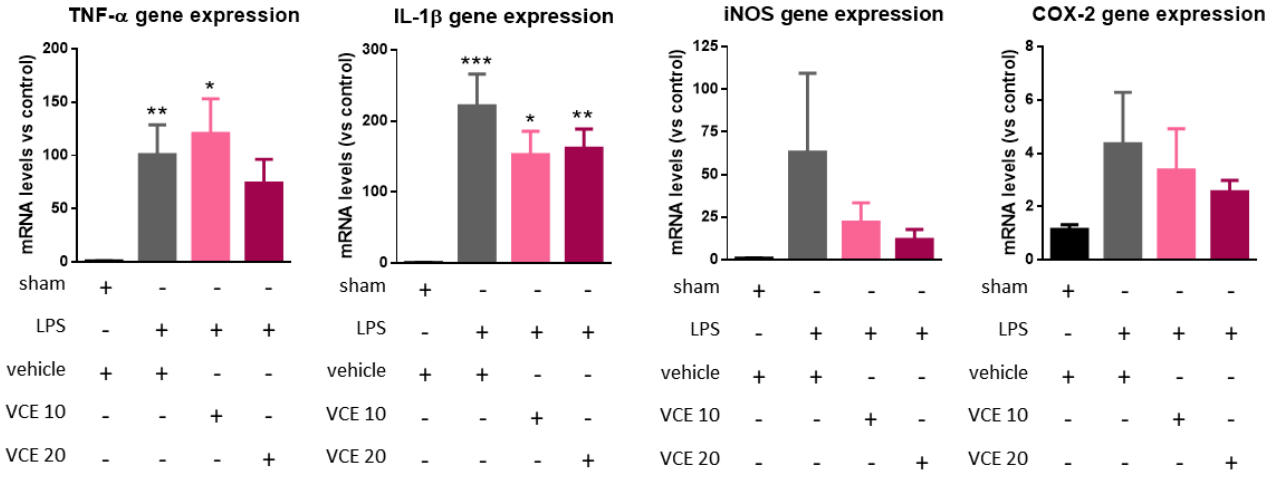

Figure 7. mRNA levels for TNF-α, IL-1β, iNOS, and COX-2 in the striatum of control and LPS-lesioned mice orally treated for 28 days after lesion with vehicle (corn oil) or VCE-003.2 at the doses of 10 (VCE 10) or 20 (VCE 20) mg/kg. Values were normalized versus control (sham) mice and correspond to means ± SEM of more than six subjects per group. Data were assessed using the one-way analysis of variance followed by the Bonferroni test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ versus vehicle-treated control (sham) mice).

3. Discussion

To determine whether glia-driven inflammation is a cause or a consequence of the degeneration of nigrostriatal neurons has remained an unsolved issue for years. At present, however, there is a general consensus assuming that glial activation may play an important pathogenic role in PD, contributing to the progressive degeneration of nigral dopaminergic neurons even in early phases of the disease [5,26]. This can be experimentally reproduced using LPS injection into the striatum [27], as well as in other experimental models of PD generated by classic mitochondrial neurotoxins [28], and it can also be seen in post-mortem PD brains at autopsy [4]. These observations prompted the investigation of anti-inflammatory agents as a potential disease-modifying therapy in PD. Thus, inhibitors of iNOS or COX-2, purinergic P2X receptor antagonists, pioglitazone, and other PPAR-γ activators, nonsteroidal anti-inflammatory drugs, the antibiotic minocycline, and immunosuppressants have been investigated at the preclinical and clinical levels in PD with variable success [4,5].

Cannabinoids have also been investigated, in particular those that activate the CB_2 receptor [6,29], which experiences an intense up-regulatory response, predominantly in microglial cells and infiltrated macrophages recruited at Central Nervous System (CNS)-lesioned areas, in PD patients and experimental animal models [8,9]. The activation of this receptor in these experimental models has been associated with anti-inflammatory and neuroprotective effects [8–10]. More recent studies have proposed an alternative anti-inflammatory target for cannabinoids in PD, the PPAR-γ receptor, which has been found to play a relevant role in the control of inflammation in numerous pathological conditions [30,31]. In fact, classic activators of this receptor such as glitazones are currently under investigation in PD [14], whereas certain cannabinoids have been already investigated for their PPAR-γ-mediated anti-inflammatory activity in different models of central and peripheral inflammation [11]. With this idea in mind, we recently investigated a CBG derivative, VCE-003.2, which was previously found to activate PPAR-γ receptors, as anti-inflammatory and neuroprotectant in the classic inflammatory model of PD generated by intrastriatal application of LPS in mice [20]. LPS provoked an intense reactive microgliosis in the substantia nigra, in parallel to an elevated expression of proinflammatory markers (e.g., TNF-α, IL-1β, iNOS) in the striatum, resulting in the deterioration of TH-containing nigral neurons. The i.p. administration of VCE-003.2 reduced LPS-induced reactivity and toxicity of microglial cells with a partial recovery in the losses of TH-positive neurons in the substantia nigra [20]. We could confirm the involvement of PPAR-γ receptors in VCE-003.2 effects in LPS-lesioned mice, although our data demonstrate, using different experimental strategies (antagonism experiments, cell cultures, docking analysis), that the effects of VCE-003.2 are exerted through its binding to a regulatory site at the PPAR-γ receptor, which differs from the canonical binding site [20].

Given the therapeutic interest in VCE-003.2 against inflammation-driven neuronal deterioration, we attempted to further progress its validation as a potential neuroprotective agent in PD, by evaluating whether these beneficial effects are also found after oral administration, which is a more relevant route of administration for its clinical development compared to the i.p. route used in the previous study [20]. An oral lipid formulation of this compound was developed for this

purpose. We used this formulation at two doses, 10 and 20 mg/kg, with the idea to reproduce the positive effects in the same experimental model of PD used in our previous experiment (LPS-lesioned mice) conducted with only one dose level (10 mg/kg) administered i.p. [20]. The dose of 10 mg/kg given orally was generally not active, with the only exception of GFAP labeling and reduction of IL-1β levels, and some small trends in behavioral tests, whereas oral administration of 20 mg/kg exhibited activity on many markers. VCE-003.2 at 20 mg/kg reduced LAMP-1 immunostaining, reflecting a positive effect on LPS-induced autophagy impairment, glial reactivity (mainly astrogliosis but also somewhat microgliosis), and the generation of pro-inflammatory markers. All these effects resulted in a partial recovery in the degeneration of TH-containing nigral neurons, as well as in a functional recovery reflected in the cylinder rearing test. Therefore, our data confirmed that oral VCE-003.2 was active, although a 2-fold higher dose of 20 mg/kg, or even higher, would be necessary to produce similar beneficial effects compared to the 10 mg/kg i.p. dose used in our previous study [20], and to progress towards the clinical development of this CBG derivative in PD. The differences between both studies may be due to the differences in bioavailability between oral and i.p. routes of administration, as well as to the fact that the present study was conducted in female mice in contrast to male mice used in the previous study [20]. The latter will require additional research.

4. Materials and Methods

4.1. Synthesis and Formulation of VCE-003.2

The aminoquinone derivative of CBG (6-(3,7)-dimethyl-octa-2,6-dienyl)-5-hydroxy-3-pentyl-2-ethylamino-[1,4]benzoquinone), so-called VCE-003.2 was synthesized as described previously [19] (see chemical structures in Figure 8). Its activity as a PPAR-γ activator has also been previously characterized [19], as well as to its negligible affinity at the CB_1 and CB_2 receptors [20]. A lipid formulation of VCE-003.2 in corn oil was used for oral administration.

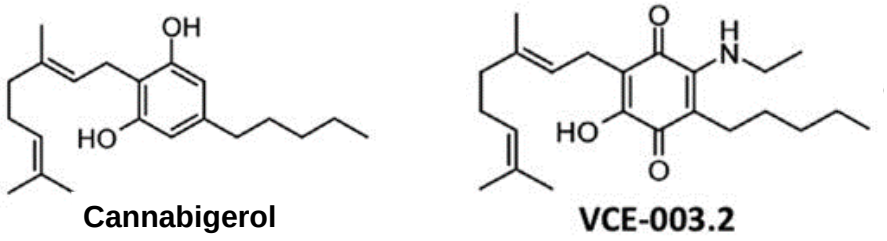

Figure 8. Chemical structures of cannabigerol (CBG) and VCE-003.2 (adapted from [19]).

4.2. Animals and Surgical Lesions

Female C57BL/6 mice (>7 month-old; 25–30 g weight) were housed in a room with controlled photoperiod (08:00–20:00) and temperature (22 ± 1 °C). They had free access to standard food and water. All experiments were conducted according to European guidelines (directive 2010/63/EU) and approved by the "Comité de Experimentación Animal" of our university (ref. PROEX 059/16). For in vivo experiments, mice (7–11 month-old) were anaesthetized (ketamine 40 mg/kg + xylazine

4 mg/kg, i.p.) and subjected to unilateral injections of S. Minnesota LPS (Sigma-Aldrich, Madrid, Spain) at two points of the right striatum following the procedure developed by Hunter et al. [27]. We used the following stereotaxic coordinates from bregma: +1.1 mm AP, −1.8 mm ML, and −3.5 mm DV, as well as −0.3 mm AP, −2.5 mm ML, and −3.2 mm DV (see details in [27]). At each intrastriatal coordinate, 5 μg of LPS in a volume of 1 μL of saline was injected slowly (0.5 μL/60 s) and the needle was left in place for 5 min before being slowly withdrawn. This avoids generating reflux and a rapid increase in intracranial pressure. Controls were sham-operated and injected with 1 μL of saline using the same coordinates. After the application of LPS or saline, mice were also subjected to pharmacological treatments as described in the following section. The lesions were generated using unilateral administration, allowing the contralateral structures to serve as controls for the different analyses.

4.3. Pharmacological Treatments and Sampling

LPS-lesioned mice were distributed into 3 groups, and administered orally with vehicle (corn oil) or 10 mg/kg or 20 mg/kg of VCE-003.2. The experiment included a fourth group consisting of sham-operated mice also treated orally with the same vehicle. The treatment was initiated approximately 16 h after the LPS lesion and was repeated daily for 28 days. Animals were subjected to behavioral analysis (rotarod, computer-aided actimeter) one week after the onset of treatment (post-lesion), and one day after the last administration (before autopsy). Immediately after the last behavioral testing, mice were killed by rapid and careful decapitation and their brains were rapidly removed and frozen in 2-methylbutane cooled in dry ice, then stored at −80 °C for subsequent immunohistochemical analysis in the substantia nigra and qPCR analysis in the striatum. In an additional experiment, the same treatment schedule was repeated using VCE-003.2, but only at the highest dose (20 mg/kg), with the purpose of exploring possible beneficial effects of this phytocannabinoid derivative at an additional behavioral measure, the cylinder rearing test, which may be more adequate for unilateral models of nigrostriatal damage.

4.4. Behavioral Analysis

Rotarod test: we used a LE8200 device (Panlab, Barcelona, Spain). After a period of acclimation and training (first session: 0 rpm for 10 s; second and third sessions: 4 rpm for 10 s), animals were tested with an acceleration from 4 to 40 rpm over a period of 300 s. Mice were tested for 3 consecutive trials and the mean of the 3 trials was calculated.

Computer-aided actimeter: motor activity was analyzed in a computer-aided actimeter (Actitrack, Panlab, Barcelona, Spain). This apparatus consisted of a 45 × 45 cm area, with infra-red beams all around, spaced 2.5 cm, coupled to a computerized control unit that analyzes the following parameters: (i) distance run in the actimeter (ambulation); (ii) time spent in inactivity; (iii) frequency of vertical activity (rearing); (iv) mean and maximal velocity developed during the running; and (v) time spent in fast (>5 cm/s) and slow (<5 cm/s) movements. Animals remained for a period of 10 min in the actimeter, but measurements were only recorded during the final 5 min (first 5 min was used only for animal acclimation).

Cylinder rearing test: given that the lesion with LPS was unilateral, this test attempts to quantify the degree of forepaw (ipsilateral, contralateral, or both) preference for wall contacts after placing the mouse in a methacrylate transparent cylinder of 15.5 cm in diameter and 12.7 cm in height [32]. Each score was made out of a 3 min trial with a minimum of 4 wall contacts.

4.5. Real Time qRT-PCR Analysis

Brain coronal slices (around 500 μm thick) were made at levels containing the striatum, according to Palkovits and Brownstein Atlas [33]. Subsequently, such structure was dissected and frozen at −80 °C (using 2-methylbutane) up to be used for qRT-PCR analysis. Total RNA was isolated from the different samples using Trizol reagent (Sigma-Aldrich, Madrid, Spain). The total amount of RNA extracted was quantitated by spectrometry at 260 nm and its purity determined from the ratio between the absorbance values at 260 and 280 nm. After genomic DNA was removed (to eliminate DNA contamination), single stranded complementary DNA was synthesized from up to 1 μg of total RNA using the commercial kits RNeasy Mini Quantitect Reverse Transcription (Qiazen, Hilgen, Germany) and iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The reaction mixture was kept frozen at −20 °C until enzymatic amplification. Quantitative RT-PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) to quantify mRNA levels for TNF-α (ref. Mm99999068_m1), IL-1β (ref. Mm00434228_m1), iNOS (ref. Mm01309902_m1), and COX-2 (ref. Mm00478372_m1), using GAPDH expression (reference Mm99999915_g1) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the threshold cycle (Ct) was calculated by the instrument's software (7300 Fast System, Applied Biosystems, Foster City, CA, USA). Expression levels were calculated using the 2-ΔΔCt method.

4.6. Immunohistochemical Procedures

Brains were sliced in coronal sections (containing the substantia nigra) in a cryostat (30 μm thick) and collected on antifreeze solution (glycerol/ethylene glycol/PBS; 2:3:5) and stored at −20 °C until used. Sections were mounted on gelatin-coated slides, and, once adhered, washed in 0.1 M potassium PBS (KPBS) at pH 7.4. Then endogenous peroxidase was blocked by 30 min incubation at room temperature in peroxidase blocking solution (Dako Cytomation, Glostrup, Denmark). After several washes with KPBS, sections were incubated overnight at room temperature with the following primary antibodies: (i) polyclonal rabbit anti-mouse TH antibody (Chemicon-Millipore, Temecula, CA, USA) used at 1/200; (ii) polyclonal rat anti-mouse CD68 antibody (AbD Serotec, Oxford, UK) used at 1/200; or (iii) polyclonal rabbit anti-mouse GFAP antibody (Dako Cytomation, Glostrup, Denmark) used at 1/200. In the case of LAMP-1 immunostaining, we used the hybridoma monoclonal rat antimouse LAMP-1 antibody 1D4B, which was deposited by Dr. J. Thomas in the Developmental Studies Hybridoma Bank (DSHB; Hybridoma Product 1D4B), created by the NICHD (NIH, Bethesda, MD, USA) and maintained at The University of Iowa, Department of Biology, Iowa City, IA, USA. In all cases, dilutions were performed in KPBS containing 2% bovine serum albumin and 0.1% Triton X-100 (Sigma Chem., Madrid, Spain). After incubation, sections were washed in

KPBS, followed by incubation with the corresponding biotinylated secondary antibody (1/200) (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine substrate–chromogen system (Dako Cytomation, Glostrup, Denmark) were used to obtain a visible reaction product. Negative control sections were obtained using the same protocol with omission of the primary antibody. A Leica DMRB microscope and a DFC300FX camera (Leica, Wetzlar, Germany) were used for the observation and photography of the slides, respectively. For quantification of the intensity of TH, LAMP-1, CD68, or GFAP immunostaining either in the substantia nigra (both ipsilateral and contralateral sides), we used the Image Processing and Analysis software ImageJ (U.S. NIH, Bethesda, MD, USA, http://imagej.nih.gov/ij/, 1997-2012) using 4 or 5 sections, separated approximately by 200 μm, and observed with 5–20x objectives depending on the method and the brain area under quantification. In all sections, the same area of the substantia nigra was analyzed. Analyses were always conducted by researchers who were blinded to all animal characteristics. Data were expressed as percentage of immunostaining intensity in the ipsilateral (lesioned) side over the contralateral (non-lesioned) side.

4.7. Data Analysis

Data were assessed using one-way ANOVA followed by the Bonferroni multiple comparison test.

5. Conclusions

In summary, our data confirm the neuroprotective potential of an oral formulation of VCE-003.2 against inflammation-driven neuronal damage in an in vivo model of neuroinflammation reminiscent of PD. Our data also indicate the need for higher doses in the case of oral administration compared to our previous study with i.p. administration [20]. Although some additional confirmatory data are still required (e.g., evaluation in another model of PD), the present data support further development of such an oral product towards the clinic, to address the lack of disease-modifying therapies in PD.

Author Contributions: Study design, coordination and supervision (JFR, EM and CG); VCE-003.2 synthesis and oral formulation (EM); Studies in LPS-lesioned mice (SB, CG and MGC); Statistical analysis of the data (SB and JFR); Manuscript preparation (JFR with the revision and approval of all authors)

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Conflicts of Interest: Eduardo Muñoz is the Chief Scientific Officer of Emerald Health Pharmaceuticals Inc. Other authors declare that they have no conflict of interest in relation to this study.

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Neuroprotection with the cannabigerol quinone derivative VCE-003.2 and its analogs CBGA-Q and CBGA-Q-Salt in Parkinson's disease using 6-hydroxydopamine-lesioned mice

Sonia Burgaz[a,b,c], Concepción García[a,b,c], María Gómez-Cañas[a,b,c], Carmen Navarrete[d], Adela García-Martín[d], Alain Rolland[d], Carmen del Río[e,f,g], María J. Casarejos[c], Eva Muñoz[a], Claudia Gonzalo-Consuegra[a,b,c], Eduardo Muñoz[d,e,f,g] and Javier Fernández-Ruiz[a,b,c,*]

[a] *Instituto Universitario de Investigación en Neuroquímica, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, Madrid, Spain;*
[b] *Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain;*
[c] *Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain;*
[d] *Emerald Health Pharmaceuticals, San Diego, USA;*
[e] *Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Córdoba, Spain;*
[f] *Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, Spain;*
[g] *Hospital Universitario Reina Sofía, Córdoba, Spain.*

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ABSTRACT

The quinone derivative of the non-psychotropic cannabinoid cannabigerol (CBG), so-called VCE-003.2, has been recently investigated for its neuroprotective properties in inflammatory models of Parkinson's disease (PD) in mice. Such potential derives from its activity at the peroxisome proliferator-activated receptor-γ (PPAR-γ). In the present study, we investigated the neuroprotective properties of VCE-003.2 against the parkinsonian neurotoxin 6-hydroxydopamine (6-OHDA), in comparison with two new CBG-related derivatives, the cannabigerolic acid quinone (CBGA-Q) and its sodium salt CBGA-Q-Salt, which, similarly to VCE-003.2, were found to be active at the PPAR-γ receptor, but not at the cannabinoid CB_1 and CB_2 receptors. First, we investigated their cytoprotective properties *in vitro* by analyzing cell survival in cultured SH-SY5Y cells exposed to 6-OHDA. We found an important cytoprotective effect of VCE-003.2 at a concentration of 20 μM, which was not reversed by the blockade of PPAR-γ receptors with GW9662, supporting its activity at an alternative site (non-sensitive to classic antagonists) in this receptor. We also found CBGA-Q and CBGA-Q-Salt being cytoprotective in this cell assay, but their effects were completely eliminated by GW9662, thus indicating that they are active at the canonical site in the PPAR-γ receptor. Then, we moved to *in vivo* testing using mice unilaterally lesioned with 6-OHDA. Our data confirmed that VCE-003.2 administered orally (20 mg/kg) preserved tyrosine hydroxylase (TH)-positive

*Corresponding autor at: Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040-Madrid, Spain. *E-mail adress*: jjfr@med.ucm.es (J. Fernández-Ruiz).

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nigral neurons against 6-OHDA-induced damage, whereas it completely attenuated the astroglial (GFAP) and microglial (CD68) reactivity found in the substantia nigra of lesioned mice. Such neuroprotective effects caused an important recovery in the motor deficiencies displayed by 6-OHDA-lesioned mice in the pole test and the cylinder rearing test. We also investigated CBGA-Q, given orally (20 mg/kg) or intraperitoneally (10 mg/kg, i.p.), having similar benefits compared to VCE-003.2 against the loss of TH-positive nigral neurons, glial reactivity and motor defects caused by 6-OHDA. Lastly, the sodium salt of CBGA-Q, given orally (40 mg/kg) to 6-OHDA-lesioned mice, also showed benefits at behavioral and histopathological levels, but to a lower extent compared to the other two compounds. In contrast, when given i.p., CBGA-Q-Salt (10 mg/kg) was poorly active. We also analyzed the concentrations of dopamine and its metabolite DOPAC in the striatum of 6-OHDA-lesioned mice after the treatment with the different compounds, but recovery in the contents of both dopamine and DOPAC was only found after the treatment with VCE-003.2. In summary, our data confirmed the neuroprotective potential of VCE-003.2 in 6-OHDA-lesioned mice, which adds to its previous activity found in an inflammatory model of PD (LPS-lesioned mice). Additional phytocannabinoid derivatives, CBGA-Q and CBGA-Q-Salt, also afforded neuroprotection in 6-OHDA-lesioned mice, but their effects were lower compared to VCE-003.2, in particular in the case of CBG-Q-Salt. *In vitro* studies confirmed the relevance of PPAR-γ receptors for these effects.

1. Introduction

Phytocannabinoids, the active constituents of the cannabis plant, and also endocannabinoids and synthetic cannabinoids have been proposed as promising neuroprotective agents, a property derived from their pleiotropism and ability to activate numerous pharmacological targets within the so-called endocannabinoid system, but also outside this signaling system (reviewed in Fernández-Ruiz, 2019).

Such neuroprotective potential is exerted by the combination of different cannabinoid capabilities, in particular the inhibition of several insults that damage neurons, for example excitotoxicity, oxidative stress and glial reactivity/inflammatory events (reviewed in Aymerich et al., 2018), but also the promotion of prosurvival events such as the elimination of protein aggregates (reviewed in Costa et al., 2016) and the replacement of neurons and other neural cells (reviewed in Oddi et al., 2020). All those events are critical in the pathogenesis of those CNS disorders involving neuronal deterioration and death (reviewed in Fernández-Ruiz et al., 2015; Paloczi et al., 2018; Aymerich et al., 2018). These activities enable cannabinoids to serve as neuroprotective agents in accidental brain damage, e.g., stroke (Kolb et al., 2019), brain trauma (Shohami et al., 2011), spinal injury (Arévalo-Martín et al., 2016), but also in chronic progressive disorders such as Alzheimer's disease (Maroof et al., 2013), amyotrophic lateral sclerosis (Giacoppo and Mazzon, 2016), Huntington's disease (Sagredo et al., 2012), multiple sclerosis (Mecha et al., 2020), cerebellar ataxias (Gómez-Ruiz et al., 2019), prion diseases (Iuvone et al., 2009), and Parkinson's disease (PD) (Junior et al., 2020).

PD is one of the disorders that has attracted most research with cannabinoids to date, with the objective to generate new therapies based on these compounds aimed at alleviating specific parkinsonian symptoms and/or at delaying disease progression (reviewed in Fernández-Ruiz et al., 2015; Aymerich et al., 2018; Antonazzo et al., 2019; Baul et al., 2019). For example, cannabinoids targeting the cannabinoid receptor type-1 (CB_1) have been found to reduce, mainly in preclinical studies, parkinsonian signs such as bradykinesia and immobility (Fernández-Espejo et al., 2005;

González et al., 2006; Kelsey et al., 2009), tremor (Sañudo-Peña et al., 1999) and/or L-DOPA-induced dyskinesia (Junior et al., 2020). Targeting the CB_1 receptor has been also proposed as neuroprotective therapy (Iuvone et al., 2007; Chung et al., 2011; Pérez-Rial et al., 2011; Nguyen et al., 2016), but most of cannabinoids proposed as disease modifiers work: (i) against inflammatory events by targeting the cannabinoid receptor type-2 (CB_2) (Price et al., 2009; García et al., 2011; Javed et al., 2016; Gómez-Gálvez et al., 2016; Chung et al., 2016; Aymerich et al., 2016; Wang et al., 2018), but also the peroxisome proliferator-activated receptor-γ (PPAR-γ) (Carroll et al., 2012; García et al., 2018; Burgaz et al., 2019) and even an orphan receptor, GPR55, which has been recently associated with the endocannabinoid system (Celorrio et al., 2017); and (ii) against oxidative injury based on cannabinoid receptor-independent effects (Lastres-Becker et al., 2005; Jiménez-Del-Río et al., 2008) and possibly on their proposed ability to modulate the signaling of the antioxidant transcription factor Nrf-2 (Gugliandolo et al., 2018).

Based on this previous experimental evidence, a family of cannabinoid analogs that have been recently investigated for their therapeutic profile for PD are the quinone derivatives of cannabigerol (CBG) (García et al., 2018; Burgaz et al., 2019). The lead compound in this series is VCE-003.2, which behaves as a PPAR-γ activator (Díaz-Alonso et al., 2016), while retaining the lack of CB_1/CB_2 activity of the CBG template (Granja et al., 2012; Carrillo-Salinas et al., 2014). We have been particularly interested in exploring the potential of this non-thiophilic CBG quinone derivative against inflammation-driven neuronal deterioration in LPS-lesioned mice (García et al., 2018; Burgaz et al., 2019), the experimental model of PD that better reproduces inflammation as a pathogenic event in this disease. VCE-003.2 worked as an anti-inflammatory and neuroprotective agent in this *in vivo* PD model after both i.p. (García et al., 2018) and oral (Burgaz et al., 2019) administration, and its effects were confirmed to be mediated by the activation of the PPAR-γ receptor (García et al., 2018). We also demonstrated its anti-inflammatory profile in cultured BV2 cells stimulated with LPS, as well as its neuroprotective effects in cultured M-213 neuronal cells incubated with conditioned media generated from cultured BV2 cells stimulated with LPS (García et al., 2018). In these *in vitro* studies (García et al., 2018), we observed that PPAR-γ-mediated effects of VCE-003.2 implied its binding to a functional alternative binding site different from the canonical binding site used by glitazones (Hughes et al., 2014). In the same study (García et al., 2018), VCE-003.2 was also found to apparently serve as neuroprotectant after i.p. administration in 6-OHDA-lesioned mice, a model of PD characterized by mitochondrial dysfunction and oxidative stress but having a more modest inflammatory response. However, the results of this experiment were preliminary and needed further replication. This is one of the objectives of our present study, focusing on the neuroprotective properties of VCE-003.2, but using 6-OHDA-lesioned mice instead of LPS-lesioned animals, as well as using an oral administration instead of the i.p. route used in the previous study (García et al., 2018). We also investigated new compounds, CBGA-Q and a water-soluble CBGA-Q-Salt, which should be active at the PPAR-γ too. The inclusion of these additional compounds had the objective to determine whether, compared to VCE-003.2, they could have advantages, for example being more potent than VCE-003.2 at the PPAR-γ receptor, or having additional activities at the CB_1/CB_2 receptors which are also useful in PD. Even, in the case of CBGA-Q-Salt, which is a more water soluble derivative, whether this may facilitate its administration and pharmacokinetics resulting in a better bioavailability. Another potential advantage for the two new derivatives is the synthesis method, as VCE-003.2 requires two-step synthesis and CBGA-Q only one step.

2. Materials and Methods

2.1 Chemical synthesis of the different compounds investigated

The aminoquinone derivative of CBG, 6-(3,7)-dimethyl-octa-2,6-dienyl)-5-hydroxy-3-pentyl-2-ethylamino-[1,4]benzoquinone, so-called VCE-003.2, was synthesized as described previously (Díaz-Alonso et al., 2016). In this study, we also included two new compounds, CBGA-Q and CBGA-Q-Salt (see chemical structures in Figure 1).

For the synthesis of CBGA-Q, cannabigerol acid ((*Z*)-3-(3,7-dimethylocta-2,6-dienyl)-2,4-dihydroxy-6-pentylbenzoic acid) (0.995 g; 2.76 mmol) was dissolved in THF (10 mL) at 20°C and KO*t*Bu (0,867 g; 7.73 mmol) was added. The mixture was stirred open to the air for 3 h, and the reaction mixture was dissolved in AcOEt (50 mL) and H_2O (50 mL), layers were separated, and the aqueous layer was washed with AcOEt (50 mL). Organic layers were discarded, and the aqueous layer was acidified to pH = 5.5 to 6.0 and extracted with AcOEt (2 x 50 mL). The acidic organic layers were dried (Na_2SO_4) and concentrated under vacuum to obtain compound CBGA-Q as a red oil. NMR-^{1}H ($CDCl_3$, 300 MHz) δ ppm: 5.08 (m, 2H), 3.18 (d, *J* = 7.0 Hz, 2H), 2.80 (t, *J* = 7.6 Hz, 2H), 2.00 (m, 4H), 1.73 (s, 3H), 1.65 (s, 3H), 1.57 (s, 3H), 1.50 (m, 2H), 1.36 (m, 4H), 0.89 (t, *J* = 7.0 Hz, 3H).

For the synthesis of the CBGA-Q-Salt, CBGA-Q (0.086 g, 0.23 mmol) was dissolved in AcOEt (1 mL). A solution of NaOH 1N (0.045 mL, 0.23 mmol, Sigma Aldrich) was added and the mixture was distilled to residue. It was then slurried in AcOEt (1 mL), stirred at room temperature and filtered to obtain the sodium salt as a brownish solid (18 mg, purity 99.34%). NMR-^{1}H ($CDCl_3$, 300 MHz) δ ppm: 5.04 (m, 2H), 3.04 (d, *J* = 7.0 Hz, 2H), 2.40 (t, *J* = 8.2 Hz, 2H), 2.00 (m, 2H), 1.79 (m, 2H), 1.67 (s, 3H), 1.63 (s, 3H), 1.55 (s, 3H), 1.47 (m, 2H), 1.28 (m, 4H), 0.84 (t, *J* = 7.0 Hz, 3H).

2.2. Analysis of the CB_1/CB_2 receptor binding profile of the different compounds investigated

To determine the affinity at the cannabinoid receptors of CBGA-Q and CBGA-Q-Salt, we conducted radioligand-binding assays using membranes purified from cells transfected with human CB_1 or CB_2 receptors (RBHCB1M400UA and RBXCB2M400UA; Perkin-Elmer Life and Analytical Sciences, Boston, MA, USA). The protein concentration was 8 µg/well for the CB_1 receptor membranes and 4 µg/well for the CB_2 receptor. The binding buffer was 50 mM TrisCl, 5 mM $MgCl_2$, 2.5 mM EDTA, 0.5 mg/mL bovine serum albumin (pH = 7.4) for CB_1, and 50 mM TrisCl, 5 mM $MgCl_2$, 2.5 mM EGTA, 1 mg/mL bovine serum albumin (pH = 7.5) for CB_2. The radioligand was [^{3}H]-CP55940 (PerkinElmer) used at a concentration of 0.4 nM for CB_1 and 0.53 nM for CB_2, with a final incubation volume of 200 µL and 600 µL, respectively. All plastic materials necessary for the experiment were previously siliconized with Sigmacote (Sigma-Aldrich, Madrid, Spain). Membranes were resuspended in the corresponding buffer and were incubated (90 min at 30ºC) under continuous stirring with the radioligand and CBGA-Q or CBGA-Q-Salt at a high concentration (10 µM) with the purpose of determining the percentage of radioligand displacement. Non-specific binding was determined with 10 µM WIN55212-2 and total radioligand binding by incubation with the membranes in absence of CBGA-Q or CBGA-Q-Salt. [^{3}H]-CP55940, WIN55212-2, CBGA-Q and CBGA-Q-Salt were dissolved in dimethyl sulfoxide (DMSO), stored in stock solutions and diluted at the necessary concentrations when used for the assays (DMSO being always lower than 0.1%).

Filtration was performed by a Harvester® filtermate (Perkin-Elmer) with Filtermat A GF/C filters pretreated with polyethylenimine 0.05%. After filtering, the filter was washed nine times with binding buffer, dried and a melt-on scintillation sheet (Meltilex™ A, Perkin Elmer) was melted onto it. Then, radioactivity was quantified by a liquid scintillation spectrophotometer (Wallac MicroBeta Trilux, Perkin-Elmer). Data were expressed as radioligand displacement percentage, and they corresponded to at least 3 experiments performed in triplicate for each point.

2.3. PPAR-γ binding and transcriptional assays for the different compounds investigated

PPAR-γ binding activity was studied using the PolarScreen™ PPARγ Competitor Assay kit (Life Technologies, CA, USA). Experiments were performed in triplicates and IC_{50} values calculated using GraphPad Prism. To investigate PPAR-γ transcriptional activity HEK-293T cells were seeded in 24-well plates and transiently co-transfected with the expression vector GAL4-PPAR-γ and the luciferase reporter vectors GAL4-luc (firefly luciferase) using Roti®-Fect (Carl Roth, Karlsruhe, Germany) following the manufacturer's instructions. After stimulation, the luciferase activities were quantified using Luciferase Assay (Promega, Madison, WI, USA).

2.4. Cultures of SH-SY5Y neuronal cells

Cultures of SH-SY5Y neuronal cell line (kindly provided by Dr. Ana Martínez, CIB-CSIC, Madrid, Spain) were used to induce cell death with 6-OHDA and to investigate *in vitro* the mechanisms of cytoprotection of the different cannabinoid derivatives under study, following a procedure described previously (Ko et al., 2019). To this end, SH-SY5Y cells were maintained in DMEM supplemented with 10% FBS, 2 mM Ultraglutamine, and 1% antibiotics (Lonza, Verviers, Belgium) and under a humidified 5% CO_2 atmosphere at 37°C. For cytotoxicity experiments, cells were seeded at 120,000 cells/well in 96-well plates and maintained under a humidified atmosphere (5% CO_2) at 37°C overnight. In a first experiment, 24 hours after seeding, cells were treated with the vehicle (saline) or with four different concentrations of 6-OHDA (50, 100, 200 and 400 μM), and were incubated during 24 hours before the neuronal death was analyzed with the MTT assay (Panreac AppliChem., Barcelona, Spain). This experiment served to select the best 6-OHDA concentration for the following experiments. In the second experiment, cells were treated with the vehicle (DMEM + 0.1% DMSO) or with four different concentrations of VCE-003.2 (2, 10, 20 and 40 μM; selected according to Díaz-Alonso et al. (2016), which described an IC_{50} = 1.2 μM for the binding of VCE-003.2 to PPAR-γ receptors), just 60 minutes before being exposed to 200 μM 6-OHDA (or saline). Cells were again incubated during 24 hours before the neuronal death was analyzed with the MTT assay (Panreac AppliChem., Barcelona, Spain). This experiment served to select the best VCE-003.2 concentration for the last experiment, in which cells were treated with the vehicle (saline) or with the PPAR-γ antagonist GW9662 (20 μM) followed, 30 minutes later, by a new treatment with VCE-003.2 (20 μM), CBGA-Q (20 μM), CBGA-Q-Salt (20 μM) or vehicle (DMEM + 0.1% DMSO), just 60 minutes before being exposed to 200 μM 6-OHDA (or saline). Cells were again incubated during 24 hours before the neuronal death was analyzed with the MTT assay (Panreac AppliChem., Barcelona, Spain). In all cases, the data of cell viability were normalized in relation to the corresponding control group (cells exposed to vehicle for 6-OHDA).

2.5. Animals and surgical lesions

Male C57BL/6 mice were housed in a room with a controlled photoperiod (06:00-18:00 light) and temperature (22 ± 1ºC). They had free access to standard food and water and were used at adult age (3-4 month-old; 25-30 g weight). All experiments were conducted according to European guidelines (directive 2010/63/EU) and approved by the "Comité de Experimentación Animal" of our university (ref. CEA-UCM 56/2012). For *in vivo* experiments, mice were anaesthetized (ketamine 40 mg/kg + xylazine 4 mg/kg, i.p.) 30 min after pretreatment with desipramine (25 mg/kg, i.p.), and then 6-OHDA free base (2 μL at a concentration of 2 μg/μL saline in 0.2% ascorbate to avoid oxidation) or saline (for control mice) were injected stereotaxically into the right striatum at a rate of 0.5 μL/min, using the following coordinates: +0.4 mm AP, ±1.8 mm ML and −3.5 mm DV, as described in Alvarez-Fischer et al. (2008). Once injected, the needle was left in place for 5 min before being slowly withdrawn. This avoided generating reflux and a rapid increase in intracranial pressure. Control animals were sham-operated and injected with 2 μL of saline using the same coordinates. After the application of 6-OHDA or saline, mice were subjected to pharmacological treatments as described in the following section. The lesions were generated using unilateral injection, the contralateral structures serving as controls for the different analyses.

2.6. Pharmacological treatments and sampling

Three different series of pharmacological treatments were carried out with 6-OHDA-lesioned mice and their controls. In the first series, 6-OHDA-lesioned mice were treated with VCE-003.2 (20 mg/kg), CBGA-Q (20 mg/kg) or vehicle (sesame oil) administered orally. In a second experiment, 6-OHDA-lesioned mice were daily treated with CBGA-Q-Salt (40 mg/kg) or vehicle (saline) also administered orally. The third experiment was conducted with CBGA-Q (10 mg/kg) and CBGA-Q-Salt (10 mg/kg) prepared in saline as vehicle and administered i.p. to 6-OHDA-lesioned mice. The doses and routes for administration used in the three experiments were selected from the previous experience of authors with similar cannabinoids, including the previous studies conducted with VCE-003.2 (García et al., 2018; Burgaz et al., 2019), as well as from the data obtained in this study in relation with the analysis of CB_1/CB_2 receptor binding and PPAR-γ binding and transcriptional activity of the two new derivatives.

In the three experiments, control mice (sham-operated) were also administered with the corresponding vehicle. The first administration, in all cases, was done approximately 16 hours after the lesion and the treatment was prolonged for two weeks (one administration per day, always at the same time). One day after the last administration, all animals were analyzed in the pole test and the cylinder rearing test; then, the animals were killed by rapid and careful decapitation and their brains were rapidly removed and frozen in 2-methylbutane cooled in dry ice and stored at -80ºC for subsequent immunohistochemical analysis in the substantia nigra and HPLC analysis and qRT-PCR in the striatum.

2.7. Behavioral procedures

2.7.1. Pole test. Mice were placed head-upward on the top of a vertical rough-surfaced pole (diameter 8 mm; height 55 cm) and the time until animals descended to the floor was recorded with a maximum duration of 120 s. When the mouse was not able to turn downward and instead dropped from the pole,

the time was taken as 120 s (default value).

2.7.2. *Cylinder rearing test*. Given that the lesions were unilateral, this test attempts to quantify the degree of forepaw (ipsilateral, contralateral, or both) preference for wall contacts after placing the mouse in a methacrylate transparent cylinder (diameter: 15.5 cm; height: 12.7 cm; Fleming et al., 2013). Each score was made out of a 3 min trial with a minimum of 4 wall contacts.

2.8. Immunohistochemical procedures

Brains were sliced in coronal sections (containing the substantia nigra) in a cryostat (30 µm thick) and collected in antifreeze solution (glycerol/ethylene glycol/PBS; 2:3:5) and stored at -20ºC until used. Sections were mounted on gelatin-coated slides, and, once adhered, washed in 0.1M potassium PBS (KPBS) at pH 7.4. Then endogenous peroxidase was blocked by 30 min incubation at room temperature in peroxidase blocking solution (Dako Cytomation, Glostrup, Denmark). After several washes with KPBS, sections were incubated overnight at room temperature with the following primary antibodies: (i) polyclonal rabbit anti-TH (Chemicon-Millipore, Temecula, CA, USA) used at 1/200; (ii) polyclonal rat anti-mouse CD68 antibody (AbD Serotec, Oxford, UK) used at 1/200; or (iii) polyclonal rabbit anti-mouse GFAP antibody (Dako Cytomation, Glostrup, Denmark) used at 1/200. Dilutions were carried out in KPBS containing 5% normal horse serum and 0.1% Triton X-100 (Sigma Chem., Madrid, Spain). After incubation, sections were washed in KPBS, followed by incubation with the corresponding biotinylated secondary antibody (1/400) (Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature. Avidin–biotin complex (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine substrate–chromogen system (Dako Cytomation, Glostrup, Denmark) were used to obtain a visible reaction product. Negative control sections were obtained using the same protocol with omission of the primary antibody. A Leica DMRB microscope and a DFC300FX camera (Leica, Wetzlar, Germany) were used for the observation and photography of the slides, respectively. For quantification of the intensity of TH, Iba-1 or CD68 immunostaining in the substantia nigra (both ipsilateral and contralateral sides), we used the NIH Image Processing and Analysis software (ImageJ; NIH, Bethesda, MD, USA) using 4-5 sections, separated approximately by 200 µm, and observed with 5x-20x objectives depending on the immunostaining and the brain area under quantification. In all sections, the same area of the substantia nigra *pars compacta* was analyzed. Analyses were always conducted by experimenters who were blinded to all animal characteristics. Data were expressed as percentage of immunostaining intensity in the ipsilateral (lesioned) side over the contralateral (non-lesioned) side.

2.9. HPLC analysis

Brain coronal slices (around 500 µm thick) were made at levels containing the striatum, according to Palkovits and Brownstein Atlas (1988). Subsequently, such structure was dissected and used for the analysis of DA and DOPAC concentrations using a HPLC equipment with an ESA Coulochem Detector. Each striatum (ipsilateral and contralateral separately) collected in the different pharmacological experiments was sonicated in 6 vol. (weight/volume) of 0.4 N perchloric acid (PCA) with 0.5 mM $Na_2S_2O_5$ and 2% EDTA and then centrifuged at 10,000 *g* at 4ºC for 20 min. DA and DOPAC levels were determined from 20 µL of the resulting supernatant. The conditions for the chromatography (Nucleosil 5C18 column used with citrate/acetate buffer 0.1 M, pH 3.9 with 10%

methanol, 1 mM EDTA, and 1.2 mM heptane sulfonic acid as mobile phase) and the electrochemical detection (analytical cell #1: +0.05 V; analytical cell #2: -0.39 V; and guard cell: +0.40 V) were previously described (Mena et al., 1993). Data were expressed as % of DA or DOPAC concentrations in the ipsilateral (lesioned) striatum *versus* the contralateral (non-lesioned) side.

2.10. qRT-PCR analysis

Striatal samples obtained from control (sham) and 6-OHDA-lesioned mice were also used for qRT-PCR analysis of the PPAR-γ receptor. Total RNA was isolated from the different samples using Trizol reagent (Sigma-Aldrich, Madrid, Spain). The total amount of RNA extracted was quantitated by spectrometry at 260 nm and its purity determined from the ratio between the absorbance values at 260 and 280 nm. After genomic DNA was removed (to eliminate DNA contamination), single stranded complementary DNA was synthesized from up to 1 μg of total RNA using the commercial kits RNeasy Mini Quantitect Reverse Transcription (Qiazen, Hilgen, Germany) and iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The reaction mixture was kept frozen at −20 °C until enzymatic amplification. Quantitative RT-PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) to quantify mRNA levels for PPAR-γ (ref. Mn01184322_m1), using GAPDH expression (reference Mm99999915_g1) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the threshold cycle (Ct) was calculated by the instrument's software (7300 Fast System, Applied Biosystems, Foster City, CA, USA). Expression levels were calculated using the 2-ΔΔCt method.

2.11. Data analysis

Data were normally distributed (tested with the Shapiro-Wilk normality test) and were assessed by one-way analysis of variance followed by the Bonferroni test or by the unpaired Student's t-test, as required, using GraphPad Prism® software (version 5.01; GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Determination of PPAR-γ/CB$_1$/CB$_2$ receptor binding for CBGA-Q and CBGA-Q-Salt

One important objective of this study was to generate novel analogs of VCE-003.2 with possibly a better pharmacological profile. Thus, we synthesized the compounds CBGA-Q and CBGA-Q-Salt. CBGA-Q is derived from CBGA in a one-step synthesis and CBGA-Q-Salt is a water-soluble sodium salt CBGA-Q derivative. Both derivatives were first analyzed by their activity at the PPAR-γ receptor showing similar binding and transcriptional activities compared to VCE-003.2 (Table 1). We also conducted radioligand binding assays using membranes purified from cells transfected with human CB$_1$ or CB$_2$ receptors to determine the affinity of CBGA-Q and CBG-Q-Salt at these receptors. Our data demonstrated that a high concentration of both derivatives (10 μM) displaced radioligand binding to both CB$_1$ and CB$_2$ receptors with a percentage in both cases lower than 30%, then indicating negligible affinity for both receptors with predicted Ki values in the micromolar range in both cases (>10 μM; see Table 1).

Table 1

Binding affinity (predicted Ki values) of CBGA-Q and CBGA-Q-Salt at the CB_1 and CB_2 receptors measured in competition studies, as well as their binding and transcriptional activity at the PPAR-γ receptors. Values correspond to data from at least 3 experiments performed in triplicate. Data for VCE-003.2 (*already published in García et al., 2018) and for WIN55,212-2 and rosiglitazone (as control compounds for CB_1/CB_2 and PPAR-γ receptors, respectively) were included for comparative purposes.

Compound	CB_1 receptor- Ki (nM)	CB_2 receptor- Ki (nM)	PPAR-γ receptor	
			Binding IC50 (μM)	Fold induction
CBGA-Q	> 10.000	> 10.000	0.77	4.1 (25 μM)
CBGA-Q Salt	> 10.000	> 10.000	2.23	6.2 (25 μM)
VCE-003.2*	> 40.000	> 40.000	1.2	19.4 (25 μM)
WIN55,212-2 (CB_1/CB_2 control)	28.8	3.7	---	---
Rosiglitazone (PPAR-γ control)	---	---	0.15	90.2 (1 μM)

3.2. Effects of VCE-003.2, CBGA-Q and CBGA-Q-Salt against 6-OHDA insult in cultured SH-SY5Ycells

The second part of our study consisted in a series of experiments conducted in a cell-based assay (cultured SH-SY5Y cells exposed to 6-OHDA) that is frequently used as an *in vitro* model of PD (Lopes et al., 2010; Ko et al., 2019). We used this experimental tool to obtain a first proof-of-concept on the cytoprotective properties of the three derivatives under investigation and, in particular, for exploring the target (PPAR-γ) involved in such beneficial effects. First, we carried out a dose-response experiment to determine the best 6-OHDA concentration to be used for the remaining *in vitro* experiments. Thus, we analyzed cell viability in a range of 6-OHDA concentrations (50-400 μM); a clear concentration-dependent effect was observed ($F(4,29)=26.12$, $p<0.0001$; Figure 1A), leading to the selection of 200 μM (~40% of death) as the optimal concentration. Second, we carried out a new dose-response experiment to determine the best VCE-003.2 concentration to increase cell survival against the 6-OHDA (200 μM) insult. VCE-003.2 showed cytoprotection in a concentration-dependent manner ($F(5,59)=22.68$, $p<0.0001$; Figure 1B) in the 2-20 μM range. We selected 20 μM for the last experiment as higher concentrations (40 μM) showed lower cytoprotective effect (Figure 1B).

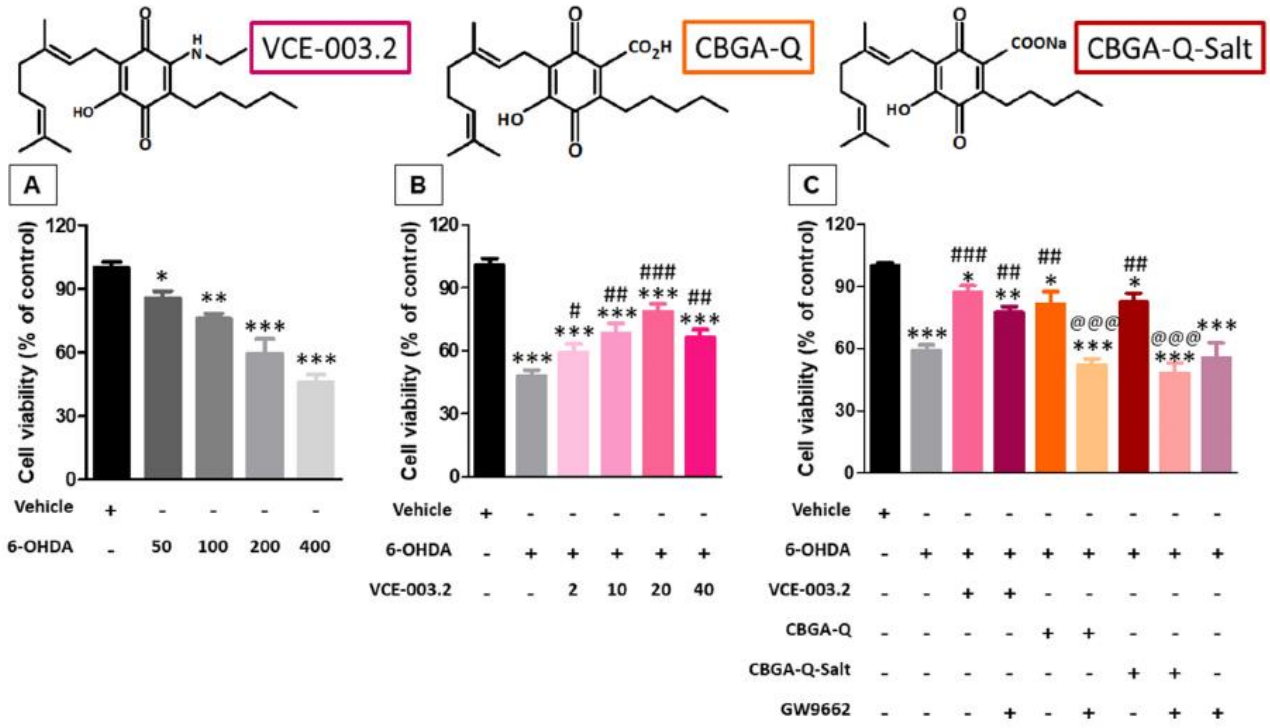

Figure 1. Chemical structures of VCE-003.2, CBGA-Q and CBGA-Q-Salt, and cell viability measured with the MTT assay in cultured SH-SY5Y cells at 24 hours to be treated with: **A:** different concentrations of 6-OHDA (50-400 µM) or vehicle (saline), **B:** different concentrations of VCE-003.2 (2, 10, 20 and 40 µM) against 6-OHDA (200 µM), and **C:** VCE-003.2 (20 µM), CBGA-Q (20 µM), CBGA-Q-Salt (20 µM) against 6-OHDA (200 µM), in the absence or the presence of GW9662 (20 µM). In all cases, a group with cells exposed to vehicle (saline) was also included to determine the 100% of cell viability. Values are means ± SEM of at least 4 independent experiments each performed in triplicate. Data were assessed by the one-way analysis of variance followed by the Bonferroni test (*p<0.05, **p<0.01, ***p<0.005 *versus* control cells; #p<0.05, ##p<0.01, ###p<0.005 *versus* cells exposed to 6-OHDA+vehicle; @@@p<0.005 *versus* cells treated with VCE-003.2, CBGA-Q or CBGA-Q-Salt).

Lastly, we investigated the cytoprotective effect of 20 µM VCE-003.2, and also similar concentrations for CBGA-Q and CBGA-Q-Salt, against 200 µM 6-OHDA, in the presence or absence of the PPAR-γ receptor inhibitor GW9662 (at 20 µM). Our data confirmed that 200 µM 6-OHDA reduced cell viability to 60%, which was significantly elevated (up to 80-90%) with VCE-003.2, CBGA-Q or CBGA-Q-Salt ($F(8,98)=17.49$, $p<0.0001$; Figure 1C). Interestingly, the cytoprotective effects of CBGA-Q and CBGA-Q-Salt were completely eliminated by GW9662 (it had no activity in this bioassay when administered alone; data not shown), supporting the involvement of the canonical site of the PPAR-γ receptor in these effects (Figure 1C). This did not happen with the cytoprotective effect of VCE-003.2 (Figure 1C), as described in García et al. (2018).

3.3. Neuroprotective effects of VCE-003.2, CBGA-Q and CBGA-Q-Salt in 6-OHDA-lesioned mice

The third part of this study consisted of analysis of the neuroprotective effects of these three compounds in 6-OHDA-lesioned mice. Given that the previous literature on VCE-003.2 in PD (García et al., 2018), together with the data obtained in this study in relation with the pharmacological profile of the two new derivatives, as well as the results derived from the above *in vitro* experiment, indicated an important role for PPAR-γ receptors in these effects, we wanted first to determine the

status of these receptors in the experimental model used for the *in vivo* studies. Thus, we analyzed gene expression for PPAR-γ in the striatum detecting elevated mRNA levels in 6-OHDA-lesioned mice compared to controls (see Supplementary Figure 1).

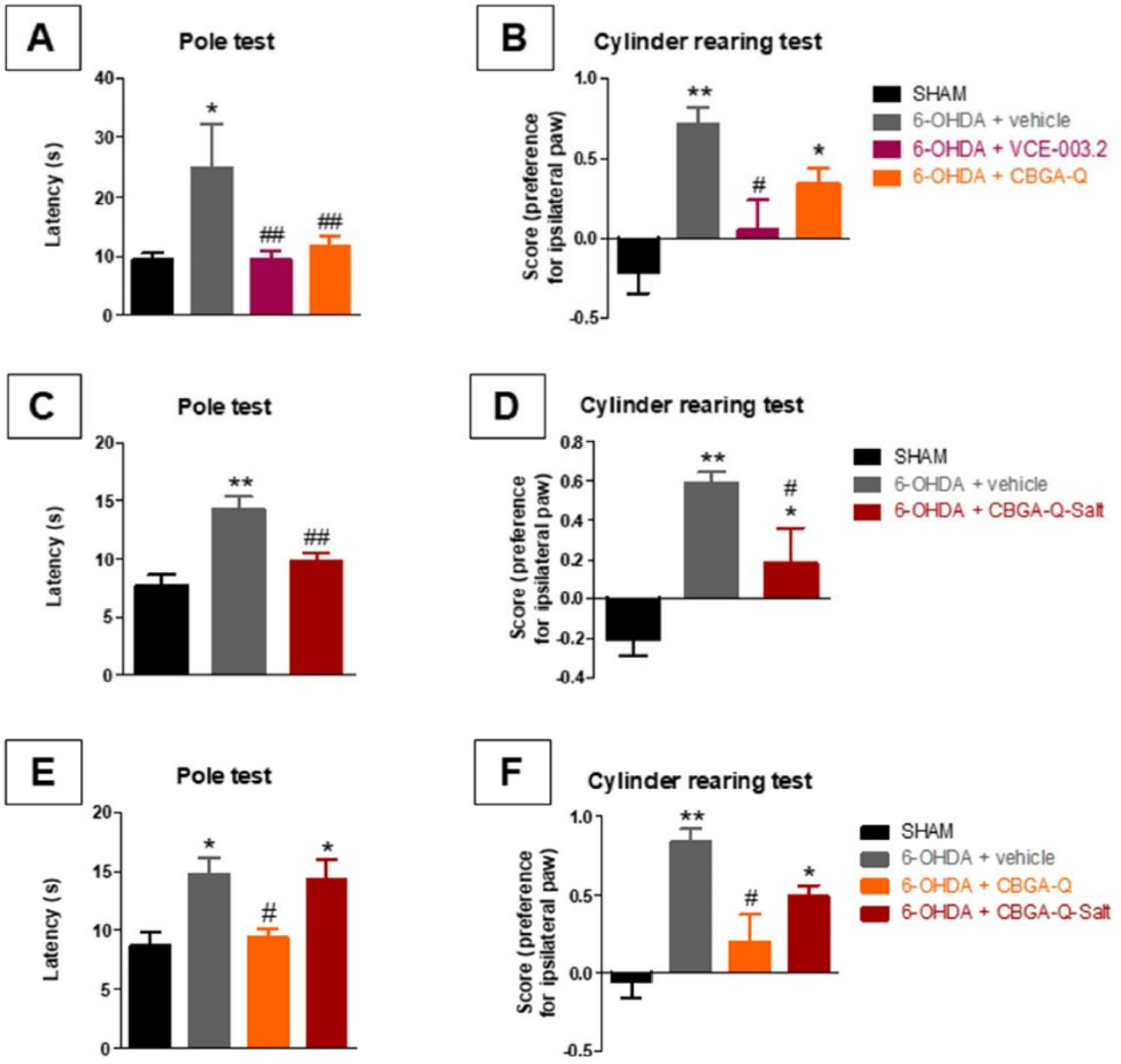

Figure 2. Behavioral analysis of control (sham) mice and unilaterally 6-OHDA-lesioned animals treated with VCE-003.2 (20 mg/kg), CBGA-Q (20 mg/kg) or vehicle (sesame oil) given orally (**A:** response in the pole test; **B:** response in the cylinder rearing test), with CBGA-Q-Salt (40 mg/kg) or vehicle (saline) given orally (**C:** response in the pole test; **D:** response in the cylinder rearing test), and with CBGA-Q (10 mg/kg), CBGA-Q-Salt (10 mg/kg) or vehicle (saline) given i.p. (**E:** response in the pole test; **F:** response in the cylinder rearing test). Treatments were daily and prolonged for 2 weeks. Data corresponded to 24 hours after the last dose and were expressed as means ± SEM of more than 5 animals *per* group. They were analyzed by one-way ANOVA followed by the Bonferroni test (*p<0.05, **p<0.01 *versus* sham; #p<0.05, ##p<0.01 *versus* 6-OHDA + vehicle).

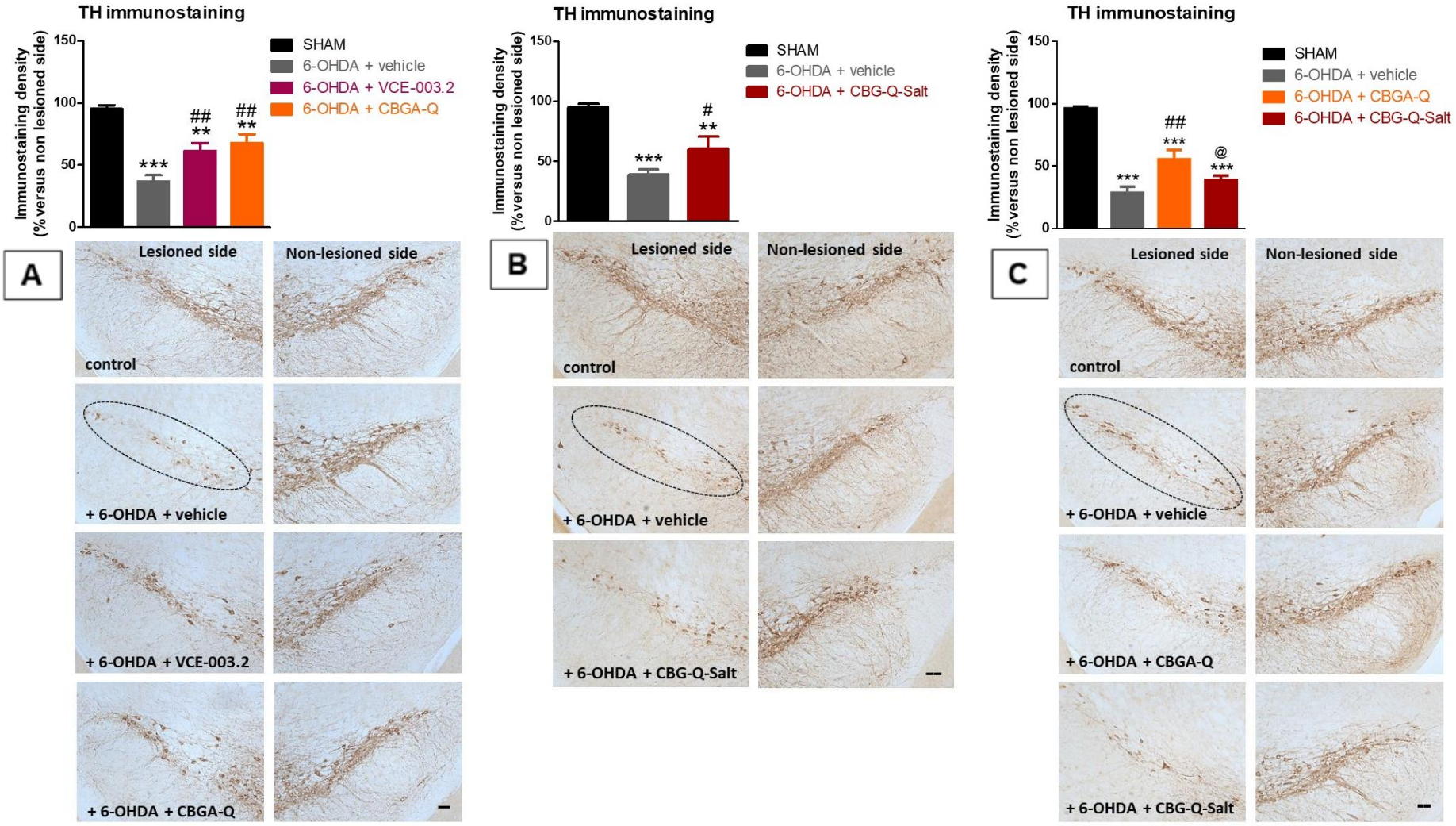

Figure 3. Intensity of the immunostaining for TH measured in a selected area of the substantia nigra pars compacta of control (sham) mice and unilaterally 6-OHDA-lesioned animals treated with VCE-003.2 (20 mg/kg), CBGA-Q (20 mg/kg) or vehicle (sesame oil) given orally (A), with CBGA-Q-Salt (40 mg/kg) or vehicle (saline) given orally (B), and with CBGA-Q (10 mg/kg), CBGA-Q-Salt (10 mg/kg) or vehicle (saline) given i.p. (C). Treatments were daily and prolonged for 2 weeks. Data corresponded to % of the ipsilateral lesioned side versus contralateral non-lesioned side at 24 hours after the last dose and were expressed as means ± SEM of more than 5 animals per group. They were analyzed by one-way ANOVA followed by the Bonferroni test (**p<0.01, ***p<0.005 versus sham; #p<0.05, ##p<0.01 versus 6-OHDA + vehicle; @p<0.05 versus 6-OHDA + CBGA-Q). Representative immunostaining images for each experimental group, with indication of the approximate area quantified, are shown in the bottom panels (scale bar = 100 µm).

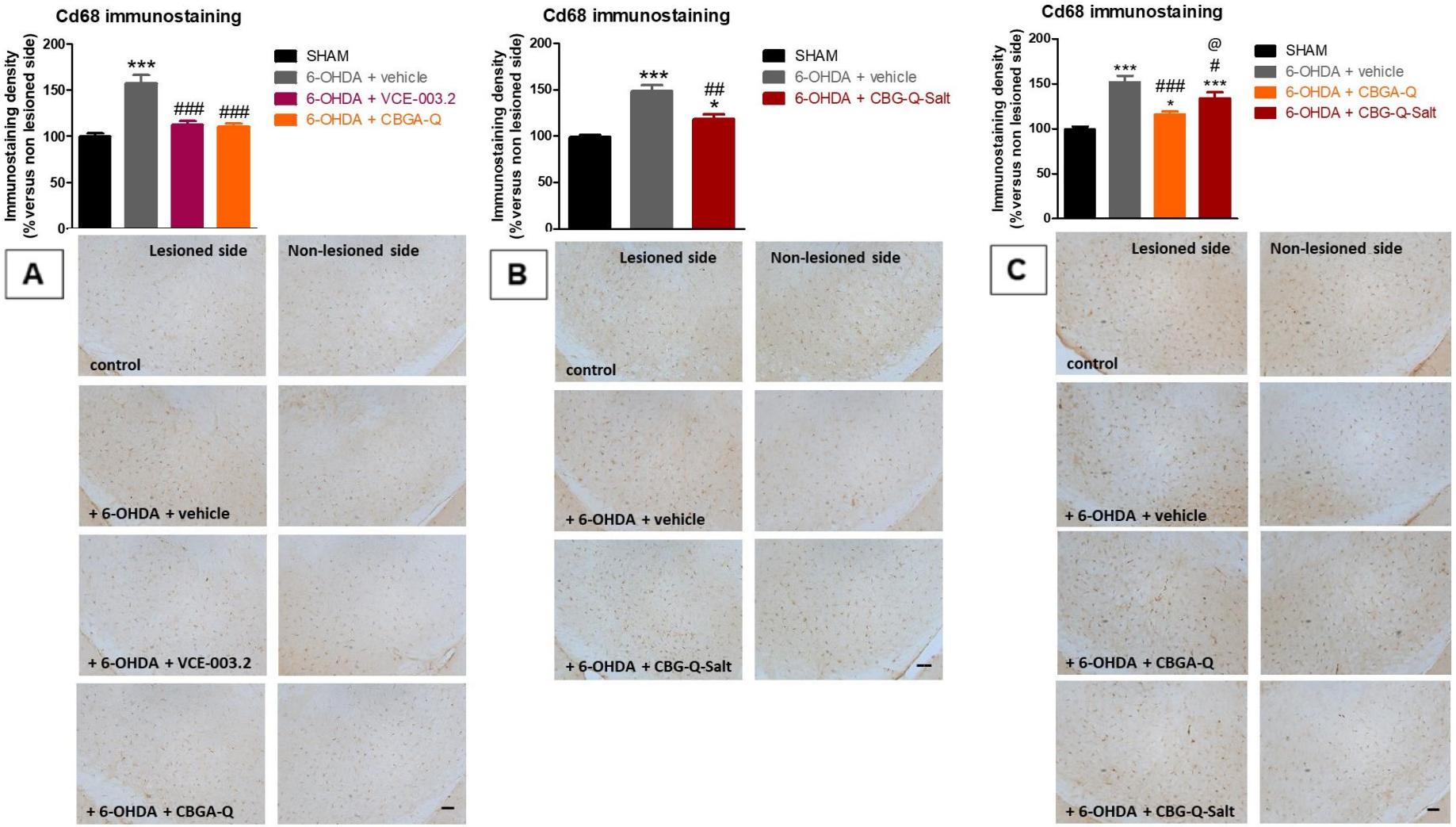

Figure 4. Intensity of the immunostaining for CD68, a marker of microglia and infiltrated macrophages, measured in a selected area of the substantia nigra pars compacta of control (sham) mice and unilaterally 6-OHDA-lesioned animals treated with VCE-003.2 (20 mg/kg), CBGA-Q (20 mg/kg) or vehicle (sesame oil) given orally (A), with CBGA-Q-Salt (40 mg/kg) or vehicle (saline) given orally (B), and with CBGA-Q (10 mg/kg), CBGA-Q-Salt (10 mg/kg) or vehicle (saline) given i.p. (C). Treatments were daily and prolonged for 2 weeks. Data corresponded to % of the ipsilateral lesioned side versus contralateral non-lesioned side at 24 hours after the last dose and were expressed as means ± SEM of more than 5 animals per group. They were analyzed by one-way ANOVA followed by the Bonferroni test (*p<0.05, ***p<0.005 versus sham; #p<0.05, ##p<0.01, ###p<0.005 versus 6-OHDA + vehicle; @p<0.05 versus 6-OHDA + CBGA-Q). Representative immunostaining images for each experimental group are shown in the bottom panels (scale bar = 100 µm).

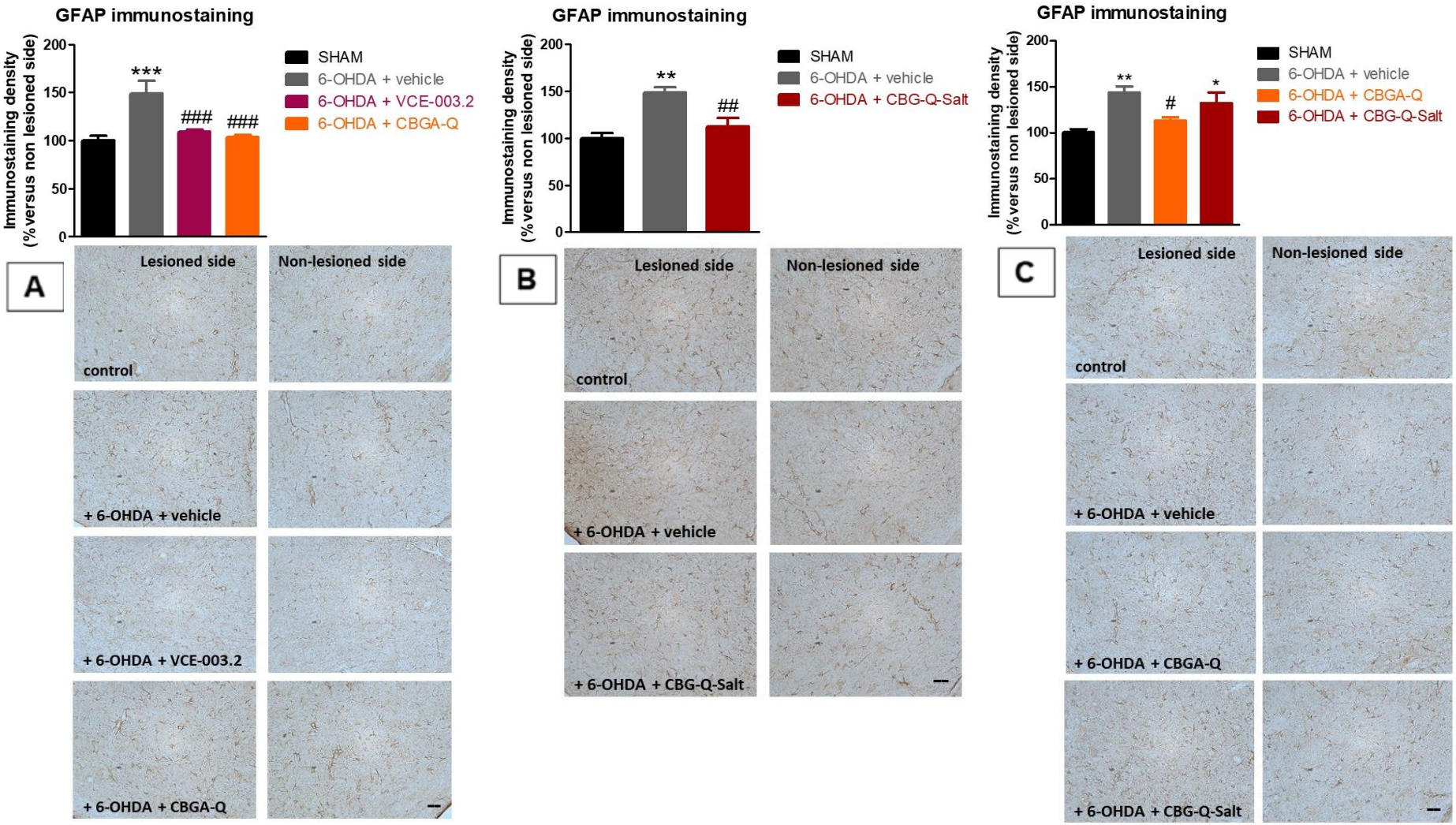

Figure 5. Intensity of the immunostaining for GFAP, a marker of astrocytes, measured in a selected area of the substantia nigra pars compacta of control (sham) mice and unilaterally 6-OHDA-lesioned animals treated with VCE-003.2 (20 mg/kg), CBGA-Q (20 mg/kg) or vehicle (sesame oil) given orally (A), with CBGA-Q-Salt (40 mg/kg) or vehicle (saline) given orally (B), and with CBGA-Q (10 mg/kg), CBGA-Q-Salt (10 mg/kg) or vehicle (saline) given i.p. (C). Treatments were daily and prolonged for 2 weeks. Data corresponded to % of the ipsilateral lesioned side versus contralateral non-lesioned side at 24 hours after the last dose and were expressed as means ± SEM of more than 5 animals per group. They were analyzed by one-way ANOVA followed by the Bonferroni test (*p<0.05, **p<0.01, ***p<0.005 versus sham; #p<0.05, ##p<0.01, ###p<0.005 versus 6-OHDA + vehicle). Representative immunostaining images for each experimental group are shown in the bottom panels (scale bar = 50 µm).

3.4. Treatments with VCE-003.2

The behavioral analysis of these mice revealed their defects in the pole test (they show a strong immobility spending much more time to descend the pole than controls; Figures 2A, 2C and 2E) and, in particular, in the cylinder rearing test (they showed a strong contralateral paresis derived from the unilateral lesion; Figures 2B, 2D and 2F). These motor defects were associated with an important decrease (>50%) in TH immunoreactivity in the lesioned substantia nigra compared to the contralateral side (Figures 3A, 3B and 3C), which likely reflects the expected death in this model of nigral dopaminergic neurons projecting to the striatum (García et al., 2011). We also found microgliosis, reflected in elevated immunoreactivity levels for CD68, a marker of microglial cells and infiltrated macrophages (>40%; Figures 4A, 4B and 4C), and astrogliosis, reflected in elevated GFAP immunostaining (>40%; Figures 5A, 5B and 5C) in the substantia nigra of 6-OHDA-lesioned mice, despite that this is not a model in which glial reactivity plays an important pathogenic role (García et al., 2011). Lastly, we also detected low levels of dopamine and its major intraneuronal metabolite DOPAC (>50%) in the striatum of 6-OHDA-lesioned mice (Figure 6 and Table 2), an observation compatible with the loss of TH-containing nigral neurons and the expected denervation of their terminals in the striatum, as found in previous studies (Lastres-Becker et al., 2005).

The treatment with VCE-003.2 administered orally (20 mg/kg) to 6-OHDA-lesioned mice caused an important improvement in the motor defects shown by these animals in the pole test (strong immobility), reducing the time spent in descending the pole to values similar to controls ($F(3,21)=5,302$, $p<0.01$; Figure 2A). The same happened with the hemiparesis shown by 6-OHDA-lesioned mice in the cylinder rearing test, which was completely reversed by the treatment with VCE-003.2 ($F(3,21)=6.829$, $p<0.005$; Figure 2B). To produce these behavioral improvements, VCE-003.2 administration partially preserved TH-positive nigral neurons against 6-OHDA insult, as reflected the elevation (>50%) in TH immunoreactivity measured in the lesioned substantia nigra after the treatment with this derivative ($F(3,22)=14.56$, $p<0.0001$; Figure 3A). As mentioned above, glial reactivity is not an important pathogenic event in 6-OHDA-lesioned mice, but a presumably secondary response caused by the death of TH-positive neurons (García et al., 2011, 2018), a fact we confirmed in our study, and it was completely attenuated by the treatment with VCE-003.2. Thus, the elevated levels of immunoreactivity for CD68 found in the substantia nigra of 6-OHDA-lesioned mice were reduced to the levels detected in controls ($F(3,23)=20.24$, $p<0.0001$; Figure 4A). The same response to VCE-003.2 was also found for the astroglial marker GFAP ($F(3,23)=13.15$, $p<0.0001$; Figure 5A). Lastly, with regard to the reduction in the concentrations of dopamine and its metabolite DOPAC observed in the striatum of 6-OHDA-lesioned mice, the treatment with VCE-003.2 elevated both dopamine (up to 50% of control values; $F(2,14)=11.0$, $p<0.005$) and DOPAC (up to the same control values; $F(2,15)=8.37$, $p<0.005$) contents (Figure 6), which is compatible with the recovery in the number of TH-positive neurons and supports that this recovery was functional in terms of transmission activity.

It is important to indicate that VCE-003.2 was not administered to control animals in our experiment, despite the interest of detecting additional effects of this derivative in absence of lesion. The reason was the ethical recommendations, based on the 3R criteria, indicated by the corresponding committee in our institution. However, given that the lesions carried out in this model were unilateral, these potential effects of VCE-003.2 in absence of lesion can be detected by looking specifically at

the non-lesioned contralateral side of all animals in this study. The Supplementary Figure 2 shows the immunostaining data for TH, CD68 and GFAP in all non-lesioned contralateral sides of the animals used for the experiment with VCE-003.2, proving no variation due to the treatment with this derivative in absence of lesion. The same analysis was also carried out in the experiments with the other two derivatives, CBGA-Q and CBGA-Q-Salt, that will be described below, then resulting in the same absence of any variation for TH, CD68 and GFAP in absence of lesion (see data in the non-lesioned contralateral structures in the Supplementary Figure 2).

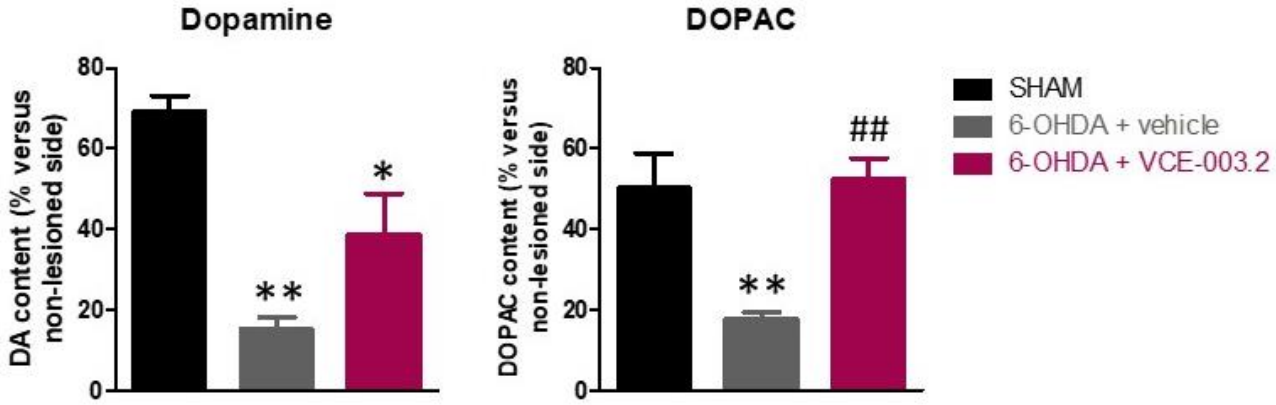

Figure 6. Dopamine and DOPAC concentrations in the striatum of control (sham) mice and unilaterally 6-OHDA-lesioned animals treated with VCE-003.2 (20 mg/kg) or vehicle (sesame oil) given orally. Treatments were daily and prolonged for 2 weeks. Data corresponded to % of the ipsilateral lesioned side versus contralateral non-lesioned side at 24 hours after the last dose and were expressed as means ± SEM of more than 5 animals per group. They were analyzed by one-way ANOVA followed by the Bonferroni test (*p<0.05, **p<0.01 versus sham; ##p<0.01 versus 6-OHDA + vehicle).

3.5. Treatment with CBGA-Q

We also treated 6-OHDA-lesioned mice with CBGA-Q using two routes for administration. First, it was administered orally (20 mg/kg) in the same experiment as VCE-003.2, where it showed very similar effects, with an almost complete normalization of the response in the pole test (Figure 2A), although with an apparently lower efficacy in the cylinder rearing test (Figure 2B). CBGA-Q also elevated TH immunoreactivity (>50%) in the 6-OHDA-lesioned substantia nigra (Figure 3A) and reduced the elevated levels of immunoreactivity for CD68 in the substantia nigra to the levels detected in controls (Figure 4A), with a similar response for the astroglial marker GFAP (Figure 5A). However, the treatment with CBGA-Q failed to recover the concentrations of dopamine and its metabolite DOPAC in the striatum of 6-OHDA-lesioned mice (Table 2), despite the recovery found in the number of TH-positive neurons, possibly indicating lower functional activity, although this had no reflection in the normalization of motor parameters (Figures 2A and 2B).

CBGA-Q was also given to 6-OHDA-lesioned mice by i.p. administration using a lower dose (10 mg/kg), but, in general, the results were relatively similar to those found after the oral administration, again with a complete normalization of the response in the pole test ($F(3,18)=5.96$, $p<0.01$; Figure 2E), a partial recovery of the hemiparesis in the cylinder rearing test ($F(3,17)=8.07$, $p<0.005$; Figure 2F), a partial preservation of TH-positive nigral neurons ($F(3,18)=36.77$, $p<0.0001$; Figure 3C), and important reductions in CD68 immunoreactivity ($F(3,18)=17.22$, $p<0.0001$; Figure 4C) and GFAP immunostaining ($F(3,18)=7.151$, $p<0.005$; Figure 5C). Also in agreement with the oral

administration, the i.p. treatment with CBGA-Q also failed to recover the concentrations of dopamine and its metabolite DOPAC in the striatum of 6-OHDA-lesioned mice (Table 2).

Table 2.

Dopamine and DOPAC concentrations in the striatum of control (sham) mice and unilaterally 6-OHDA-lesioned animals treated with: (i) CBGA-Q (20 mg/kg) or vehicle (sesame oil) given orally; (ii) CBGA-Q-Salt (40 mg/kg) or vehicle (saline) given orally; and (iii) CBGA-Q (10 mg/kg), CBGA-Q-Salt (10 mg/kg) or vehicle (saline) given i.p.. Treatments were daily and prolonged for 2 weeks. Data correspond to % of the ipsilateral lesioned side *versus* contralateral non-lesioned side 24-hour post last dose and are expressed as means ± SEM of more than 5 animals *per* group. They were analyzed by one-way ANOVA followed by the Bonferroni test (**p<0.01, ***p<0.005 *versus* sham).

Route	Treatments	DA (% *versus* non-lesioned side)	DOPAC (% *versus* non-lesioned side)
Oral	Sham	69.1 ± 3.9 (5)	50.2 ± 8.7 (5)
	+ 6-OHDA + vehicle	15.2 ± 3.0 (4)***	17.7 ± 1.7 (4)**
	+ 6-OHDA + CBGA-Q	27.8 ± 6.0 (6)***	26.3 ± 4.1 (7)**
	Statistics	F(2,14)=29.9, p<0.0001	F(2,15)=7.8, p<0.01
Oral	Sham	95.7 ± 0.9 (4)	109.6 ± 11.3 (4)
	+ 6-OHDA + vehicle	31.3 ± 5.4 (4)**	52.4 ± 12.7 (4)
	+ 6-OHDA + CBGA-Q-Salt	40.1 ± 12.4 (6)**	73.3 ± 22.4 (6)
	Statistics	F(2,13)=11.2, p<0.005	F(2,13)=2.0, ns
i.p.	Sham	82.0 ± 4.7 (5)	117.4 ± 13.0 (4)
	+ 6-OHDA + vehicle	13.4 ± 4.0 (4)***	25.3 ± 5.6 (4)***
	+ 6-OHDA + CBGA-Q	19.0 ± 4.6 (5)***	38.6 ± 7.1 (5)***
	+ 6-OHDA + CBGA-Q-Salt	11.2 ± 3.2 (5)***	25.7 ± 5.8 (5)***
	Statistics	F(3,18)=66.1, p<0.0001	F(3,17)=27.7, p<0.0001

** p < 0.01
*** p < 0.005 *versus* sham

3.6. Treatment with CBGA-Q-Salt

The third derivative, CBGA-Q-Salt, was also investigated in 6-OHDA-lesioned mice using the same two routes for administration. As for CBGA-Q, CBGA-Q-Salt was first administered orally (40 mg/kg). It showed an important reduction in the time spent to descend in the pole test (F(2,14)=12.64, p<0.005; Figure 2C) and a partial reduction in the hemiparesis shown in the cylinder rearing test (F(2,10)=16.66, p<0.005; Figure 2D). CBGA-Q-Salt also elevated TH immunoreactivity in the 6-OHDA-lesioned substantia nigra (F(2,13)=15.61, p<0.001; Figure 3B), whereas it reduced the elevated levels of immunoreactivity for CD68 (F(2,14)=20.15, p<0.0001; Figure 4B) and for GFAP (F(2,14)=10.29, p<0.005; Figure 5B). In the three cases, CBGA-Q-Salt showed an apparent lower effect compared to VCE-003.2 and CBGA-Q. However, by contrast with VCE-003.2 and similarly to CBGA-Q, the treatment with CBGA-Q-Salt failed to recover the concentrations of dopamine and its metabolite DOPAC in the striatum of 6-OHDA-lesioned mice (Table 2).

CBGA-Q-Salt was also investigated after i.p. administration at a lower dose (10 mg/kg) in the same experiment carried out with CBGA-Q, but contrarily to this acid derivative, CBGA-Q-Salt was poorly active by this route of administration. We did not find any recovery in the pole test (Figure 2E), although the compound was partially active in the cylinder rearing test (Figure 2F). In concordance with this poor behavioral improvement, it did not elevate the low immunoreactivity for TH found in 6-OHDA-lesioned mice (Figure 3C), and showed only small effects in reducing the elevated glial reactivity found in 6-OHDA-lesioned mice, which were statistically significant only in the case of Cd68 (Figure 4C) but not for GFAP (Figure 5C). Lastly, the i.p. treatment with CBGA-Q-Salt failed to recover the concentrations of dopamine and its metabolite DOPAC in the striatum of 6-OHDA-lesioned mice (Table 2). It is important to note that we used the same dose, 10 mg/kg than for CBGA-Q, which was, in general, active to a similar extent as a 2-fold higher dose given orally. In the case of CBGA-Q-Salt, the dose used for oral administration was 4-fold higher than the dose used for i.p. administration. It is possible that we would have needed a dose of at least 20 mg/kg for i.p. administration.

4. Discussion

Our current study is an extension of previous reports aimed at exploring the neuroprotective potential of the non-thiophilic CBG quinone derivative VCE-003.2 in experimental models of PD (García et al., 2018; Burgaz et al., 2019). The novelties of this follow-up study are: (i) the use of the 6-OHDA model, which was explored before only in a pilot study (García et al., 2018, supplementary data); (ii) the use of an oral route for administration, which may facilitate the clinical development of the most active compounds; and (iii) the testing of new analogs of CBGA in search of advantages (e.g., higher potency at the PPAR-γ receptor, additional activities at other receptors, easier and faster synthesis, and/or better bioavailability) for future clinical development. Our data confirmed first that VCE-003.2 was also highly active in the 6-OHDA model as pointed out in our pilot study (García et al., 2018), showing an important recovery in the number of TH-positive nigral neurons with a parallel recovery in the striatal contents of dopamine and its major intraneuronal metabolite DOPAC. This had an important impact in the recovery of the motor defects typical of 6-OHDA-lesioned mice, which showed values similar to controls. These data support that VCE-003.2 was active not only against inflammation-driven neuronal deterioration, as found in our previous studies in LPS-lesioned mice (García et al., 2018; Burgaz et al., 2019), but also in a 6-OHDA-lesioned mouse model with a more modest glial reactivity. This has been found to be secondary to neuronal death, and it was also completely attenuated by the treatment with VCE-003.2, presumably as a result of its capability to preserve TH-positive nigral neurons. This observation confirms our preliminary data also obtained with VCE-003.2 (administered i.p.) in 6-OHDA-lesioned mice (García et al., 2018, supplementary data), and confirms the activity of this CBG aminoquinone derivative when given orally to mice using a dose two-fold higher than in the previous i.p. administration (García et al., 2018, supplementary data). This is an important observation in the direction of a future clinical development of VCE-003.2 for PD that was also observed in the LPS model (Burgaz et al., 2019). Moreover, oral VCE-003.2 was found to be neuroprotective and to induce neurogenesis in a preclinical model of Huntington´s disease (Aguareles et al., 2019).

This study was also designed to explore the neuroprotective potential of new CBGA analogs, with the objective to determine whether they may improve the profile shown by VCE-003.2 in

experimental PD. VCE-003.2 is a two-step synthesis aminoquinone derivative of CBG that is highly lipophilic with a limited oral absorption (10-12% oral bioavailability) when formulated in lipid solvents (Aguareles et al., 2019). Since the conversion of the resorcinol to paraquinone is essential for the increased PPAR-γ activity of cannabinoids (Granja et al., 2012), we developed the new analogs CBGA-Q and CBGA-Q-Salt. CBGA-Q has the advantage that it can be synthetized in one-step starting from CBGA, and CBGA-Q-Salt is a water-soluble derivative, so having *a priori* possibly better bioavailability. We demonstrated that these two new compounds, CBGA-Q and CBGA-Q-Salt, have a similar mechanism of action, activation of PPAR-γ receptors, using transcriptional assays. As VCE-003.2, they are also devoid of any activity at the CB_1 and CB_2 receptors. We found CBGA-Q, given both orally and i.p. (at the same doses as VCE-003.2), to be the most active of these two new CBGA derivatives in preserving TH-positive neurons, attenuating glial reactivity and improving motor performances, in some cases to a similar extent than VCE-003.2. However, we did not find recoveries in the striatal contents of dopamine and DOPAC similar to VCE-003.2, which indicates that, in addition to PPAR-γ, VCE-003.2 may activate other signaling pathways involved in neuroprotection and perhaps neurogenesis in PD, which would not be present for CBGA-Q. This difference between the compounds may be related to their differences in the binding to PPAR-γ, as will be described below. This possibility remains to be further investigated. Accordingly, we have recently shown that VCE-003.2 is also capable to induce a transient activation of ERK and mTORC1 signaling pathways in HiB5 hippocampal progenitor cell line (Diaz-Alonso et al., 2016) and promote neuronal-like differentiation as revealed by CTIP2 reporter activation in neuralized mouse embryonic teratocarcinoma P19 cells (Aguareles et al., 2019). Thus, VCE-003.2, as many other cannabinoids, is a multitarget compound and further research on its mechanism of action is warranted.

In the case of the water-soluble CBGA analog, CBGA-Q-Salt, our data reflect that its neuroprotective activity is not comparable to VCE-003.2 and CBGA-Q. CBGA-Q-Salt was only active when given orally at a dose two-fold higher than the doses used for VCE-003.2 and CBGA-Q, although to a lower extent, but was completely inactive given intraperitoneally. It is possible that this complete absence of activity given i.p. (it only showed a small effect in the cylinder rearing test and in CD68 immunostaining) may be due to a too low dose for this compound, and higher doses (≥ 20 mg/kg) may be active, at least to the extent found with the oral dose, but this will require additional studies.

Our study also investigated the potential mechanism(s) involved in the effects of these compounds against 6-OHDA insult. We concentrated in the PPAR-γ given that the previous literature (Díaz-Alonso et al., 2016; García et al., 2018), the receptor profile shown by these three compounds, and the data obtained with the analysis of the PPAR-γ mRNA levels in the striatum that proved an elevation in 6-OHDA-lesioned mice compared to controls. The confirmation of the role of PPAR-γ was obtained by using an *in vitro* model, SH-SY5Y cells. First, we characterized the best concentration of 6-OHDA to kill the cells and the best concentration of VCE-003.2 to promote cell survival. We used VCE-003.2 at this concentration (20 μM), as well as CBGA-Q and CBGA-Q-Salt at the same concentration, for confirming their cytoprotective profile in this cell assay. They all induced increased cell viability significantly, which was completely reversed by a classic PPAR-γ inhibitor (GW9662) only for CBGA-Q and CBGA-Q-Salt but not VCE-003.2. This confirms that CBGA-Q and CBGA-Q-Salt would be acting in this cell assay to promote cell survival by the

activation of the canonical site in the PPAR-γ receptor. However, our data confirmed that this was not the case for VCE-003.2, which, in the previous study was found to act at both the canonical and, in particular, alternative regulatory (non-sensitive to classic antagonists such as GW9662) sites in the PPAR-γ receptor (García et al., 2018), although this difference could be a dose issue given the reversal found with GW9662 against the effect of VCE-003.2 in cultured neural progenitor cells observed at concentrations significantly lower than those used here (Díaz-Alonso et al., 2016). Anyway, our result suggests that for VCE-003.2 the quinone modification at positions 1,4 is essential for the binding to the canonical site and the modification at position 2 (ethylamine) is required for the binding at the alternative site of PPAR-γ and perhaps to other signaling pathways activated by this cannabinoid. Although this will require further research, these subtle mechanistic differences may have an important influence in the different extent found for the neuroprotective activity of these three compounds: VCE-003.2 > CBGA-Q > CBGA-Q-Salt.

In summary, our data confirmed the neuroprotective potential of VCE-003.2 in 6-OHDA-lesioned mice, which adds to its previously identified activity found in an inflammatory model of PD (LPS-lesioned mice). VCE-003.2 was more active than the new cannabinoid derivatives investigated, CBGA-Q and CBGA-Q-Salt. Those new CBGA derivatives afforded neuroprotection in 6-OHDA-lesioned mice but with effects lower compared to VCE-003.2, in particular in the case of CBGA-Q-Salt. *In vitro* studies confirmed the relevance of PPAR-γ receptors for these effects. With this study, we expect to add experimental support to the idea that a pharmaceutical formulation, using a pleiotropic cannabinoid derivative targeting PPAR-γ receptors, and perhaps other relevant targets for PD, may be of great interest as a disease-modifying agent in PD.

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Declaration of potential conflict of interest

The authors declare that they have not any conflict of interest in relation with this study.

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Oral VCE-003.2 as a disease-modifying treatment in AAV-α-synuclein model of Parkinson's disease

Sonia Burgaz[1-3], [...] and Javier Fernández-Ruiz[1-3].

[1] Instituto Universitario de Investigación en Neuroquímica, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, Madrid, Spain;
[2] Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain;
[3] Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain;

Abstract

The therapeutic potential of the phytocannabinoid derivative VCE-003.2 (administered both i.p. and orally) has been proved in two different experimental models of Parkinson's disease (PD), 6-hydroxydopamine(6-OHDA)-lesioned mice, a model predominantly based on mitochondrial dysfunction and oxidative stress, and LPS-lesioned mice in which neuronal damage is associated with an intense glial reactivity and neuroinflammation. Our data have indicated important benefits with this compound in the two models through anti-inflammatory and neuroprotective effects that appeared to be mediated by the activation of a regulatory site within the peroxisome proliferator-activated receptor-γ (PPAR-γ). No evidence exists that VCE-003.2 may be also active in experimental models of PD based on α-synuclein dysregulation. To this end we explored again VCE-003.2, using a model of PD based on local overexpression of mutant (A53T) α-synuclein (α-SYN) administered using adeno-associated viral vector (AAV) technology. VCE-003.2 was administered orally during 14 days at the dose of 20 mg/kg, as in previous studies. We found a behavioural improvement based on expected disease-modifying effects in the motor tests, which however was not correlated with the histological analysis of the substantia nigra (SN). In this respect, we found no recovery in the loss of TH-positive neurons, as well as no attenuation in elevated microglial and astroglial reactivities in the SN by VCE-003.2 treatment. However, RNA sequencing analysis in the striatum of these animals revealed important changes in the expression of immune response, inflammatory and lysosomal genes in the α-SYN mice, with some of them reversed after VCE-003.2 treatment (eg. E2F1 regulon). In summary, our results show potential benefits with VCE-003.2 in the model of α-SYN aggregation, although future mechanistic studies are needed.

Key words: cannabinoids; VCE-003.2; α-synuclein; neuroprotection; neuroinflammation; PPAR-γ; Parkinson's disease.

1. Introduction

Cannabinoids are pleiotropic compounds that have multiple pharmacological targets within the endocannabinoid system, as well as in other signalling systems, which makes them promising neuroprotective agents (Fernández-Ruiz, 2019). Such neuroprotective potential has been preclinically investigated in different neurological conditions, such as accidental brain damage (e.g., stroke, brain trauma) and in chronic progressive disorders (e.g., Alzheimer's

disease (AD), amyotrophic lateral sclerosis, Huntington's disease (HD)) (Fernández-Ruiz et al., 2015). One of the disorders that has recruited to date more research with cannabinoids is PD, searching for new therapies based on these compounds aimed at delaying disease progression but also at alleviating specific parkinsonian symptoms (Antonazzo et al., 2019; Baul et al., 2019). There are several preclinical studies which give evidence of the potential of cannabinoid compounds over different targets. The cannabinoid receptor type 1 (CB1) has been studied for reducing parkinsonian signs as bradykinesia and immobility (Kelsey et al., 2009), tremor (Sañudo-Peña et al., 1998) and/or L-DOPA-induced dyskinesia (Espadas et al., 2020), as well as a neuroprotective therapy (Chung et al., 2011; Pérez-Rial et al., 2011). However, most of cannabinoids proposed as disease modifiers target the cannabinoid receptor type 2 (CB2) whose activation entails anti-inflammatory effects in different PD models (García et al., 2011, Javed et al., 2016; Gómez-Gálvez et al., 2016; Wang et al., 2018). Similar effects have been found for the activation of PPAR-γ (García et al., 2018; Burgaz et al., 2019) and even by targeting an orphan receptor, GPR55, which has been recently associated with the endocannabinoid system (Celorrio et al., 2017). Also, there are receptor-independent effects of these compounds, as they have antioxidant properties (Lastres-Becker et al., 2005) and they have been proposed as modulators of the antioxidant transcription factor Nrf2 signalling (Gugliandolo et al., 2018).

These studies have situated several cannabinoid compounds in a promising position for serving to generate a cannabinoid-based therapy for specific symptoms and, in particular, for disease progression in patients affected by PD. An interesting cannabinoid compound in PD is the non-thiophilic CBG quinone derivative VCE-003.2, which behaves as a PPAR-γ activator with no activity at the CB1/CB2 receptors (Díaz-Alonso et al., 2016). This study investigated the effect of VCE-003.2 in murine models of HD, confirming its neuroprotectant profile exerted by activating PPAR-γ and its ability to cross the blood–brain barrier after systemic administration. Moreover, oral VCE-003.2 was found to be neuroprotective and to induce neurogenesis in a preclinical model of HD (Aguareles et al., 2019). Regarding PD, VCE-003.2 has been found to be active as an anti-inflammatory and neuroprotective agent against inflammation-driven neuronal deterioration in LPS-lesioned mice (García et al., 2018; Burgaz et al., 2019), but also in 6-OHDA-lesioned mice (Burgaz et al., 2021). These effects were found to be mediated by its binding at a functional alternative site (different from the canonical binding site used by glitazones) in the PPAR-γ receptor in *in vitro* studies carried out in cell-based assays (García et al., 2018; Burgaz et al., 2021).

Now, we are interested in further investigating the neuroprotective profile of VCE-003.2 in PD models based on α-synuclein dysregulation and accumulation, as the lack of aggregates is an important drawback of the other PD models we used to date. We recently carried out some experiments in the A53T α-synuclein transgenic mouse model, but results were failed due to a poor pathological PD phenotype in these mice. Then, we initiated experiments in an alternative α-synuclein-based murine model, which is based on AAV-mediated overexpression of mutant A53T α-synuclein, following previous studies of some of our collaborators that confirmed the relevance of this model for investigating PD therapeutics (Rodríguez-Pérez et al., 2018; Castro-Sánchez et al., 2018). This AAV model is much more advantageous compared with the classic α-synuclein transgenic mice. It generates a pathological phenotype much closer to PD (motor

defects, α-synuclein dysregulation, loss of dopaminergic neurons, autophagy induction, glial reactivity) and these pathological features are reached in a shorter time (a strong phenotype is visible at 2 weeks after viral inoculation, whereas this takes up to one year in transgenic mice resulting in a very poor pathological phenotype). Therefore, our objective in this study was to investigate the neuroprotective effects of a chronic oral administration of 20 mg/kg of VCE-003.2 in this model using several behavioural, biochemical, and histopathological markers for confirming these potential benefits.

2. Materials and methods

2.1. Animals and surgical lesions

Male C57BL/6 mice were housed in a room with a controlled photoperiod (08:00-20:00 light) and temperature (22 ± 1ºC). They had standard food and water *ad libitum* and were used at adult age (7–8-month-old; 28-33 g weight). All experiments were conducted according to national and European guidelines (directive 2010/63/EU), as well as conformed to ARRIVE guidelines and approved by the "Comité de Experimentación Animal" of our university (PROEX: 056/19).

Mice were anaesthetized (ketamine 40 mg/kg + xylazine 4 mg/kg, i.p.) and subjected to unilateral injections of viral particles (rAAV9-α-SYN^{A53T}; kindly provided by Dr. Lanciego from University of Navarra, Spain), into the SN of the right hemisphere following the procedure described in Lastres-Becker et al., 2012. In brief, we injected 1 µl of a viral suspension containing 3.33 x 10^{13} GC/ml at the following stereotaxic coordinates from bregma: +2.5 mm AP, −1.4 mm ML and −4.5 mm DV, formulated in phosphate-buffered saline (PBS), pH 7.4, supplemented with 200nM NaCl and 0.001% pluronic F-68. The volume of 1 µl was injected slowly (0.2 µl/min) and the needle was left in place for 5 min before being slowly withdrawn. This avoids generating reflux and a rapid increase of intracranial pressure. Control animals were SHAM-operated and injected with 1 µl of a viral suspension of AAV9-CMV-null containing 1.07 x 10^{13} GC/ml using the same coordinates. The lesions were generated using unilateral administration, so that contralateral structures serve as controls for the different analyses.

2.2. Pharmacological treatments and sampling

After the application of rAAV9-α-SYN^{A53T} or null (empty) virus, animals were distributed into 3 groups in each experiment, and were subjected to a daily treatment with VCE-003.2 (20 mg/kg), or vehicle (sesame oil) by oral administration. The first administration, in all cases, was done approximately 24 hours after the lesion and the treatment was prolonged for two weeks. At the end of the treatment (24 hours after the last injection), they were analysed in different behavioural tests just before being sacrificed by decapitation, and their brains were rapidly removed and divided coronally in two parts. The anterior halves were used to dissect the striatum (both ipsilateral and contralateral sides separately) and tissues were rapidly frozen by immersion in cold 2-methylbutane and stored at -80ºC for RNA sequencing analysis (RNAseq). The posterior halves containing the midbrains were fixed for one day at 4°C in fresh 4% paraformaldehyde prepared in 0.1 M PBS, pH 7.4. Samples were cryoprotected by

immersion in a 30% sucrose solution for a further day, and finally stored at -80°C for immunohistochemical analysis in the substantia nigra.

2.3. Behavioural tests

2.3.1. Cylinder rearing test (CRT)

Given that the lesions were unilateral, this test attempts to quantify the degree of forepaw (ipsilateral, contralateral, or both) preference for wall contacts after placing the mouse in a methacrylate transparent cylinder (diameter: 15.5 cm; height: 12.7 cm; Fleming et al., 2013). Each score was made from a 3 min trial with a minimum of 4 wall contacts.

2.3.2. Elevated-body swing test (EBST)

Mice were placed head-downward hanging by their tail in a vertical axis (about 15 cm from the surface) and they were recorded for 60 s (Borlongan and Sanberg, 1995; Bentea et al., 2015). A swing was recorded whenever the animal moved its head out of the vertical axis to either side, and for the next swing to be counted, the animal must have returned to the vertical position first.

2.4. Immunohistochemical procedures

Brains were sliced in coronal sections (containing the SN) in a cryostat (30 µm thick) and collected on antifreeze solution (glycerol/ethylene glycol/PBS; 2:3:5) and stored at -20 ºC until used. Brain sections were mounted on gelatine-coated slides, and once adhered, washed in 0.1M potassium PBS (KPBS) at pH 7.4. Endogenous peroxidase was blocked by 30 min incubation at room temperature in peroxidase blocking solution (Dako Cytomation, Glostrup, Denmark). After several washes with KPBS, sections were incubated overnight at room temperature with the following polyclonal antibodies: (i) rabbit anti-α-synuclein (Chemicon-Millipore, Temecula, CA, USA) used at 1:2000; (ii) rabbit anti-tyrosine hydroxylase (TH) (Chemicon-Millipore, Temecula, CA, USA) used at 1:200; (iii) rat anti-mouse CD68 antibody (AbD Serotec, Oxford, UK) used at 1:200; or (iv) rabbit anti-mouse GFAP antibody (Dako Cytomation, Glostrup, Denmark) used at 1:200. Dilutions were carried out in KPBS containing 2% bovine serum albumin and 0.1% Triton X-100 (Sigma Chem., Madrid, Spain). After incubation, sections were washed in KPBS, followed by incubation with the corresponding biotinylated secondary antibody (1:1000 for α-synuclein; 1:200 for the rest) (Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature. Avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine substrate–chromogen system (Dako Cytomation, Glostrup, Denmark) were used to obtain a visible reaction product. Negative control sections were obtained using the same protocol with omission of the primary antibody. A Leica DMRB microscope and a DFC300FX camera (Leica, Wetzlar, Germany) were used for the observation and photography of the slides, respectively. For quantification of α-synuclein, TH, CD68 or GFAP immunostaining in the SN, we used the NIH Image Processing and Analysis software (ImageJ; NIH, Bethesda, MD, USA) using 4-5 sections, separated approximately by 200 µm, and observed with 5x-20x objectives depending on the method and the brain area under quantification. In all sections, the same areas of the SN were analysed. The analyses were always conducted by experimenters who were blinded to all animal

characteristics. Data were expressed as percentage of immunostaining intensity in the ipsilateral (lesioned) side over the contralateral (non-lesioned) side.

2.5. Transcriptomic analysis

2.5.1. RNA isolation

RNA was isolated from the striatum samples using the RNeasy Lipid Tissue Mini kit (Qiagen) following the manufacturer's instructions, including the optional DNase I step. Once RNA was isolated, samples were stored at −80°C. RNA concentration and RNA integrity number (RIN) was assessed using Nanodrop and bioanalyzer. Average RIN values for the samples was 8.10.

2.5.2. Library preparation, sequencing, and alignment

Library preparation and sequencing was outsourced to the Centre for Genomic Regulation (CRG). Libraries were prepared and sequenced by 50 bp sequencing using HighSeq. Sequences were aligned against the mouse genome (~99% alignment rate) with 60% of reads aligned to exonic regions. Individual gene counts and transcripts per million (TPM) used for downstream analyses were generated using RSEM and assembled to a matrix via the tximport R package. Then, counts per million (CPM) were calculated using cpm() function from the edgeR packing in R. Lowly expressed genes were filtered out, which were defined as having less than one CPM in at least 50% of the samples leading to a total of 14,018 genes. Principal Component Analysis (PCA) was used to identify sources of variation and outliers, leading to 9 samples that were maintained for downstream analysis.

2.5.3. Differential expression analysis

Differential expression analysis was performed using the R package Limma. The inputs included the covariate file and the count matrix. edegR was used for data normalization using TMM values and followed by voom transformation. Two independent analyses were performed: (i) α-SYN vs SHAM (3 samples vs 3 samples) and (ii) α-SYN + VCE-003.2 vs α-SYN + vehicle (3 samples vs 3 samples). P-values were corrected using the Benjamini-Hochberg false discovery rate (FDR).

2.5.4. Pathway enrichment analysis

Pathway enrichment analysis was performed using pathways from Biological Process (Gene Ontology) and MSigDb. Unbiased pathway analysis was performed using 2 approaches:

- In the comparison lesioned vs non-lesioned: differentially expressed genes (DEGs) were filtered (FDR < 0.05 & absolute logFC > 0.5) and gene set enrichment analysis (GSEA) browser was used for pathway enrichment using Biological Process (BP) from Gene Ontology (GO) and MSigDb. Only pathways enriched at q-value < 0.05 were considered.
- In the comparison α-SYN + VCE-003.2 vs α-SYN + vehicle no DEGs were obtained at FDR < 0.05. Thus, pathway analysis was performed using the pre-ranked strategy in terms of t value using fGSEA (and BP from GO). Only pathways enriched at q-value < 0.05 were considered.

2.5.5. Transcription factor enrichment analysis

For exploring if genes were enriched for specific transcription factors, we used DoRothEA (Garcia-Alonso et al., 2019; Holland et al., 2020). Regulons were filtered at confidence levels "A" and "B". Regulon enrichment was runned using fGSEA with preranked data in terms of t value. Only regulons with adjusted p-value < 0.05 were considered.

2.7. Statistics

In behavioural and immunohistochemical analyses, data were assessed using one-way ANOVA, followed by the Tukey test using GraphPad Prism, version 8.00 for Windows (GraphPad Software, San Diego, CA, USA). A p value lower than 0.05 was used as the limit for statistical significance. The sample sizes in the different experimental groups were always ≥ 5. In RNAseq studies data was analysed using R. In differential expression analyses, p-values were corrected using the Benjamini-Hochberg FDR. FDR lower than 0.05 was used as the limit for statistical significance. In enrichment analyses, p-values were corrected using q-values. Pathways or regulons enriched with q-values lower than 0.05 were considered significant. The sample size was 3 animals per group.

3. Results

In this experiment, the neuroprotective potential of an oral administration of VCE-003.2 was investigated in the mutant α-synuclein overexpression (α-SYN) model of PD. We analysed first the motor behaviour of these animals using the CRT. The α-SYN overexpression produced unilateral hemiparesis, which was reduced by the treatment with VCE-003.2 ($F(2,26)=12.80$, $p < 0.0005$; **Figure 1A**). Indeed, there was a general mobility increase in the treated animals with respect to both controls (SHAM-operated) and the α-SYN overexpression group ($F(2,25)=8.354$, $p < 0.005$; **Figure 1B**). Next, we further studied their contralateral differences using the EBST. The vehicle treated animals with α-SYN presented a clear preference for ipsilateral turns, which was not affected by the treatment with VCE-003.2 ($F(2,28)=1422$, $p < 0.0001$; **Figure 1C**). However, as in CRT, we could observe a greater activity in the α-SYN treated animals, which could be quantified as the total number of turns done along the test ($F(2,28)=17.60$, $p < 0.0001$; **Figure 1D**).

The α-synuclein overexpression in the SN of these mice was analysed by a specific immunostaining procedure, and we found the extended presence of this protein all over the midbrain in both α-SYN groups, although not in the form of aggregates (**Figure 2**). The analysis of the SN revealed a complete loss of TH-positive neurons in the α-SYN mice ($F(2,28)=235$, $p < 0.0001$; **Figure 3**), which, surprisingly, was not affected by the VCE-003.2 chronic treatment.

Also, the overexpression of α-SYN caused microglial reactivity in the SN, measured by CD68 immunostaining, which was not reduced by the chronic treatment with VCE-003.2 ($F(2,28)=112.9$, $p < 0.0001$; **Figure 4A**). Similarly, the presence of astrocytes in the SN was measured by GFAP immunostaining, showing an increase of these cells in the α-SYN groups, again irrespective of their treatment ($F(2,28)=65.35$, $p < 0.0001$; **Figure 4B**).

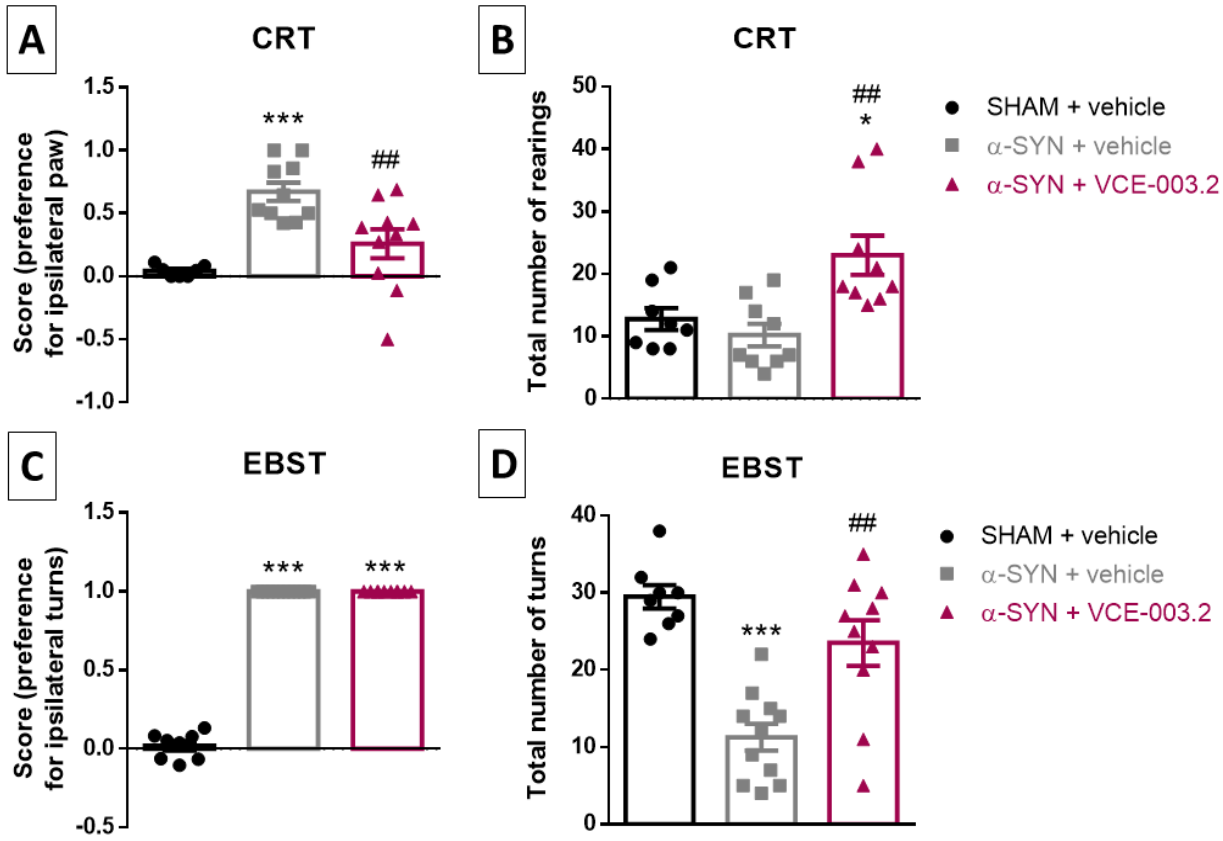

Figure 1. Response in the cylinder rearing test, as a preference score (**A**) or total number of contacts (**B**) and in the elevated-body swing test as a preference score (**C**) or total number of swings (**D**) of male mice subjected to unilateral AAV-α-SYN-injections or sham-operated and daily treated with VCE-003.2 (20 mg/kg, orally) for two weeks. Values represent means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Tukey test (*p<0.05; ***p<0.001 vs sham-operated group; ##p<0.01 vs vehicle-treated α-SYN mice).

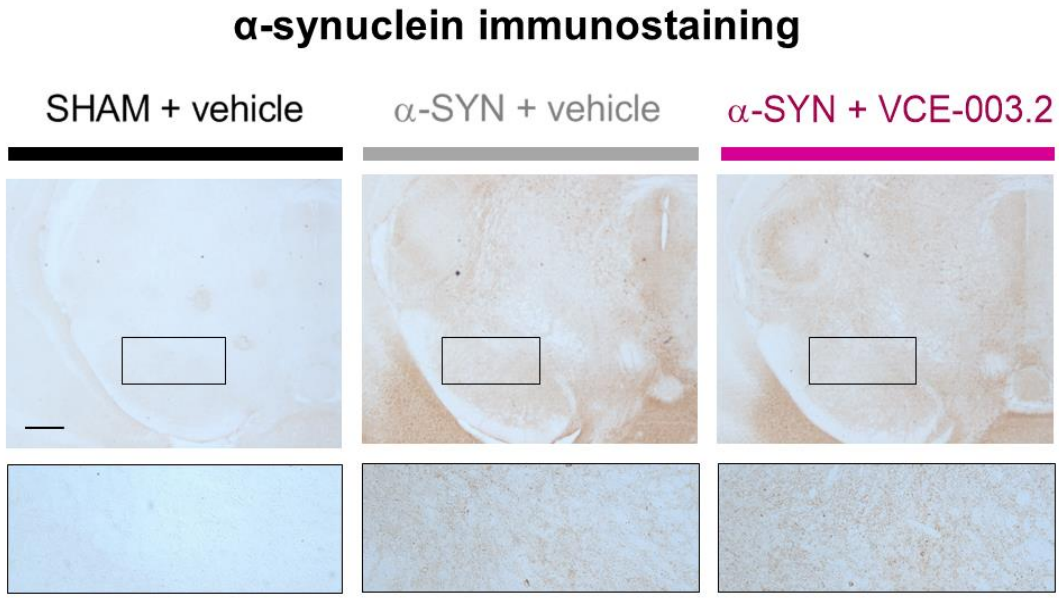

Figure 2. Representative images of the immunostaining of α-synuclein in the SN of male mice subjected to unilateral AAV-α-SYN-injections or sham-operated and daily treated with VCE-003.2 (20 mg/kg, orally) for two weeks. Images have a scale bar = 200 µm, including a specific inlet showing the exact area where dopaminergic cell-bodies should be found (4x magnified).

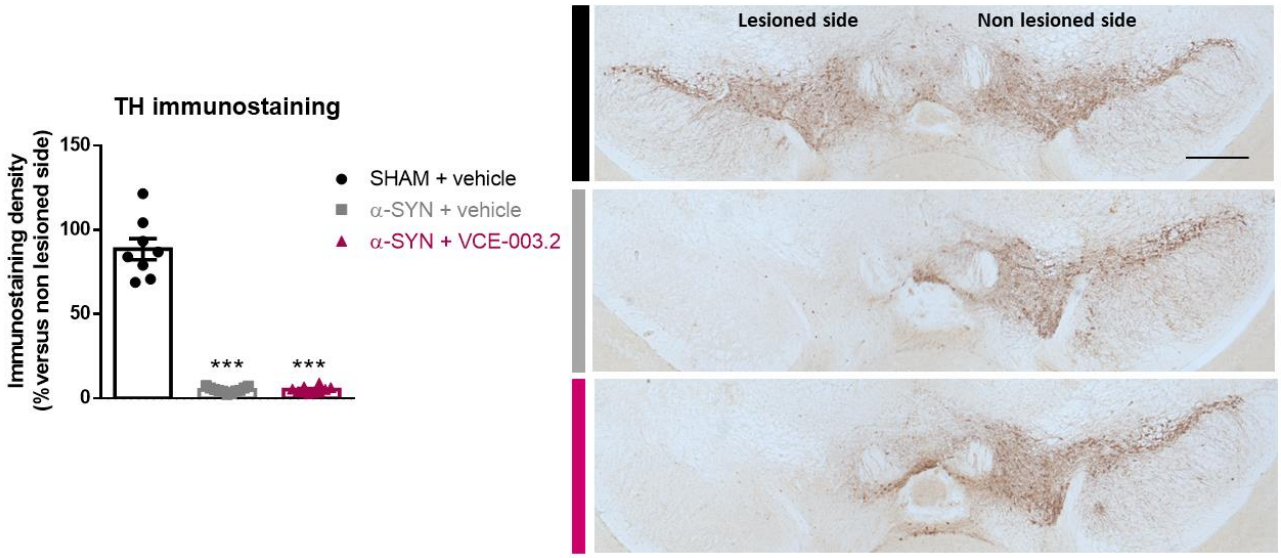

Figure 3. Quantification of TH, including representative images (scale bar = 200 µm), measured in the SN of male mice subjected to unilateral AAV-α-SYN-injections or sham-operated and daily treated with VCE-003.2 (20 mg/kg, orally) for two weeks. Values represent means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Tukey test (***p<0.001 vs sham-operated group).

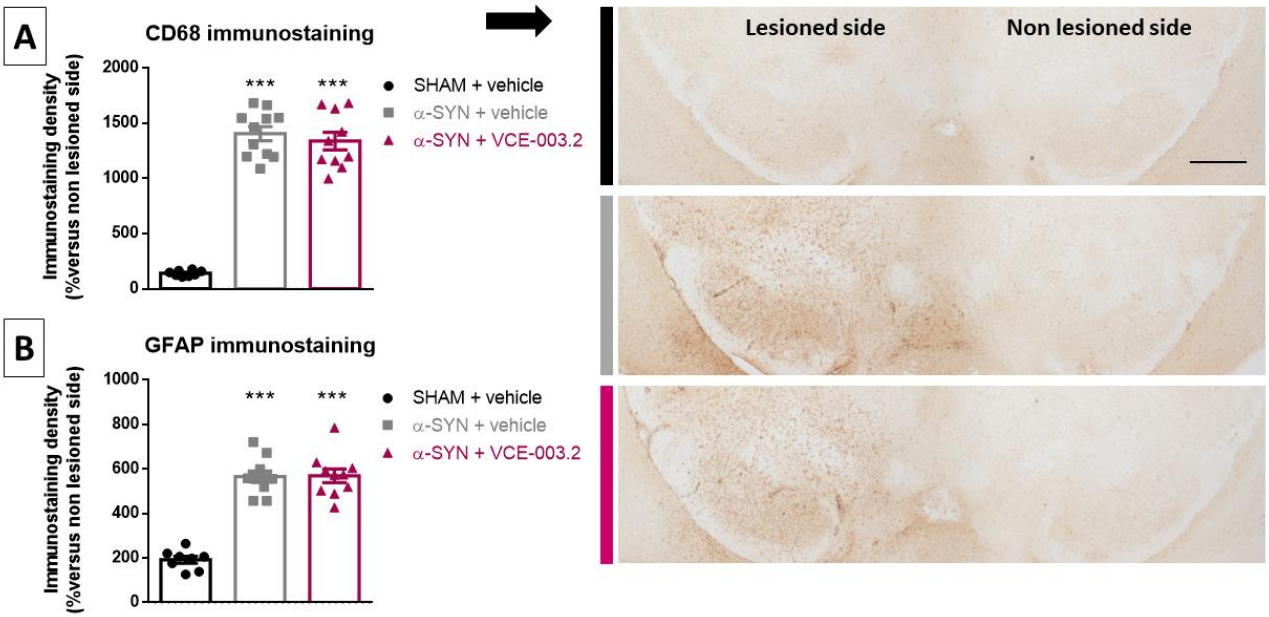

Figure 4. Quantification of CD68, including representative images (**A**; scale bar = 200 µm), and of GFAP (**B**) measured in the SN of male mice subjected to unilateral AAV-α-SYN-injections or sham-operated and daily treated with VCE-003.2 (20 mg/kg, orally) for two weeks. Values represent means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Tukey test (***p<0.001 vs sham-operated group).

The above data indicated that VCE-003.2 was active in reversing the motor deterioration of AAV-α-SYN-lesioned mice, but this occurred with no recovery of TH-positive cells and no attenuation of the elevated glial reactivity. Therefore, it is possible that the functional recovery may be related more to some improvements in the striatal functionality than in the preservation of nigral neurons. To investigate this, we performed RNAseq in striatal tissues from the three experimental groups in an attempt to find specific genes or gene families that

may be altered by the lesion and normalized by the treatment. After alignment, counts were normalized and voom-transformed (**Figure 5A**). Lowly expressed genes (<1 cpm in 50% of the samples) were eliminated, reducing the initial number of genes, 29971, to 14018. Afterwards, we conducted PCA to study the distribution of the groups through a multidimensional reduction. The PCA plot reflects that there is a clear separation of samples according to whether they received damage or not (**Figure 5B**) indicating a massive transcriptomic effect of α-synuclein injection. Regarding the effect of VCE-003.2, even though samples treated and not treated clustered together, there is a slight separation between both groups of samples.

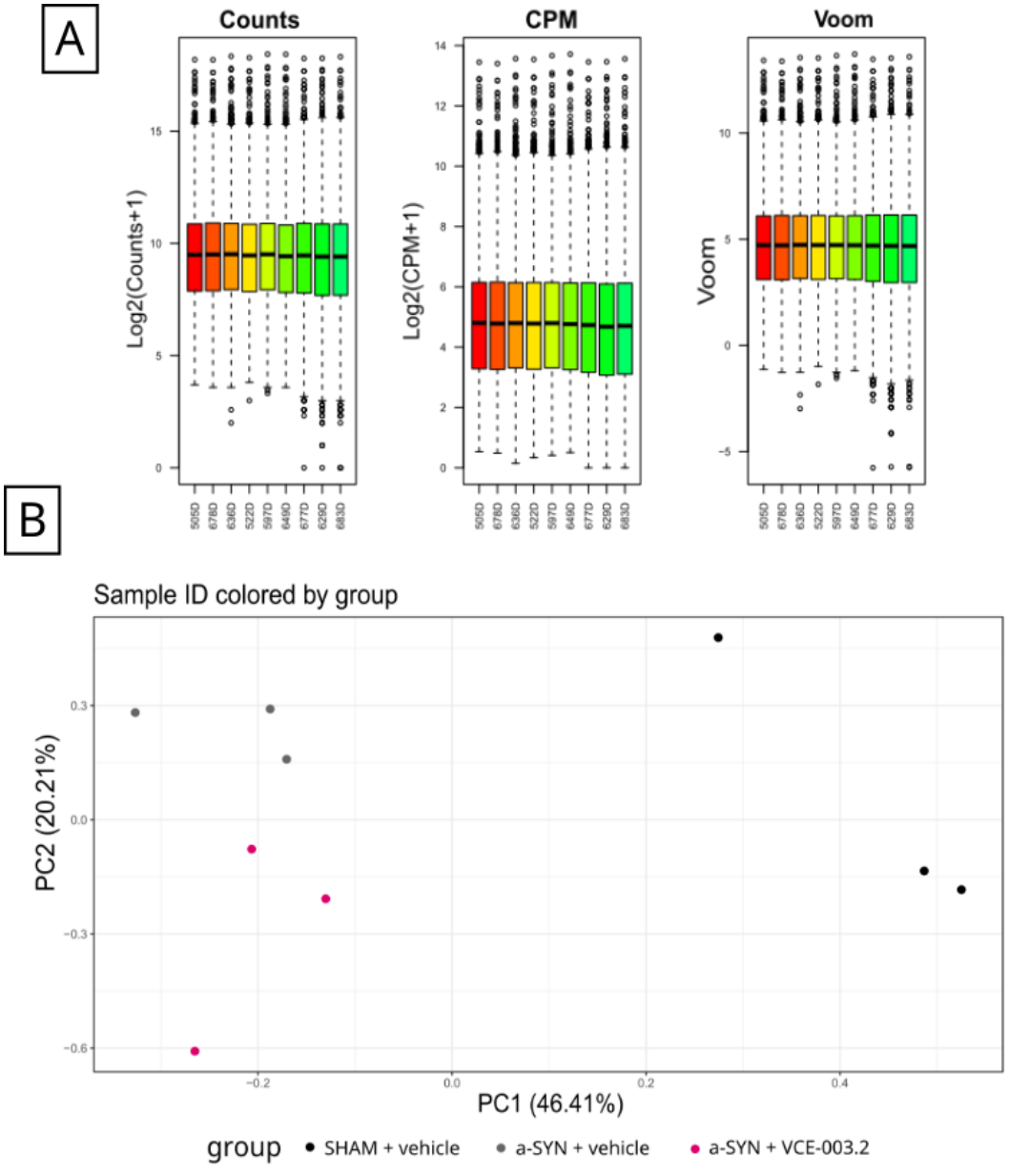

Figure 5. Quality control and data visualization. (**A**) Effect of normalization and voom-transformation. (**B**) PCA of all samples (n=3/group).

In a first set of analyses, we compared the damaged group (α-SYN) versus the non-damaged group (SHAM) in order to characterise the transcriptomic signature driven by α-synuclein injection. We performed differential expression analysis using the Limma package and obtained

1260 DEGs at FDR < 0.05 (**Figure 6A**). These results demonstrate substantial transcriptomic changes in the striatum of animals damaged with the mutated α-SYN overexpression. To understand more specifically the biological implications of these changes, we performed pathway analysis using hallmark pathways from MSigDb. Given the high amount of DEGs obtained in this analysis, we filtered those with absolute logFC higher of 0.5 and FDR < 0.05. Pathway analysis revealed that upregulated DEGs were enriched for pathways related to inflammation such as interferon signalling, complement or NFκB pathways, among others. We also observed enrichment for apoptotic genes, in line with the cell death elicited in this model. Regarding the downregulated genes, we also observed changes in pathways related to the inflammatory response such as some complement and NFκB genes, as well as hypoxia response pathway. More specific information about the deregulated pathways can be seen in **Figure 6B**.

To deepen more in this transcriptional signature, we performed targeted enrichment using Fisher-exact test. Using an inflammatory gene list from GO we obtained a significant enrichment (p-value=$1.63x10^{-7}$, Fisher's exact test), confirming the results obtained by the unbiased GSEA. More specifically, we observed a significant enrichment among DEGs for damage associated microglia (DAM) signature, such as *Csf1r*, *Tyrobp*, *Trem2*, or *Apoe*. We also observed a significant enrichment for genes related to phagocytosis (p-value=$3.6x10^{-9}$ Fisher's exact test) and lysosomal degradation (p-value=0.000177, Fisher's exact test), such as *Rab7b*, *Clu*, *Cts* family, *Grn* or *Hexb* among others. Finally, we investigated if genes that have been identified by human genome wide association analysis (GWAS) were deregulated in the α-synuclein model. Using the genes identified in the most recent study (Nalls et al., 2019) we observed an enrichment (only significant when lowering the threshold to FDR 0.10), pointing to a tendency to capture the human genetics of the disease, with representative genes such as *Snca* or *Tmem163*.

Next, we also investigated if there was enrichment for genes regulated by specific transcription factors, using regulons from Dorothea (Garcia-Alonso et al., 2019; Holland et al., 2020). In this approach, we used pre-ranked genes in terms of t value and performed the analysis using fGSEA. We observed a significant enrichment in several regulons (**Figure 6C**). Most of them were upregulated with respect to the non-damaged group, and all related to immune and inflammatory responses, such as the regulons of Spi1 and Runx1, which are in charge of microglial differentiation, or Irf1 and 9, which regulate interferons, immune response, and anti-viral responses too, together with the transcription factors Stat1 and 2. There was also an increased expression of the regulon of NFκB, which is an important mediator of cell-survival and anti-inflammatory pathways. The only downregulated regulon was the one controlled by Atf6, which regulates the unfolded protein response (UPR).

In a second set of analyses, we compared the damaged and treated group (α-SYN + VCE-003.2) versus the damaged group (α-SYN + vehicle). However, and probably given the sample size, we could not obtain significant results. Indeed, no DEGs were observed at FDR < 0.05 thus the volcano plot shows DEGs at a suggestive threshold of 0.10 (**Figure 7A**). Nevertheless, pre-ranked pathway analysis revealed that VCE-003.2 activates a transcriptomic signature enriched for hallmark pathways such as complement, coagulation or adipogenesis (**Figure 7B**). It is remarkable that the directionality of the pathways is flipped versus the previous analysis,

indicating that the treatment with VCE-003.2 could be reverting the transcriptomic damage elicited by the α-synuclein overexpression (**Figure 6B**).

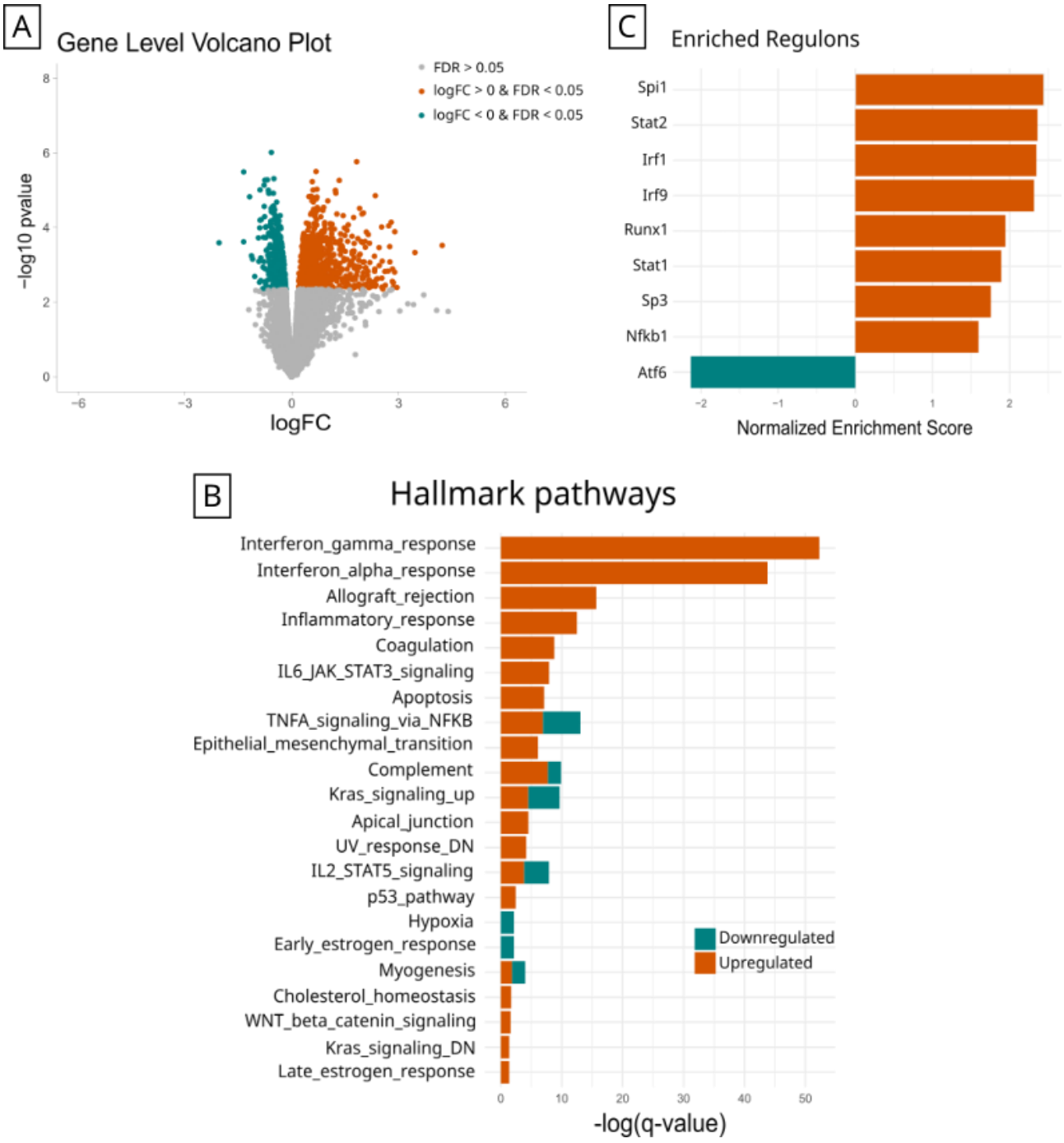

Figure 6. Differential expression analysis in α-SYN vs SHAM. (**A**) Volcano plot representing in the X axis the fold-change (logFC) of gene expression between α-SYN (n=3) vs SHAM (n=3) and in the Y axis the significance in -log10pvalue scale. DEGs at FDR<0.05 are coloured if upregulated (orange) or downregulated (blue). (**B**) Pathway enrichment analysis for the upregulated (orange) and downregulated (blue) DEGs using hallmark pathways from GSEA, with the significance as -log10 scale of the q-value. (**C**) Pre-ranked regulon enrichment analysis using Dorothea. Regulons which are significantly enriched are represented in the y-axis and x-axis represents the directionality of the expression by the Normalized Enrichment Score, being on the left the downregulated regulons (blue) and on the right the upregulated regulons (orange).

Figure 7. Differential expression analysis in α-SYN + VCE-003.2 vs α-SYN + vehicle. (**A**) Volcano plot representing in the X axis the fold-change (logFC) of gene expression between α-SYN + VCE-003.2 (n=3) vs α-SYN (n=3) and the Y axis the significance in -log10pvalue scale. DEGs at FDR<0.1 are coloured if upregulated (orange) or downregulated (blue). (**B**) Pre-ranked pathway enrichment analysis using hallmark pathways using fGSEA, with the significance represented by color, blue represents significant enrichment and grey not significant enrichment. X-axis represents the Normalized Enrichment Score, indicating the directionality of the effect. (**C**) Pre-ranked regulon enrichment analysis using Dorothea. Regulons which are significantly enriched are represented in the y-axis and x-axis represents the directionality of the expression by the Normalized Enrichment Score, being on the left the downregulated regulons (blue) and on the right the upregulated regulons (orange).

Focusing on the regulons, we observe that the expression of certain ones is even. The analysis of enrichment for certain regulons shows that VCE 003.2 elicits a potentiation of the increased expression of genes related to the viral infection, such as the regulons of Irf1 and 9 and Stat1 and 2 (**Figure 7C**). Interestingly, other regulons show the opposite changes than when comparing damaged versus non damaged, such as Atf6, which appears upregulated in the damaged and treated group, indicating an effect of VCE-003.2 over the altered UPR response (**Figure 7C**). Another interesting response, and in accordance with previous observations that were described for other EHP analysis, include the coordinated downregulation of genes related with the cell-cycle, and particular, with the E2F transcription factors (**Figure 7C**).

Finally, the effect of mutated α-synuclein injection and VCE-003.2 on the expression of certain genes and regulons is presented in representative boxplots. We observed that the overexpression of α-synuclein remains unaltered between the treated and non-treated groups (**Figure 8**), just as found in the histological analysis (**Figure 2**). *Trem2* expression is increased in the α-synuclein group, and even more in the animals who received VCE-003.2 (**Figure 8**). We also checked the expression of *C3* and *Lrp2*, which are expressed in astrocytes in relation with aggregate clearance, and we could observe that VCE-003.2 trended to reduce the overexpression caused by α-synuclein overexpression (**Figure 8**). As for the endocannabinoid system (ECS) expression, and we found a downregulation in *Cnr1*, partially reversed by VCE-003.2 (**Figure 8**). Next, we found a similar or even higher expression of the regulons related with protective immune response and inflammation, such as Irf1 and Spi1, in the group treated with VCE-003.2 (**Figure 8**). As for NFκB and Atf6 regulons, the treatment was able to partially revert the changes produced by α-synuclein overexpression (**Figure 9**). Also, we can observe an effect of the treatment for the regulon of E2F1, which appears slightly upregulated in the damaged group, and downregulated after the chronic treatment with VCE-003.2 (**Figure 9**).

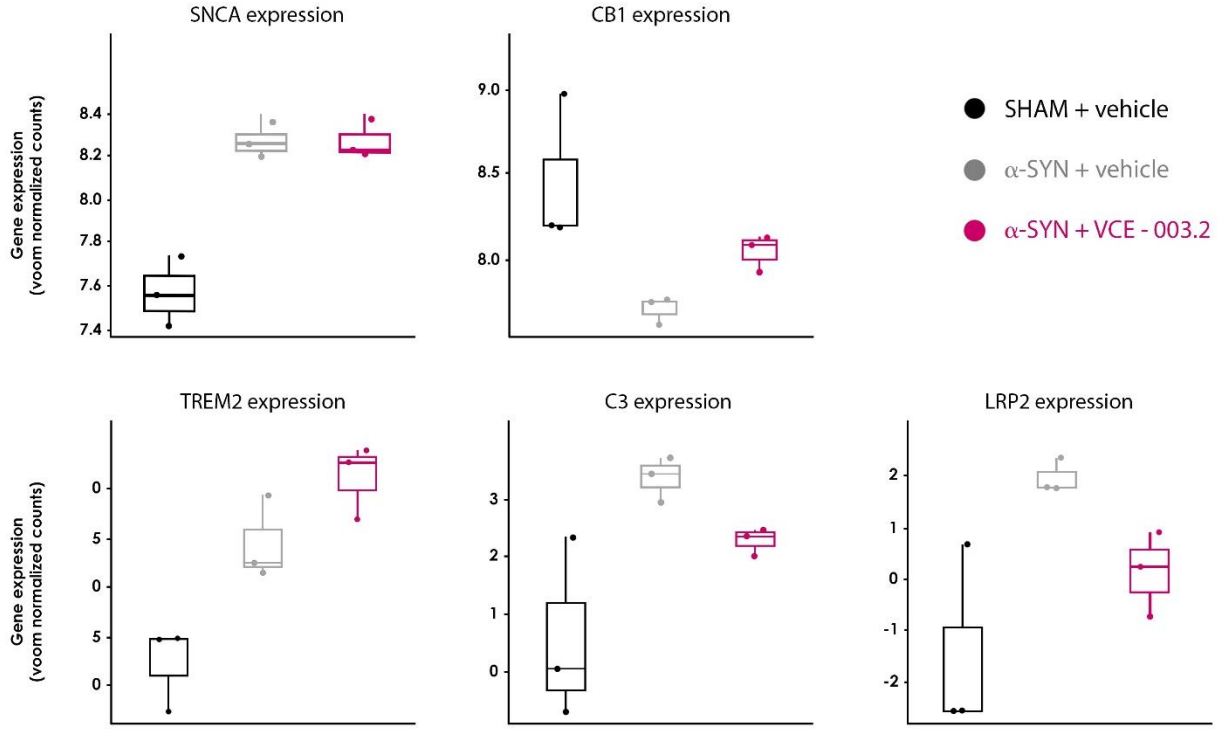

Figure 8. Expression of selected genes. Adjusted gene expression levels after normalization are shown. Boxplots: the line represents the median. The boxes extend from the 25th - 75th percentile and the lines extend 1.5 times the interquartile range (n=3/group). Statistic values of: <black and gray>/<gray and pink>: SNCA (logFC=0.7/-0.004; FDR=0.0087/0.99), TREM2 (logFC=0.59/0.33; FDR=0.032/0.49), CB1 (logFC: -0.75/-0.75; FDR: 0.041/0.041), C3 (logFC: 2.75/-1.08; FDR: 0.025/0.12), LRP2 (logFC: 3.46, -1.86; FDR: 0.02/0.11).

3. Discussion

In our research group we have deeply studied the neuroprotective potential of the non-thiophilic CBG quinone derivative VCE-003.2 in different experimental models of PD based on mitochondrial dysfunction, oxidative stress, or inflammation driven neurodegeneration, both by intraperitoneal administration (García et al., 2018) and by oral administration (Burgaz et al.,

2019; Burgaz et al., 2021), to facilitate the clinical development of this compound. However, these models reproduce only some of the neuropathological features of PD, but not all. An important feature not investigated in relation with benefits of VCE-003.2 yet is α-synuclein dysregulation and aggregation. To this end, we have used here a model based on the overexpression of mutant (A53T) α-synuclein in the SN using viral technology for administering locally (intranigral) the mutant gene. Our data showed that VCE-003.2 was active in the motor impairments caused by α-SYN, showing an important recovery in the CRT and a general increase in the mobility of those mice, as we found in our previous studies in other models (Burgaz et al., 2019; Burgaz et al., 2021). We also found neuronal death in the SN, as in our previous studies, but this neuronal death was massive, which counteracted any protective effect of VCE-003.2 being visible at this level. The same problem was also found for the neuroinflammatory events, suggesting that the behavioural improvements should not be a consequence of a direct protective effect on the SN, but to improvements at other levels (eg. correction of potential dysregulations in striatal neurons under the control of these neurons). To this end, we investigated possible transcriptomic changes in the striatum that could explain the improvement observed in the behavioural tests.

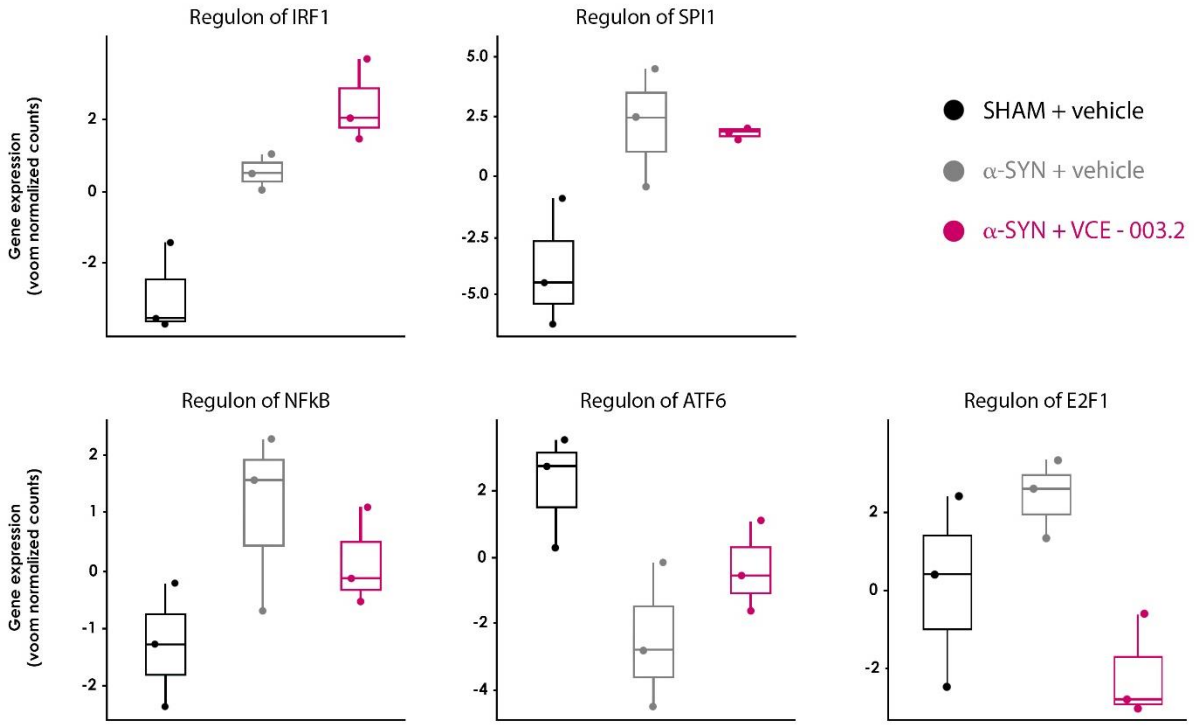

Figure 9. Expression of selected regulons. Adjusted gene expression levels of all genes included in the regulons after normalization are shown. Boxplots: the line represents the median. The boxes extend from the 25th - 75th percentile and the lines extend 1.5 times the interquartile range (n=3/group). Statistic values of: <black and gray>/<gray and pink>: IRF1 (FDR=0.0000137/0.0063), SPI1 (FDR=8.5e-9/0.56), NFκB (FDR=0.06/0.82), ATF6 (FDR=0.0013/0.0069), E2F1 (FDR=0.71/0.00015).

Firstly, we found a substantial transcriptomic change driven by the mutated α-synuclein overexpression which allowed us to study in detail this model and validate it as a tool for PD. The main upregulated pathways were related to inflammation and to immune response, such as interferon signalling, the main responsible for anti-viral responses (Muhuri et al., 2021).

Using a targeted analysis, we could identify some of the DEGs related to the inflammatory response, some classic ones as *Gfap* or *Cd14*, as well as genes associated with DAM signature, some of which could be relevant for PD. For example, *Trem2*: its mutations can be risk factors for PD (Liu et al., 2016); there are altered levels in PD patients (Wilson et al., 2020); TREM2 participates in changing the proinflammatory M1 microglia to M2 (Zhang et al., 2018); and TREM2 deficiency implied a shift to proinflammatory microglial activation, aggravating neurodegeneration in models of PD (Guo et al., 2019), reinforcing its increased expression after VCE-003.2 treatment as a protective response against the damage. Another upregulated gene is *Csf1r*, involved in microglial proliferation and that is significantly upregulated in patients and PD models (Neal et al., 2020), just as in our α-synuclein lesioned mice. Some of the other DEGs we found have been classically related to AD, such as *TyroBp* or *ApoE*. However, recent studies have shown that *ApoE* genotype is also related to synucleinopathy (Davis et al., 2020). Other DEGs related with AD but with potential relevance in PD are two astrocytic markers: *C3*, implied in the clearance of β-amyloid aggregates (Fu et al., 2012) and in the A1 phenotype of astrocytes (King et al., 2020); and *Lrp2*, that participates in regulation of neurogenesis (Gajera et al., 2010) and it is dysregulated in PD patients (Zaccaria et al., 2021). We could observe a trend to decrease in their expression in the VCE-003.2 group, which may be interesting to follow up in future experiments. The enrichment for genes related to phagocytosis and lysosomal degradation, is also related to PD pathophysiology (Bonam et al., 2021). For example, *Cd6*8 (Hopperton et al., 2018) or *Grn* (Yao et al., 2020), which encodes progranulin (PGRN), a key regulator of lysosomal function that has been related with a higher severity of PD and with reduced levels of GCase enzyme, that correlate with higher α-synuclein levels (Blandini et al., 2019), leading to dementia and PD (Rodrigues and Kale, 2021). As for the ECS, we found a downregulation of *Cb1*, which has been described in other PD models, such as 6-OHDA model (Walsh et al., 2010) and LPS model (Chapter 3; Burgaz et al., 2021c). The treatment with VCE-003.2 showed a trend to reverse this change, which may be contributing to the regulation of the proteasome system (Salgado-Mendialdúa et al., 2018).

Next, we found a significant increase in regulons of transcription factors, such as Irf1 and 9, and their coregulators Stat1 and 2, responsible for immune and anti-viral responses (Muhuri et al., 2021). These responses increased after the treatment with VCE-003.2, which could indicate a more aggressive response to the AAV injection, due to the role of PPAR-γ against viral infections in the brain (Layrolle et al., 2021). It is true that no changes have been detected in the PPAR-γ regulon, but considering its wide range of action, there may be changes in inflammatory processes in both directions. There were also changes in regulons implied in inflammatory processes, such as the regulons of Runx1 and Spi1, which participate in microglial differentiation and the regulation of the inflammatory response (Pimenova et al., 2021), as well as the regulon of NFκB, which is an important mediator of inflammatory pathways (Kempuraj et al., 2016). Interestingly, we observed an upregulation of the regulon of Spi1 after the treatment with VCE-003.2, which may imply a specialized microglial contribution (Satoh et al., 2014), as well as a decrease in the regulon of NFκB, a potential mechanism against neuroinflammation already described in relation to PPAR-γ activation (Cai et al., 2018). The only downregulated regulon in the lesioned mice was Atf6, which regulates the UPR, process which is known to be affected in PD (Wodrich et al., 2022). Specifically, Atf6 has been found to

be inhibited by α-synuclein causing the disruption of the UPR signalling (Credle et al., 2015). With the VCE-003.2 treatment, the regulon of Atf6 was upregulated, probably a beneficial response in the recovery of the UPR signalling in PD models (Gupta et al., 2021).

Finally, another interesting response after the VCE-003.2 treatment was the coordinated downregulation of genes related with the cell-cycle, such as Sox10, a crucial factor for glial differentiation; Prdm14, which plays a role in cellular pluripotency; and the E2F transcription factors. The increase in the expression of E2F1 and its role in DNA damage/repair has been proposed as a potential mechanism in cell cycle re-entry, which would be implicated in the modulation of neuronal apoptosis (Verdaguer et al., 2007). Furthermore, in neurons of the SN of PD patients there is an increase in E2F1 expression and a duplication of DNA which could be contributing to cell death (Höglinger et al., 2007). They also find this increase in the MPTP model, but the blockade of E2F1 avoids the DNA duplication and thus achieves neuroprotection. This means the downregulation of E2F1 we observe in the VCE-003.2 treated group may be one of the protective mechanisms responsible for the improved neurological state of our mice, as in that same study, mice deficient for E2F1 are protected against neuronal death in the SN. Indeed, the mechanism underlying this protective effect may be related to PD pathophysiology, as the blockade of E2F1 has been described to provoke the disinhibition of complex I of mitochondria, which ends up in a neuroprotective effect (Alvira et al., 2007). Although the signalling pathway behind these effects must be further studied, some bioinformatic and experimental analysis have newly revealed E2F1 as a target of PPAR-γ signalling, although in relation to angiogenic processes (Vattulainen-Collanus et al., 2016). Also, we found a downregulation of E2F4 regulon, which has been described to be affected by mutations in the *GBA* gene, which in turn has been related with mild cognitive impairment in PD patients (Jiang et al., 2020). Lastly, we observed a downregulation of the regulon of p53, involved in apoptosis, which has also been related with PD, as there is interplay between p53 and parkin (Checler and Alves da Costa, 2014). They suggest that p53 elevation could likely contribute to the exacerbated cell death observed in PD-affected brains, so the inactivation of this p53 could also contribute to the effect of VCE-003.2 in the α-synuclein model. For instance, p53 shares apoptotic route with E2F1, and their inhibitors have been proposed as potential neuroprotective tools (Camins et al., 2007).

In further studies, we plan to deepen in the localization and characteristics of the α-synuclein aggregates and to repeat our experiment with a lower dose of virus and a higher sample number, to elucidate which of the above proposed mechanisms may be responsible for beneficial effects of VCE-003.2 chronic treatment. Furthermore, some of the effects previously seen for VCE-003.2 in other PD models (Burgaz et al., 2019; Burgaz et al., 2021), should be reconsidered under the light of these new results, as they may not be associated specifically with α-synuclein overexpression. To this end, and as a part of this new study, we plan to use an *in vitro* model of α-synuclein-transfected cells (Nascimento et al., 2020) where we can explore the specific molecular mechanisms underlying the effects of VCE-003.2 treatment and find out if there is a relationship between them and the α-synuclein aggregation.

In conclusion, the data obtained in this new model of PD, based on α-synuclein dysregulation, and despite the need of model optimization in further studies, reinforced the beneficial effects proposed for VCE-003.2 as a pharmacological strategy for PD. Over the

years, we have demonstrated VCE-03.2 to be effective in neurotoxin-based models of PD and here in a model of synucleinopathy, which makes its potential in PD against a variety of pathological events highly robust and susceptible to progress towards the clinical studies.

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CHAPTER 2

Preclinical evaluation of compounds targeting CB2: the CBD derivative VCE-004.8, and the phytocannabinoid THCV, alone and in combination with CBD.

In this chapter, we have evaluated the neuroprotective and anti-inflammatory potential of two different compounds which are able to target CB2 receptors, although in this case both of them are known to be multitarget compounds.

In the first paper, we started the characterization of the CBD derivative VCE-004.8, which has been described as a CB2 and PPAR-γ agonist. We started its evaluation in a classical *in vivo* model of PD based on the injection of 6-OHDA in the striatum. We observed a partial recovery of the hemiparesis caused by the insult, accompanied by a small but significant preservation of the dopaminergic neurons of the SN. As for neuroinflammation, VCE-004.8 produced an important and significant reduction of both microgliosis and astrogliosis measured in the SN, which seems consistent with the activation of CB2 and PPAR-γ, as both targets are key in inflammatory processes. To confirm this, we used an *in vitro* approach, based on challenging the SH-SY5Y cells with 6-OHDA, and co-administering VCE-004.8 with CB2 and/or PPAR-γ antagonist. We found that both PPAR-γ and CB2 receptors contribute to the effect of VCE-004.8, although there was a major contribution of PPAR-γ receptors in the cytoprotective effect, as the CB2 antagonist on its own had no effect.

In the second paper, we evaluated the potential of the phytocannabinoid THCV, which has been described as a CB2 agonist, but also CB1 antagonist at low doses, and partial agonist of GPR55. In previous work of our research group, THCV showed neuroprotective properties in both LPS and 6-OHDA models of PD (García et al., 2011), although with less potency than CBD. To continue this study, we investigated whether the combination of THCV with CBD may enhance its neuroprotective effect in the same two mice models. In LPS-lesioned mice, the treatments with THCV and CBD alone, were able to reduce the hemiparesis in the CRT, and to recover the autophagy dysfunction and neuroinflammation of the SN and the striatum, but their efficacy was not increased when combined. In the 6-OHDA model, the treatment with THCV preserved the dopaminergic neurons of the SN, reduced the autophagy dysfunction and neuroinflammation to the same extent as CBD, but was less effective attenuating the motor impairments. However, the combination of both treatments worsened the effects of each of the treatments on their own, indicating a possible crosstalk between their mechanisms of action. To elucidate this interaction, we analysed the effect of these compounds *in vitro* and we observed that lower concentrations of both compounds, specially of THCV could improve the effects of the combined treatment. The effects of THCV were reversed by the addition of antagonists aimed at blocking PPAR-γ and CB2 receptors, respectively. As for CBD, we found a major contribution of PPAR-γ receptors in the cytoprotective effect of CBD, alone or in combination.

Papers included in this chapter:

Burgaz, S., García, C., Gómez-Cañas, M., Rolland, A., Muñoz, E., & Fernández-Ruiz, J. (2021). **Neuroprotection with the Cannabidiol Quinone Derivative VCE-004.8 (EHP-101) against 6-Hydroxydopamine in Cell and Murine Models of Parkinson's Disease**. *Molecules (Basel, Switzerland)*, *26*(11), 3245.

Burgaz, S., [...] & Fernández-Ruiz, J. **Investigating a combined strategy with THCV and CBD as a neuroprotective therapy in experimental models of Parkinson's disease.** (Data not published yet).

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Neuroprotection with the Cannabidiol Quinone Derivative VCE-004.8 (EHP-101) against 6-Hydroxydopamine in Cell and Murine Models of Parkinson's Disease

Sonia Burgaz [1,2,3], Concepción García [1,2,3], María Gómez-Cañas [1,2,3], Alain Rolland [4], Eduardo Muñoz [4,5,6,7] and Javier Fernández-Ruiz [1,2,3,*]

1 Department of Biochemistry and Molecular Biology, Faculty of Medicine, Institute on Neurochemistry Research, Complutense University, 28040-Madrid, Spain; soniabur@ucm.es (S.B.); conchig@med.ucm.es (C.G.); mgc@med.ucm.es (M.G.-C.)
2 Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), 28031-Madrid, Spain
3 Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), 28034-Madrid, Spain
4 Emerald Health Pharmaceuticals, San Diego, CA 92121, USA; arolland@emeraldpharma.life (A.R.); fi1muble@uco.es (E.M.)
5 Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), 14004-Córdoba, Spain
6 Department of Cellular Biology, Physiology and Immunology, University of Córdoba, 14004-Spain
7 Hospital Universitario Reina Sofía, 14004-Córdoba, Spain
* Correspondence: jjfr@med.ucm.es; Tel.: +34-913941450

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Abstract: The 3-hydroxyquinone derivative of the non-psychotrophic phytocannabinoid cannabigerol, so-called VCE-003.2, and some other derivatives have been recently investigated for neuroprotective properties in experimental models of Parkinson's disease (PD) in mice. The pharmacological effects in those models were related to the activity on the peroxisome proliferator-activated receptor-γ (PPAR-γ) and possibly other pathways. In the present study, we investigated VCE-004.8 (formulated as EHP-101 for oral administration), the 3-hydroxyquinone derivative of cannabidiol (CBD), with agonist activity at the cannabinoid receptor type-2 (CB_2) receptor in addition to its activity at the PPAR-γ receptor. Studies were conducted in both in vivo (lesioned-mice) and in vitro (SH-SY5Y cells) models using the classic parkinsonian neurotoxin 6-hydroxydopamine (6-OHDA). Our data confirmed that the treatment with VCE-004.8 partially reduced the loss of tyrosine hydroxylase (TH)-positive neurons measured in the substantia nigra of 6-OHDA-lesioned mice, in parallel with an almost complete reversal of the astroglial (GFAP) and microglial (CD68) reactivity occurring in this structure. Such neuroprotective effects attenuated the motor deficiencies shown by 6-OHDA-lesioned mice in the cylinder rearing test, but not in the pole test. Next, we explored the mechanism involved in the beneficial effect of VCE-004.8 in vivo, by analyzing cell survival in cultured SH-SY5Y cells exposed to 6-OHDA. We found an important cytoprotective effect of VCE-004.8 at a concentration of 10 μM, which was completely reversed by the addition of antagonists, T0070907 and SR144528, aimed at blocking PPAR-γ and CB_2 receptors, respectively. The treatment with T0070907 alone only caused a partial reversal, whereas SR144528 alone had no effect, indicating a major contribution of PPAR-γ receptors in the cytoprotective effect of VCE-004.8 at 10 μM. In summary, our data confirmed the neuroprotective potential of VCE-004.8 in 6-OHDA-lesioned mice, and in vitro studies confirmed a greater relevance for PPAR-γ receptors rather than CB_2 receptors in these effects.

Keywords: cannabinoids; VCE-004.8; EHP-101; PPAR-γ receptors; CB_2 receptors; mitochondrial dysfunction; 6-hydroxydopamine; Parkinson's disease.

1. Introduction

Phytocannabinoids, the active constituents of Cannabis plant, and also endocannabinoids and synthetic cannabinoids have been proposed as promising neuroprotective agents, a property derived from their pleiotropism and ability to activate numerous cytoprotective targets within the endocannabinoid system, but also outside this signaling system (reviewed in [1]). Such neuroprotective potential has been preclinically investigated in accidental brain damage (e.g., stroke, brain trauma, spinal injury) and, in particular, in chronic progressive disorders (e.g., Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, and others) [2]. This also includes Parkinson's disease (PD), which is one of the disorders that has attracted to date most of the research with cannabinoids, aimed at exploring neuroprotective therapies to delay or arrest disease progression and also alleviate specific parkinsonian symptoms (reviewed in [2–5]). Some of these studies have concentrated on compounds targeting the cannabinoid type-1 (CB_1) receptor, demonstrating neuroprotective properties in some experimental models of PD [6,7]. However, most of the experimental evidence obtained with this receptor concentrated on the capability to reduce specific parkinsonian signs such as bradykinesia and immobility [8–10], tremor [11], and/or L-dihydroxyphenylalanine (L-DOPA)-induced dyskinesia [12]. In contrast, the neuroprotective potential of cannabinoids in PD was initially associated with compounds, such as cannabidiol, having an antioxidant profile exerted by cannabinoid receptor-independent effects [13] or through modulating nuclear factor erythroid 2-related factor 2 (Nrf-2) signaling [14]. Later on, strong neuroprotective properties in PD were found for those cannabinoids active against inflammation and glial reactivity, whose effects are exerted through the activation of the cannabinoid type-2 (CB_2) receptor [15–18], but also targeting the peroxisome proliferator-activated receptor-γ (PPAR-γ) [19,20] and the G protein-coupled receptor 55 (GPR55) [21].

These studies have identified several promising cannabinoid compounds to generate a cannabinoid-based therapy for specific symptoms and, in particular, for disease progression in patients affected by PD. One such compound is the phytocannabinoid Δ^9- tetrahydrocannabivarin (Δ^9-THCV), which is active in alleviating motor inhibition [16] or delaying L-DOPA-induced dyskinesia [22] by its CB_1 receptor antagonist activity. It also displays an important anti-inflammatory and neuroprotective profile in 6-hydroxydopamine (6-OHDA)- and lipopolysaccharide (LPS)-lesioned mice exerted through multiple mechanisms, in particular by activating the CB_2 receptor [16,18]. A second interesting compound is the non-thiophilic cannabigerol (CBG) quinone derivative VCE-003.2, which behaves as a PPAR-γ activator with no activity at the CB_1/CB_2 receptors [23]. It has been found to be active as anti-inflammatory and neuroprotectant against inflammation-driven neuronal deterioration in LPS-lesioned mice [19,20], and also in 6-OHDA-lesioned mice [24]. These effects were found to be mediated by its binding at a functional alternative site different from the canonical binding site used by glitazones in the PPAR-γ receptor, as shown in in vitro studies [19,24]. A third group of promising compounds are the cannabigerolic acid analog CBGA-Q and its salt form [24]. Similarly to VCE-003.2, both exhibited a notable effect as neuroprotective agents in 6-OHDA-lesioned mice, but the neuroprotective effects were mediated by the activation of the canonical binding site in the

PPAR-γ receptor [24]. The structures of all these synthetic compounds were previously disclosed [23,24].

Another interesting compound that has not been investigated in PD yet is the cannabidiol (CBD) aminoquinone derivative, VCE-004.8 (Emerald Health Pharmaceuticals, USA; see chemical structure in Figure 1). VCE-004.8 has the ability to activate the CB_2 receptor in addition to the PPAR-γ receptor [25], which may be of interest in PD following the results found in studies selectively activating the CB_2 receptor [16,18]. This compound is presently under clinical investigation (formulated as EHP-101 oral solution) for autoimmune disorders (https://clinicaltrials.gov/ct2/show/NCT04166552), such as systemic sclerosis, a rare form of scleroderma, and for multiple sclerosis. In the present study, we first investigated the neuroprotective effect of VCE-004.8 (EHP-101) in 6-OHDA-lesioned mice using an oral administration. Second, we also investigated the potential targets for these effects (PPAR-γ and/or CB_2 receptors) using cultured SH-SY5Y cells exposed to 6- OHDA.

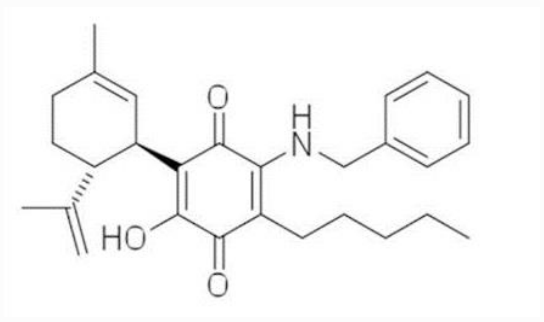

Figure 1. Chemical structure of VCE-004.8 (disclosed for the first time in [25]).

2. Results

2.1. Neuroprotective Effects of VCE-004.8 in 6-OHDA-Lesioned Mice

Our first experiment explored the neuroprotective potential of an oral administration of VCE-004.8 at the dose of 20 mg/kg in 6-OHDA-lesioned mice. After 2 weeks of daily treatment, we first investigated the neurological status of these mice using two motor tests (pole and cylinder rearing tests). In the first test, our 6-OHDA-lesioned mice spent more time in descending the pole than control (sham) mice, but the treatment with VCE-004.8 was unable to reverse this defect ($F(2,15) = 1.356$, ns; Figure 2). This was not the case in relation with the hemiparesis shown in the cylinder rearing test (this is a more reliable test for testing parkinsonism signs when unilateral models are used) by 6-OHDA-lesioned mice compared to controls, which was partially recovered after the treatment with VCE-004.8 ($F(2,12) = 13.40$, $p < 0.005$; Figure 2).

This positive effect was concordant with a small (<40%) but significant preservation in the number of tyrosine hydroxylase (TH)-positive nigral neurons ($F(2,16) = 28.42$, $p < 0.0001$; Figure 3), accompanied by a much more evident reduction in the elevated glial reactivity labelled with two proteins: cluster of differentiation (Cd68) ($F(2,16) = 17.07$, $p < 0.0005$; Figure 4) and glial fibrillary acidic protein (GFAP) (2,16) = 16.59, $p < 0.0005$; Figure 5).

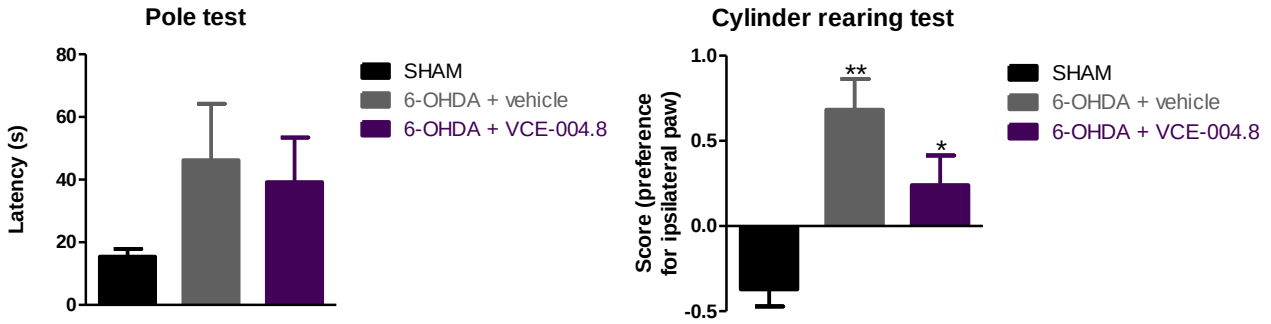

Figure 2. Response in the pole test and in the cylinder rearing test of control (sham) mice and unilaterally 6-OHDA-lesioned animals treated with VCE-004.8 (20 mg/kg) or vehicle (Maisine CC/corn oil) given orally. Treatments were daily and prolonged for 2 weeks. Data corresponded to 24 h after the last dose and were expressed as means ± SEM of more than 5 animals per group. They were analyzed by one-way ANOVA followed by the Bonferroni test (* $p < 0.05$, ** $p < 0.01$ versus sham).

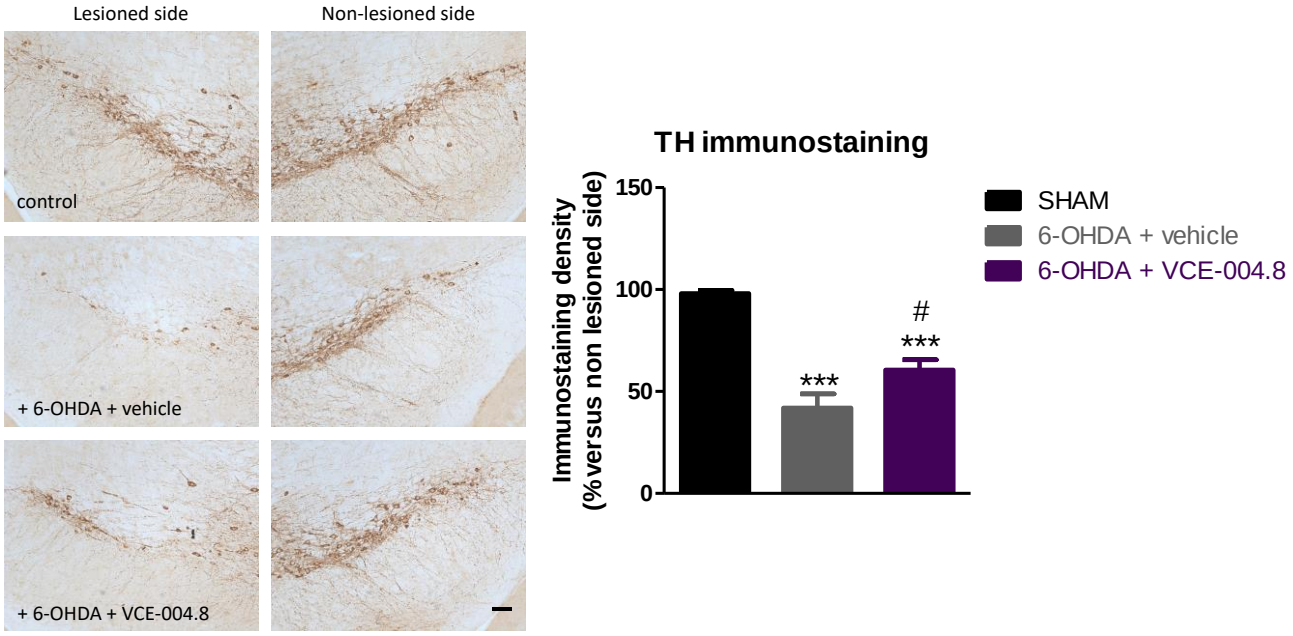

Figure 3. Intensity of the immunostaining for TH measured in a selected area of the substantia nigra pars compacta of control (sham) mice and unilaterally 6-OHDA-lesioned animals treated with VCE-004.8 (20 mg/kg) or vehicle (Maisine CC/corn oil) given orally. Treatments were daily and prolonged for 2 weeks. Data corresponded to percentage of the ipsilateral lesioned side versus contralateral non-lesioned side at 24 h after the last dose and were expressed as means ± SEM of more than 5 animals per group. They were analyzed by one-way ANOVA followed by the Bonferroni test (*** $p < 0.005$ versus sham; # $p < 0.05$ versus 6-OHDA + vehicle). Representative immunostaining images for each experimental group are shown in the left panels (scale bar = 100 μm)-

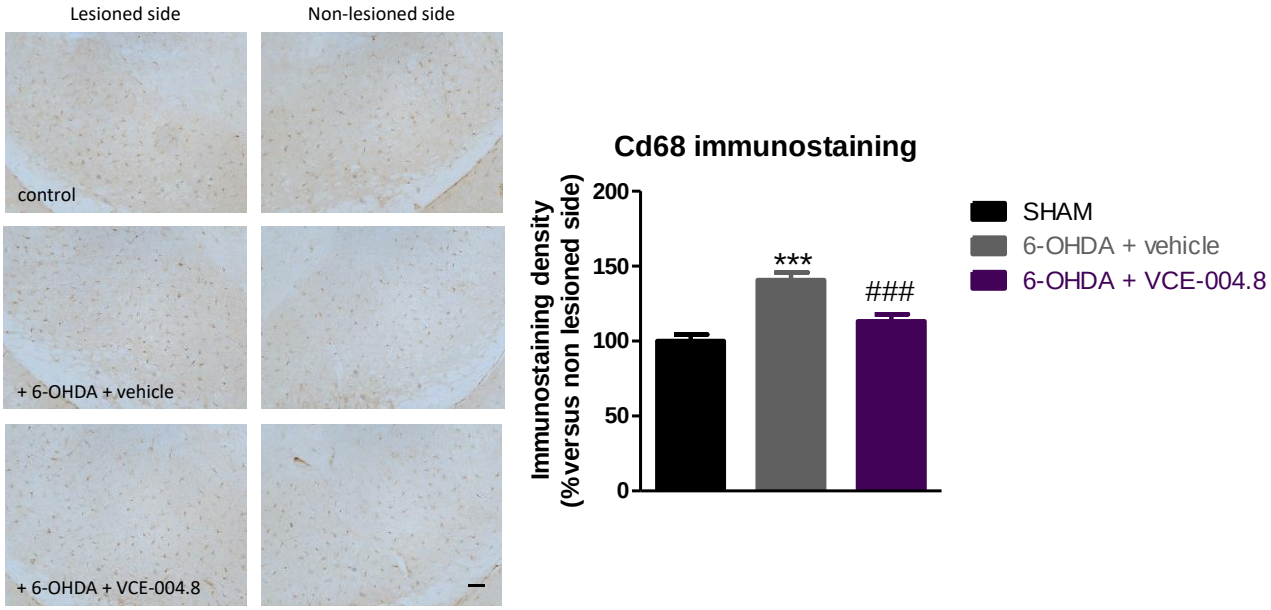

Figure 4. Intensity of the immunostaining for Cd68, a marker of microglia and infiltrated macrophages, measured in a selected area of the substantia nigra pars compacta of control (sham) mice and unilaterally 6-OHDA-lesioned animals treated with VCE-004.8 (20 mg/kg) or vehicle (Maisine CC/corn oil) given orally. Treatments were daily and prolonged for 2 weeks. Data corresponded to percentage of the ipsilateral lesioned side versus contralateral non-lesioned side at 24 h after the last dose and were expressed as means ± SEM of more than 5 animals per group. They were analyzed by one-way ANOVA followed by the Bonferroni test (*** $p < 0.005$ versus sham; ### $p < 0.005$ versus 6-OHDA + vehicle). Representative immunostaining images for each experimental group are shown in the left panels (scale bar = 100 μm).

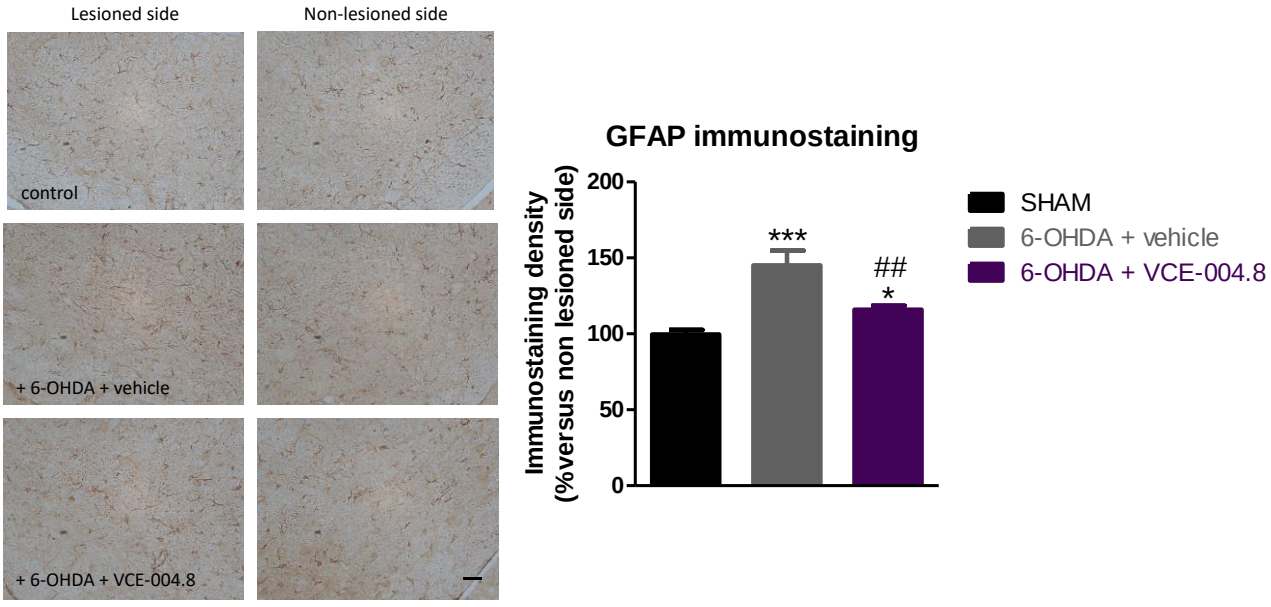

Figure 5. Intensity of the immunostaining for GFAP, a marker of astrocytes, measured in a selected area of the substantia nigra pars compacta of control (sham) mice and unilaterally 6-OHDA-lesioned animals treated with VCE-004.8 (20 mg/kg) or vehicle (Maisine CC/corn oil) given orally. Treatments were daily and prolonged for 2 weeks. Data corresponded to percentage of the ipsilateral lesioned side versus contralateral non-lesioned side at 24 h after the last dose and were expressed as means ± SEM of more than 5 animals per group. They were analyzed by one-way ANOVA followed by the Bonferroni test (* $p < 0.05$, *** $p < 0.005$ versus sham; ## $p < 0.01$ versus 6-OHDA + vehicle). Representative immunostaining images for each experimental group are shown in the left panels (scale bar = 50 μm).

2.2. Effects of VCE-004.8 against 6-OHDA Insult in Cultured SH-SY5Ycells

The second part of our study consisted of a series of experiments conducted in a cell-based assay (cultured SH-SY5Y cells exposed to 6-OHDA) that is frequently used as an in vitro model of PD [26], and that, in our case, was used as a way to confirm the in vivo data and, in particular, for exploring the potential targets (PPAR-γ and/or CB_2 receptors) involved in the beneficial effects found with this CBD derivative. First, we carried out a concentration-response experiment to determine the best VCE-004.8 concentration to increase cell survival against the 6-OHDA (200 μM) insult according to similar experiments conducted in the same cell-based model with other phytocannabinoid derivatives [24]. VCE-004.8 showed cytoprotection in a concentration-dependent manner ($F(5,35) = 116.6$, $p < 0.0001$; Figure 6) in the range 2–10 μM, with higher concentrations resulting in being less efficacious (20 μM) or even more toxic (40 μM) (Figure 6).

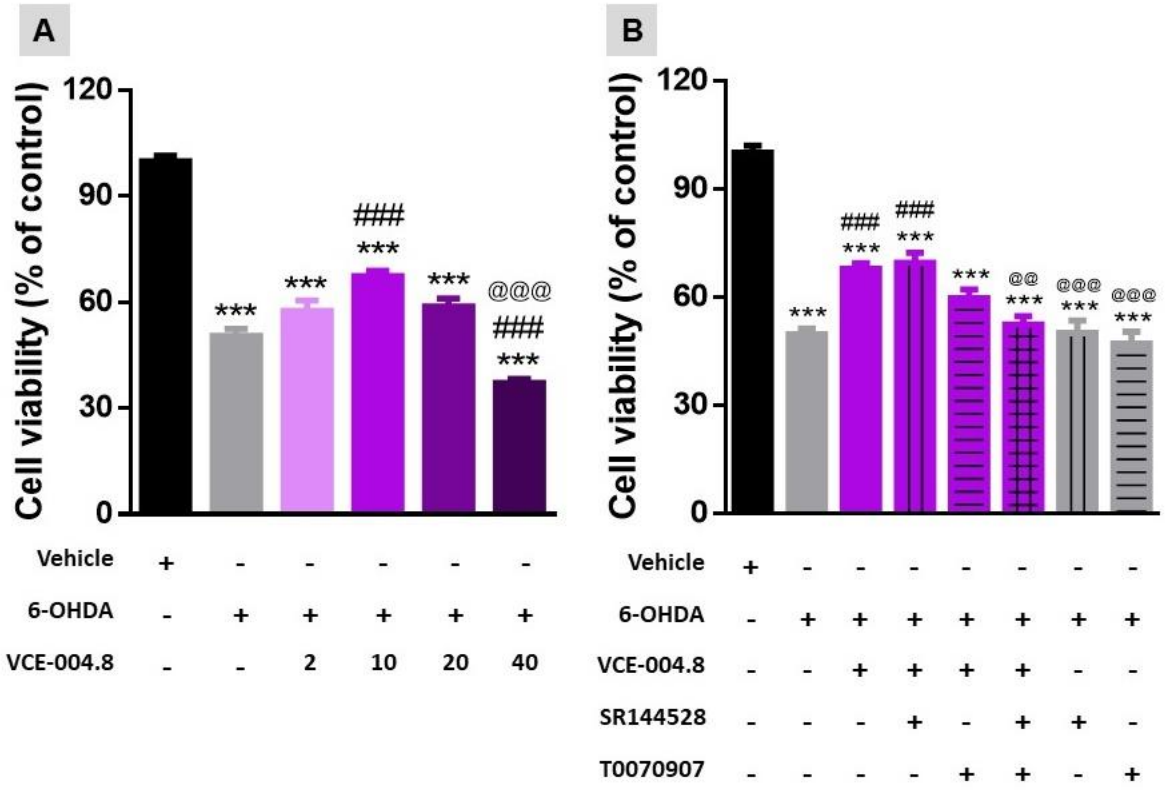

Figure 6. Cell viability measured with the MTT assay in cultured SH-SY5Y cells at 24 h to be treated with: (**A**): different concentrations of VCE-004.8 (2, 10, 20, and 40 μM) against 6-OHDA (200 μM), and (**B**): VCE-004.8 (10 μM) against 6-OHDA (200 μM), in the absence or the presence of T0070907 (10 μM), SR144528 (10 μM), or both. In all cases, a group with cells exposed to vehicles was also included to determine the 100% of cell viability. Values are means ± SEM of at least 4 independent experiments each performed in triplicate. Data were assessed by the one-way analysis of variance followed by the Bonferroni test (Both panels: *** $p < 0.005$ versus control cells; ### $p < 0.005$ versus cells exposed to 6-OHDA+vehicle; Panel A: @@@ $p < 0.005$ versus cells treated with the other VCE-004.8 concentrations; Panel B: @@ $p < 0.01$, @@@ $p < 0.005$ versus cells treated with VCE-004.8 in absence or presence or SR144528).

Once having selected 10 μM as the most adequate concentration, we investigated the cytoprotective effect of this concentration of VCE-004.8 against 200 μM 6-OHDA, in the presence or absence of the PPAR-γ receptor inhibitor T0070907 (10 μM) to inactivate PPAR-γ receptors, the selective antagonist

SR144528 (10 μM) to block CB_2 receptors, or the combination of both compounds. Our data confirmed that 200 μM 6-OHDA reduced cell viability up to 50%, which was significantly elevated (up to 70%) with VCE-004.8 ($F(7,47) = 49.0$, $p < 0.0001$; Figure 6). This cytoprotective effect was completely eliminated only with the combination of T0070907 and SR144528 together (Figure 6), thus confirming that both PPAR-γ and CB_2 receptors contribute to the effect of VCE-004.8. However, whereas the treatment with T0070907 alone caused a partial reversal, the treatment with SR144528 alone has no effect (Figure 6), thus indicating a major contribution of PPAR-γ receptors in the cytoprotective effect of VCE-004.8 at 10 μM. Both antagonists have no effect when added alone to cell cultures (Figure 6).

3. Discussion

The experiments included here follow previous experiments aimed at exploring the neuroprotective potential of different CBG or CBGA quinone derivatives (e.g., VCE-003.2, CBGA-Q, CBGA-Q-Salt) in experimental models of PD [19,20,24]. The novelty of this study was the evaluation of a 3-hydroxyquinone derivative of CBD, VCE-004.8, which, compared to the other phytocannabinoid derivatives investigated in PD [19,20,24], has the particularity to be also active as an agonist of the CB_2 receptor in addition to its activity on the PPAR-γ receptor [25]. A priori, our expectation was that this hybrid activity may provide VCE-004.8 with some advantages in terms of potency and/or broad-spectrum (multitarget) properties compared to the other compounds. Our data confirmed first that VCE-004.8 was also active in the 6-OHDA model, being able to attenuate the loss in the number of TH-positive nigral neurons caused by this neurotoxin. Our data also proved that VCE-004.8 was strongly active against the inflammatory response (elevated astrogliosis labelled with GFAP and microgliosis labelled with CD68) occurring in 6-OHDA-lesioned mice, which was completely attenuated by the treatment with this CBD derivative. A priori this effect would be presumably the result of the VCE-004.8-induced capability to preserve TH-positive nigral neurons, as glial reactivity has been found to be secondary to neuronal death in this PD model [16]. Such benefits were paralleled by recovery of the motor defects typical of 6-OHDA-lesioned mice. We used first the pole test and found no effects of VCE-004.8. Next, we used the cylinder rearing test, which is a much more adequate tool for detecting motor impairment in PD models generated by unilateral lesions, and we observed that the hemiparesis shown by 6-OHDA-lesioned mice in this test was significantly attenuated by the treatment with VCE-004.8.

Therefore, these in vivo data confirm the benefits of this CBD derivative when given orally in this experimental model of PD, as also demonstrated previously for the CBG and CBGA quinone derivatives [19,20,24]. However, our data did not show additional potency for VCE-004.8 in 6-OHDA-lesioned mice, despite its hybrid activity (CB_2 and PPAR-γ receptors) [25]. This could be related to the fact that, whereas PPAR-γ receptors are elevated in lesioned areas in 6-OHDA-treated mice [24], CB_2 receptors remain unaltered [16], which may limit the contribution of this last receptor. An alternative explanation may be that the combination of CB_2/PPAR-γ activities provided by VCE-004.8 may be reflected exclusively in potentiating its anti-inflammatory activity, a fact that may be concluded from our data in GFAP and CD68 immunostaining, but without representing a greater

improvement of its neuroprotective effects (no greater TH immunostaining recovery compared with CBG derivatives) in the 6-OHDA model in which glial reactivity is secondary to neuronal deterioration. If this is true, it is possible that VCE-004.8 may work better in more inflammatory models of PD such as LPS- or rotenone-lesioned mice, in which it is well-known that inflammatory events play more important roles in the pathogenesis. In addition, CB_2 receptors become up-regulated in lesioned structures in both LPS- [16,18] and rotenone-lesioned mice [27], which has been found to facilitate the anti-inflammatory and neuroprotective effect of compounds selectively activating this receptor in these models.

Lastly, as in our previous studies with different CBG/CBGA derivatives [19,20,24], we also investigated the potential mechanism(s) involved in the effects of the CBD derivative against 6-OHDA insult using an in vitro model, SH-SY5Y cells. We first explored the best concentration (10 μM) of VCE-004.8 for having a significant increase in cell viability against the 6-OHDA insult, and we then investigated the contribution of both CB_2 and PPAR-γ receptors to these effects using selective blockade with SR144528 and T0070907, respectively. Our in vitro data confirmed the in vivo findings that PPAR-γ receptors have a major role in the effects of VCE-004.8 in the 6-OHDA model, as reversal of its cytoprotective effects was seen only when T0070907 was added, either alone (partial reversal) or combined with SR144528 (total reversal). By contrast, the use of SR144528 alone had no effect in reversing the VCE-004.8-induced cytoprotection. Therefore, our data support that both receptors are necessary to reach the maximal reversal of this effect, but with higher relevance for PPAR-γ. In addition, our data also confirmed that the site for the effect of VCE-004.8 in the PPAR-γ was the canonical site and not the alternative regulatory site in the PPAR-γ receptor [28], which we found to be the site for the action of VCE-003.2 [19,24].

4. Materials and Methods

4.1. Synthesis and Receptor Characterization of the Different Compounds Investigated

The 3-hydroxyquinone derivative of CBD, (1′R,6′R)-3-(benzylamino)-6-hydroxy-3′-methyl-4-pentyl-6′-(prop-1-en-2-yl)-[1,1′bi(cyclohexane)]-2′,3,6-triene-2,5-dione (VCE-004.8) was synthesized as described previously (del Río et al., 2016). Its pharmacodynamic profile (PPAR-γ and CB_2 agonist) has been previously described [25] and its oral lipid formulation (EHP-101) is currently in clinical development following its benefits found for bleomycin-induced skin and lung fibrosis [29].

4.2. Animals and Surgical Lesions

Male C57BL/6 mice were housed in a room with a controlled photoperiod (06:00–18:00 light) and temperature (22 ± 1 °C). They had free access to standard food and water and were used at adult age (3–4-month-old; 25–30 g weight). All experiments were conducted according to European guidelines (directive 2010/63/EU) and approved by the "Comité de Experimentación Animal" of our university (ref. CEA-UCM 56/2012). For in vivo experiments, mice were anaesthetized (ketamine 40 mg/kg + xylazine 4 mg/kg, i.p.) 30 min after pretreatment with desipramine (25 mg/kg, i.p.), and then 6-OHDA free base (2 μL at a concentration of 2 μg/μL saline in 0.2% ascorbate to avoid oxidation) or saline (for control mice) were injected stereotaxically into the right striatum at a rate of 0.5

μL/min, using the following coordinates: +0.4 mm AP, ±1.8 mm ML and −3.5 mm DV, as described by Alvarez-Fischer and coworkers [30]. Once injected, the needle was left in place for 5 min before being slowly withdrawn, thus avoiding reflux and a rapid increase in intracranial pressure. Control animals were sham-operated and injected with 2 μL of saline using the same coordinates. After the application of 6-OHDA or saline, mice were subjected to pharmacological treatments as described in the following section. The lesions were generated using unilateral injection, the advantage of which is that contralateral structures serve as controls for the different analyses.

4.3. Pharmacological Treatments and Sampling

VCE-004.8 (20 mg/kg, according to a previous study [25]) or vehicle (Maisine CC/corn oil) were orally administered to 6-OHDA-lesioned mice. Control mice (sham-operated) were also administered with the vehicle. The first dose was administered approximately 16 h after the lesion, and the treatment was prolonged for two weeks (one dose per day, always at the same time). One day after the last administration, all animals were analyzed in the pole test and the cylinder rearing test, at the end of which animals were killed by rapid and careful decapitation and their brains were rapidly removed and fixed for one day at 4 °C in fresh 4% paraformaldehyde prepared in 0.1 M phosphate buffered-saline (PBS), pH 7.4. Samples were cryoprotected by immersion in a 30% sucrose solution for a further day, and finally stored at −80 °C for subsequent immunostaining analysis in the substantia nigra.

4.4. Behavioral Procedures

4.4.1. Pole Test

Mice were placed head-upward on the top of a vertical rough-surfaced pole (diameter 8 mm; height 55 cm) and the time until animals descended to the floor was recorded with a maximum duration of 120 s [31]. When the mouse was not able to turn downward and instead dropped from the pole, the time was taken as 120 s (default value).

4.4.2. Cylinder Rearing Test

Given that the lesion was unilateral, this test attempted to quantify the degree of forepaw (ipsilateral, contralateral, or both) preference for wall contacts after placing the mouse in a methacrylate transparent cylinder (diameter: 15.5 cm; height: 12.7 cm; [32]). Each score was made out of a 3 min trial with a minimum of 4 wall contacts.

4.5. Immunohistochemical Procedures

Brains were sliced in coronal sections (containing the substantia nigra) in a cryostat (30 μm thick) and collected on antifreeze solution (glycerol/ethylene glycol/PBS; 2:3:5) and stored at −20 °C until used. Sections were mounted on gelatin-coated slides, and, once adhered, washed in 0.1M potassium PBS (KPBS) at pH 7.4. Then endogenous peroxidase was blocked by 30 min incubation at room temperature in peroxidase blocking solution (Dako Cytomation, Glostrup, Denmark). After

several washes with KPBS, sections were incubated overnight at room temperature with the following primary antibodies: (i) polyclonal rabbit anti-TH (Chemicon-Millipore, Temecula, CA, USA) used at 1/200; (ii) polyclonal rat anti-mouse CD68 antibody (AbD Serotec, Oxford, UK) used at 1/200; or (iii) polyclonal rabbit anti-mouse GFAP antibody (Dako Cytomation, Glostrup, Denmark) used at 1/200. Dilutions were carried out in KPBS containing 2% bovine serum albumin and 0.1% Triton X-100 (Sigma Chem., Madrid, Spain). After incubation, sections were washed in KPBS, followed by incubation with the corresponding biotinylated secondary antibody (1/200) (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine substrate–chromogen system (Dako Cytomation, Glostrup, Denmark) were used to obtain a visible reaction product. Negative control sections were obtained using the same protocol with omission of the primary antibody. A Leica DMRB microscope and a DFC300FX camera (Leica, Wetzlar, Germany) were used for the observation and photography of the slides, respectively. For quantification of the intensity of TH, GFAP or CD68 immunostaining either in the substantia nigra (both ipsilateral and contralateral sides), we used the NIH Image Processing and Analysis software (ImageJ; NIH, Bethesda, MD, USA) using 4–5 sections, separated approximately by 200 μm, and observed with 5×–20× objectives depending on the method and the brain area under quantification. In all sections, the same area of the substantia nigra pars compacta was analyzed. Analyses were always conducted by experimenters who were blinded to all animal characteristics. Data were expressed as percentage of immunostaining intensity in the ipsilateral (lesioned) side over the contralateral (non-lesioned) side.

4.6. Cultures of SH-SY5Y Neuronal Cells

Cultures of SH-SY5Y neuronal cell line (kindly provided by Dr. Ana Martínez, CIB-CSIC, Madrid, Spain) were used to induce cell death with 6-OHDA and to investigate in vitro the mechanisms of cytoprotection of the different cannabinoid derivatives under study, following a procedure described previously [33]. To this end, SH-SY5Y cells were maintained in DMEM supplemented with 10% FBS, 2 mM Ultraglutamine, and 1% antibiotics (Lonza, Verviers, Belgium) and under a humidified 5% CO_2 atmosphere at 37 °C. For cytotoxicity experiments, cells were seeded at 60,000 cells/well in 96-well plates and maintained under a humidified atmosphere (5% CO_2) at 37 °C overnight. In a first experiment, 24 h after seeding, cells were treated with the vehicle (DMEM + 0.1% DMSO) or with four different concentrations of VCE-004.8 (2, 10, 20, and 40 μM; selected according to Del Río et al., 2016), just 60 min before being exposed to 200 μM 6-OHDA (or saline) following our previously published studies with different concentrations of 6-OHDA in these cells [24]. Cells were incubated over 24 h before the neuronal death was analyzed with the MTT assay (Panreac AppliChem., Barcelona, Spain). This experiment served to select the best VCE-004.8 concentration for the second experiment, in which cells were treated with the vehicle (DMEM + 0.1% DMSO), with the PPAR-γ antagonist T0070907 (10 μM), with the selective CB_2 receptor antagonist SR144528 (10 μM), or with both, followed, 30 min later, by a new treatment with VCE-004.8 (10 μM) or vehicle (DMEM + 0.1% DMSO), just 60 min before being exposed to 200 μM 6-OHDA (or saline). Cells were again

incubated over 24 h before the neuronal death was analyzed with the MTT assay (Panreac Appli-Chem., Barcelona, Spain). In all cases, the data of cell viability were normalized in relation to the corresponding control group (cells exposed to vehicles for 6- OHDA and compounds).

4.7. Data Analysis

Data were normally distributed (tested with the Shapiro–Wilk normality test) and were assessed by one-way analysis of variance followed by the Bonferroni test, using GraphPad Prism® software (version 5.01; GraphPad Software Inc., San Diego, CA, USA).

5. Conclusions

In summary, our data confirmed the neuroprotective potential of VCE-004.8 in 6-OHDA-lesioned mice, which adds to information on previously investigated cannabinoid derivatives. In vitro studies confirmed a greater relevance for PPAR-γ receptors rather than CB_2 receptors in these effects. With this study as a whole followed by additional preclinical studies in other experimental models of PD, we expect that all these data will generate further interest in cannabinoid derivatives targeting CB_2 and PPAR-γ receptors as disease-modifying agents in PD.

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Investigating a combined strategy with THCV and CBD as a neuroprotective therapy in experimental models of Parkinson's disease

Sonia Burgaz[1-3], [...] and Javier Fernández-Ruiz[1-3].

[1] Instituto Universitario de Investigación en Neuroquímica, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, Madrid, Spain;

[2] Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain;

[3] Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain;

Abstract: As found for THC, the phytocannabinoid THCV is also antioxidant and a CB2 receptor agonist, but, in contrast, at doses lower than 5 mg/kg, it behaves as a CB1 receptor antagonist rather than an agonist. Such profile is particularly attractive for the treatment of Parkinson's disease (PD), as THCV may serve to alleviate akinetic symptoms due to its action as a CB1 receptor antagonist, as well as to provide neuroprotection due to its antioxidant activity and its capability to activate CB2 receptors. Its neuroprotective properties were particularly evident in LPS-lesioned mice, but they were rather modest in 6-hydroxydopamine(6-OHDA)-lesioned mice, a model in which the phytocannabinoid CBD resulted to be highly active. In the present study, we investigated whether the combination of THCV (2 mg/kg, i.p.) with cannabidiol (CBD; 3 mg/kg, i.p.), may enhance its neuroprotective effect in the same two mouse models. First, in the LPS model, both THCV and CBD reduced the paresis of the contralateral forepaw in the cylinder test. We observed high levels of microgliosis and astrogliosis, which were importantly reduced by the three treatments to the same extent, as well as in the autophagic dysfunction. In the striatum, THCV was able to reduce the mRNA expression of proinflammatory cytokines and enzymes, whereas CBD or the combined treatment had barely any effect. Regarding the 6-OHDA model, the treatment with both compounds, but surprisingly not with the combination, completely reduced the paresis of the contralateral forepaw in the cylinder test and partially reversed the motor inhibition observed in lesioned mice in a computer-aided actimeter, effects that were likely caused by the capability of THCV and CBD to preserve nigrostriatal neurons. The inflammation and autophagy dysfunction derived from the neuronal death were partially reversed by the treatment with THCV, as well as with CBD, but the combination did not produce any improvements. Next, we explored the mechanism involved in these effects by analysing cell survival in cultured SH-SY5Y cells exposed to 6-OHDA. We found a cytoprotective effect of THCV at different concentrations, which was similar to the one found for CBD, as in the *in vivo* models. We could also observe that lower concentrations of both compounds, especially THCV could improve the effects of the combined treatment. The effects of THCV were reversed by the addition of antagonists T0070907 and SR144528, aimed at blocking PPAR-γ and CB2 receptors, respectively. As for CBD, the treatment with T0070907 alone caused a partial reversal, whereas SR144528 alone had no effect, indicating a major contribution of PPAR-γ receptors in the cytoprotective effect of CBD, alone or in combination. In summary, our data support the potential of THCV, due to its promising neuroprotective properties that, however, could not be enhanced by a combined treatment with CBD at the studied doses. Although further studies are clearly required to determine the clinical significance of these data in humans, these results situate THCV by itself (not when combined with CBD) in a promising position for developing a cannabinoid-based therapy for PD patients.

Key words: cannabinoids; THCV; CBD; neuroprotection; neuroinflammation; 6-hydroxydopamine; LPS; Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disorder with an unknown etiology and a fatal course. The main treatment is only symptom-alleviating and consists of replacing with levodopa and/or dopaminergic agonists the lack of dopamine tone caused by the death of the dopaminergic neurons of the substantia nigra (SN). Thus, the current research is aimed at exploring neuroprotective therapies to delay or arrest disease progression as well as to alleviate the motor symptoms (Aymerich et al., 2018). Inflammation has been suggested as a key etiologic factor and pathogenic event in PD, which has promoted the investigation of neuroinflammatory strategies to limit neurodegeneration in this disease (Hirsh and Hunot, 2009). Also, oxidative stress has been proposed as a main feature of the disease as the selective death of nigral dopaminergic neurons could answer to a higher vulnerability of these neurons to metabolic and oxidative stress (Pissadaki and Bolam, 2013). Thus, a potential neuroprotective treatment for PD could be one with both anti-inflammatory and antioxidant profile.

Phytocannabinoids, the active constituents of *Cannabis sativa*, together with endocannabinoids and synthetic cannabinoids have been proposed as promising neuroprotective agents, as a result of their pleiotropism and ability to activate different targets both inside and outside the endocannabinoid system (ECS; Fernández-Ruiz, 2019). Cannabinoids are known to have an antioxidant profile due to their chemical structure (Lastres-Becker et al., 2005) and anti-inflammatory properties due to their activity through CB2 receptor (Chiurchiù et al., 2015), and their potential has been widely demonstrated in experimental PD models, such as MPTP-treated rats (Price et al., 2009) and in LPS-lesioned mice (García et al., 2011; Gómez-Gálvez et al., 2016). These studies show how the deletion of CB2 receptors implies more vulnerability to a LPS insult, as well as the efficacy of different CB2-activating cannabinoids against local inflammation and gliosis. Activation of CB2 was also effective in MPTP-lesioned mice, reducing neuroinflammatory events and preserving dopaminergic neurons (Chung et al., 2016; Shi et al., 2017). Also, CB2 agonists were beneficial in 6-OHDA-treated rats (a model based on oxidative stress and mitochondrial dysfunction), though to a lesser extent, by partially reducing dopamine depletion in the striatum (García-Arencibia et al., 2007). The anti-inflammatory potential of cannabinoids has been reinforced with the observation that many of these compounds are also active at the PPAR-γ receptors, which are involved in the control of neuroinflammatory responses (Agarwal et al., 2017). This different signalling pathway enables these compounds to have anti-inflammatory effects besides their CB2 receptor-mediated activity. Among other neurodegenerative diseases, the beneficial effects of PPAR-γ-acting cannabinoid agonists have already been proved in different PD models (García et al., 2018), and have been further studied in Chapter 1 (Burgaz et al., 2019; Burgaz et al., 2021a) and in Chapter 2 (Burgaz et al., 2021b).

Based on this previous experimental evidence, one phytocannabinoid with a highly interesting profile for PD may be Δ^9-tetrahydrocannabivarin (THCV). It has antioxidant properties and activates CB2 receptor with significant potency (García et al., 2011). Additionally, it works as a CB1 antagonist (when used at doses lower than 3 mg/kg (Pertwee, 2008)), which is interesting against hypokinetic signs in PD (González et al., 2006) and has

certain agonist activity at the GPR55 receptor (Morales et al., 2017). Using 6-OHDA-lesioned mice, we demonstrated that THCV shows anti-hypokinetic effects by blocking CB1 receptors at low doses (García et al., 2011). THCV was also able to preserve nigral neurons against 6-OHDA- and LPS insults in mice due to its antioxidant activity and CB2 agonism, respectively (García et al., 2011). The effects in 6-OHDA-lesioned mice were equivalent to those observed with the antioxidant phytocannabinoid cannabidiol (CBD) (Lastres-Becker et al., 2005; García-Arencibia et al., 2007), whereas those found in LPS-lesioned mice were like the effects found with HU-308, a selective CB2 agonist (Gómez-Gálvez et al., 2016). Recently, we also demonstrated the anti-dyskinetic potential of THCV, both to delay the occurrence and to attenuate the magnitude of dyskinetic signs (Espadas et al., 2020).

Taken together, this evidence has situated THCV in a promising position for serving to generate a cannabinoid-based therapy for specific symptoms and for disease progression in patients affected by PD. In the present study, we evaluated if the combination of both phytocannabinoids could imply an additive or synergistic effect to their individual respective effects (García-Fuente et al., 2018; Anand et al., 2021). To this end, we investigated whether the combination of THCV (2 mg/kg, i.p.) with cannabidiol (CBD; 3 mg/kg, i.p.) may enhance its neuroprotective effects, both in the LPS and 6-OHDA models, to check their anti-inflammatory and antioxidant effects respectively, and to find the possible mechanism of action behind this multitarget therapy using an *in vitro* approach.

2. Materials and methods

2.1. Animals and surgical lesions

Male C57BL/6 mice were housed in a room with a controlled photoperiod (08:00-20:00 light) and temperature (22 ± 1ºC). They had standard food and water *ad libitum* and were used at adult age (3–4-month-old; 25-30 g weight). All experiments were conducted according to national and European guidelines (directive 2010/63/EU), as well as conformed to ARRIVE guidelines and approved by the "Comité de Experimentación Animal" of our university (PROEX: 056/19).

In a first experiment, mice were anaesthetised (ketamine 40 mg/kg + xylazine 4 mg/kg, i.p.) and subjected to unilateral injections of *S. Minnesota* LPS (Sigma-Aldrich, Madrid, Spain) into two points of the right striatum following the procedure developed by Hunter et al. 2009. We used the following stereotaxic coordinates from bregma: +1.1 mm AP, −1.8 mm ML and −3.5 mm DV, as well as −0.3 mm AP, −2.5 mm ML and −3.2 mm DV. At each intrastriatal coordinate, 5 µg of LPS in a volume of 1 µl of saline was injected slowly (0.5 µl/min) and the needle was left in place for 5 min before being slowly withdrawn. This avoids generating reflux and a rapid increase of intracranial pressure. Control animals were sham-operated and injected with 1 µl of saline using the same coordinates. The lesions were generated using unilateral administration, so that contralateral structures serve as controls for the different analyses.

In a second experiment, male C57BL/6 mice were subjected to stereotaxic unilateral application of 6-OHDA or saline. To do that, mice were anaesthetized (ketamine 40 mg/kg + xylazine 4 mg/kg, i.p.) 30 min after pre-treatment with desipramine (25 mg/kg, i.p.) to avoid

noradrenergic injury. Then 6-OHDA free base (2 µl at a concentration of 2 µg/µl saline in 0.2% ascorbate to avoid oxidation) or saline (for control mice) were injected stereotaxically into the right striatum at a rate of 0.5 µl/min, using the following coordinates: +0.4 mm AP, −1.8 mm ML and −3.5 mm DV, as described in Alvarez-Fischer et al. (2008). Once injected, the needle was left in place for 5 min before being slowly withdrawn, thus avoiding reflux and a rapid increase of intracranial pressure. Control animals were sham-operated and injected with 2 µl of saline using the same coordinates. Again, the lesions were generated using unilateral injection, the advantage of which is that contralateral structures serve as controls for the different analyses.

2.2. Pharmacological treatments and sampling

After the application of LPS/6-OHDA or saline, animals were distributed into 5 groups in each experiment, and were subjected to i.p. daily treatment with THCV (2 mg/kg), CBD (3 mg/kg), both THCV and CBD at the same doses, or vehicle (Tween80: saline, 1:16). The first administration, in all cases, was done approximately 24 h after the lesion and the treatment was prolonged for three weeks in the model of LPS and two weeks in the model of 6-OHDA. At the end of the treatment (24 hours after the last injection), they were analysed in different behavioural tests just before being killed by rapid and careful decapitation. Their brains rapidly removed and divided coronally in two parts. The anterior halves were used to dissect the striatum (both ipsilateral and contralateral sides separately) and tissues were rapidly frozen by immersion in cold 2-methylbutane and stored at -80ºC for qPCR analysis. The posterior halves containing the midbrains were fixed for 24 hours at 4°C in fresh 4% paraformaldehyde prepared in 0.1 M PBS, pH 7.4. Samples were cryoprotected by immersion in a 30% sucrose solution for other 24 hours, and finally stored at -80°C for immunohistochemical analysis.

2.3. Behavioural tests

2.3.1. Computer-aided actimeter

Motor activity was analysed in a computer-aided actimeter (Actitrack, Panlab, Barcelona, Spain). This apparatus consisted of a 45x45 cm area, with infra-red beams all around, spaced 2.5 cm, coupled to a computerized control unit that analyses the following parameters: (i) distance run in the actimeter (ambulation); (ii) time spent in inactivity (resting time); and (iii) mean and velocity developed during the running. Animals remained for a period of 10 min in the actimeter, but measurements were only recorded during the final 5 min (first 5 min was used only for animal acclimation).

2.3.2. Cylinder rearing test (CRT)

Given that the lesions were unilateral, this test attempts to quantify the degree of forepaw (ipsilateral, contralateral, or both) preference for wall contacts after placing the mouse in a methacrylate transparent cylinder (diameter: 15.5 cm; height: 12.7 cm; Fleming et al., 2013). Each score was made from a 3 min trial with a minimum of 4 wall contacts.

2.3.3. Pole test

Mice were placed head-upward on the top of a vertical rough-surfaced pole (diameter 8 mm; height 55 cm) and the time until animals descended to the floor was recorded with a

maximum duration of 120 s (Matsuura et al., 1997). When the mouse was not able to turn downward and instead dropped from the pole, the time was taken as 120 s (default value).

2.4. Immunohistochemical analysis

Brains were sliced in coronal sections (containing the SN) in a cryostat (30 μm thick), collected on antifreeze solution (glycerol/ethylene glycol/PBS; 2:3:5) and stored at -20ºC until used. Brain sections were mounted on gelatine-coated slides, and once adhered, washed in 0.1M KPBS at pH 7.4. Endogenous peroxidase was blocked by 30 min incubation at room temperature in peroxidase blocking solution (Dako Cytomation, Glostrup, Denmark). After several washes with KPBS, sections were incubated overnight at room temperature with the following polyclonal antibodies: (i) rabbit anti-TH (Chemicon-Millipore, Temecula, CA, USA) used at 1:200; (ii) rat anti-mouse CD68 antibody (AbD Serotec, Oxford, UK) used at 1:200; or (iii) rabbit anti-mouse GFAP antibody (Dako Cytomation, Glostrup, Denmark) used at 1:200. In the case of LAMP-1 immunostaining, we used the hybridoma monoclonal rat antimouse LAMP-1 antibody 1D4B, which was deposited by Dr. J. Thomas in the Developmental Studies Hybridoma Bank (DSHB; Hybridoma Product 1D4B), created by the NICHD (NIH, Bethesda, MD, USA) and maintained at The University of Iowa, Department of Biology, Iowa City, IA, USA. Dilutions were carried out in KPBS containing 2% bovine serum albumin (BSA) and 0.1% Triton X-100 (Sigma Chem., Madrid, Spain). After incubation, sections were washed in KPBS, followed by incubation with the corresponding biotinylated secondary antibody (1:200) (Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature. Avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine substrate–chromogen system (Dako Cytomation, Glostrup, Denmark) were used to obtain a visible reaction product. Negative control sections were obtained using the same protocol with omission of the primary antibody. A Leica DMRB microscope and a DFC300FX camera (Leica, Wetzlar, Germany) were used for the observation and photography of the slides, respectively.

For quantification of TH, GFAP or CD68 immunostaining in the SN, we used the NIH Image Processing and Analysis software (ImageJ; NIH, Bethesda, MD, USA) using 4-5 sections, separated approximately by 200 μm, and observed with 5x-20x objectives depending on the method and the brain area under quantification. In all sections, the same area of the SN was analysed. Analyses were always conducted by experimenters who were blinded to all animal characteristics. Data were expressed as percentage of immunostaining intensity in the ipsilateral (lesioned) side over the contralateral (non-lesioned) side.

2.5. Real time q-PCR (RT-qPCR) analyses

Total RNA was isolated from the different striatum samples using Trizol reagent (Sigma-Aldrich, Madrid, Spain). The total amount of RNA extracted was quantitated by spectrometry at 260 nm and its purity from the ratio between the absorbance values at 260 and 280 nm. After genomic DNA was removed (to eliminate DNA contamination), single-stranded complementary DNA was synthesized from up to 1 μg of total RNA using the commercial kits RNeasy Mini Quantitect Reverse Transcription (Qiazen, Hilgen, Germany) and iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The reaction mixture was kept frozen at -20ºC until enzymatic amplification. Quantitative RT-PCR assays were performed using TaqMan Gene Expression

Assays (Applied Biosystems, Foster City, CA, USA) to quantify mRNA levels for TNF-α (ref. Mm99999068_m1), IL-1β (ref. Mm00434228_m1), iNOS (ref. Mm01309902_m1), COX-2 (ref. Mm00478372_m1), Arginase-1 (ref. Mm00475988_m1), using GAPDH expression (ref. Mm99999915_g1) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the threshold cycle (Ct) was calculated by the instrument's software (7300 Fast System, Applied Biosystems, Foster City, CA, USA). Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. Cell cultures

SH-SY5Y neuronal cell line (kindly provided by Dr. Ana Martínez, CIB-CSIC, Madrid, Spain) was used to induce cell death with 6-OHDA and to investigate *in vitro* the possible cytoprotective effects of THCV and CBD, following a procedure described previously (Ko et al., 2019). To this end, SH-SY5Y cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% FBS, 2 mM Ultraglutamine, and 1% antibiotics (Lonza, Verviers, Belgium) and under a humidified 5% CO_2 atmosphere at 37°C. For cytotoxicity experiments, cells were seeded at 60,000 cells/well in 96-well plates and maintained under a humidified atmosphere (5% CO_2) at 37°C overnight. 24 hours after seeding, cells were treated with the vehicle (DMEM + 0.1% DMSO) or with different concentrations of THCV and CBD (50, 100 and 500 nM and 1, 5 and 10 μM), just 60 minutes before being exposed to 200 μM 6-OHDA (or saline) following our previously published studies (Burgaz et al., 2021a). Cells were incubated during 24 hours before the neuronal death was analysed with the MTT assay (Panreac AppliChem., Barcelona, Spain). Data of cell viability were normalized in relation to the corresponding control group (cells exposed to vehicles for 6-OHDA, THCV and CBD).

Next, the same procedure was followed for studying the co-treatment with both compounds, choosing three combinations of the optimal concentrations of THCV and CBD (100 and 500; 50 and 100; and 50 and 500 nM, respectively). Once selected the optimal concentration of the combination of THCV and CBD, the same procedure was followed, but including the pre-treatment with antagonists to determine the mechanism of action. We used a CB2 antagonist, SR144528 (Cayman Chemical, Ann Arbor, Michigan, USA), and a PPAR-γ antagonist, T0070907 (Cayman Chemical, Ann Arbor, Michigan, USA), both at a 10 μM concentration, selected according to our previously published studies (Burgaz et al., 2021b). The cells were treated with the antagonists 24 hours after seeding and 30 mins before the treatment with THCV (100 nM), CBD (500 nM) or with both. Again, cells were incubated during 24 hours before the neuronal death was analysed with the MTT assay and data of cell viability were normalized in relation to the corresponding control group.

2.7. Statistics

Data were assessed using one-way ANOVA, followed by the Bonferroni test using GraphPad Prism, version 8.00 for Windows (GraphPad Software, San Diego, CA, USA). A p value (p) lower than 0.05 was used as the limit for statistical significance. The sample sizes in the different experimental groups were always > 5.

3. Results

In a first experiment we explored the anti-inflammatory and neuroprotective potential of the i.p. administration of THCV at the dose of 2 mg/kg in LPS-lesioned mice, as well as its combination with CBD at the dose of 3 mg/kg, for three weeks. We analysed the neurological status of these mice using the pole test, in which LPS-lesioned mice tended to spend more time in descending the pole than sham mice, and the treatment with THCV, CBD and their combination were unable to reverse this defect (F(4,37)=1.644, ns; **Figure 1A**). This was not the case in the CRT, a more reliable for testing parkinsonism signs in unilateral models. The hemiparesis shown by LPS-lesioned mice compared to controls was significantly attenuated after the treatment with THCV, CBD and their combination to the same extent (F(4,37)=5.476, p<0.005; **Figure 1B**).

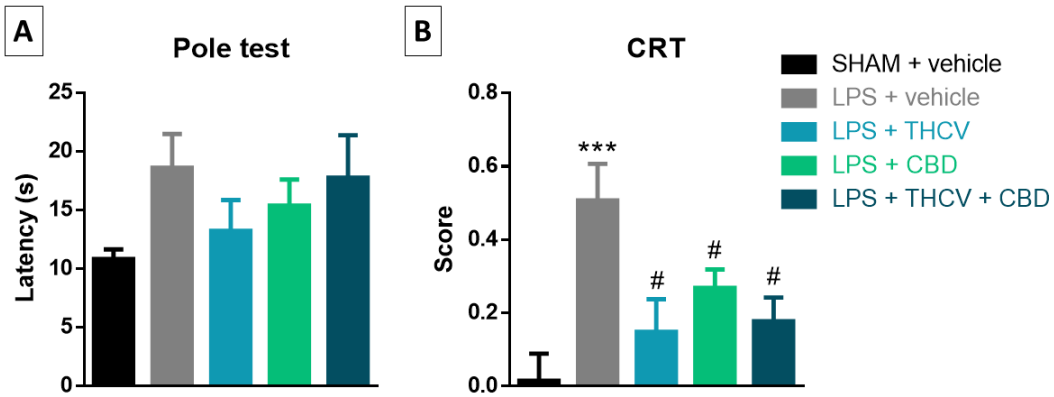

Figure 1. Response in the pole test (**A**) and in the cylinder rearing test (**B**) of male mice subjected to unilateral LPS lesions or sham-operated and daily treated with THCV (2 mg/kg, i.p.), CBD (3 mg/kg, i.p.) or both treatments for three weeks. Values represent means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Bonferroni test (***p<0.005 vs sham-operated group; ##p<0.01 vs vehicle-treated LPS lesioned mice).

The histopathological analysis of the SN revealed no significant loss of TH-containing nigrostriatal neurons caused by the LPS lesion (F(4,37)=0.4902, ns; **Figure 2A**), as expected for this model, were the neurodegeneration in this area is in general scarce or very low. We also analyzed LAMP-1, a marker of autophagy, which has been found to be elevated in experimental models of PD, including LPS-lesioned mice, and in human samples of patients, reflecting autophagy dysregulation. Our data indicated a significant elevation in LAMP-1 expression in LPS-lesioned mice and an important recovery after the treatment with THCV, alone or in combination with CBD (F(4,38)=44.67, p<0.0001; **Figure 2B**).

The microglial reactivity provoked by LPS in the SN of lesioned mice, measured by CD68 immunostaining, was reduced by the chronic treatment with THCV, although it did not reach the levels of the sham-operated mice (F(4,36)=16.15, p<0.0001; **Figure 3A**). Again, these effects were not intensified by the combination of the treatment with CBD. These changes were associated with an important reduction, triggered by THCV in the intense astroglial reactivity provoked by LPS in the SN, measured by GFAP immunostaining (F(4,39)=64.59, p<0.0001; **Figure 3B**). The groups treated with CBD, alone or combined with THCV, also showed a reduction in the astrogliosis, to the same extent than animals only treated with THCV.

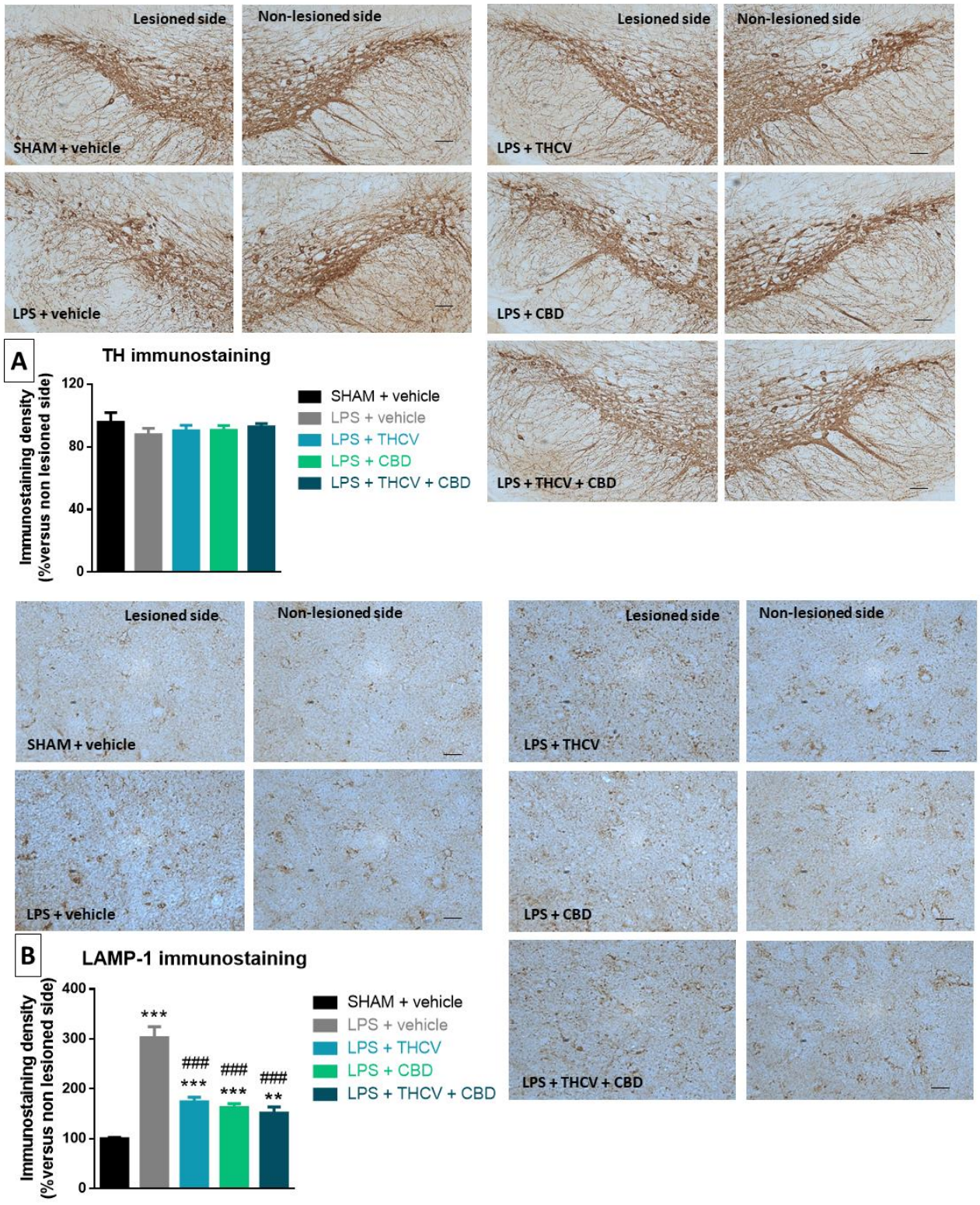

Figure 2. Quantification of TH (**A**) and LAMP-1 immunoreactivity (**B**), including representative images (scale bar = 100 µm and 25 µm, respectively), measured in the SN of male mice subjected to unilateral LPS lesions or sham-operated and daily treated with THCV (2 mg/kg, i.p.), CBD (3 mg/kg, i.p.) or both treatments for three weeks. Values correspond to % of the ipsilateral lesioned side vs contralateral non-lesioned side and were expressed as means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Bonferroni test. (**p<0.01, ***p<0.001 vs sham-operated group; ###p<0.001 vs vehicle-treated LPS lesioned mice).

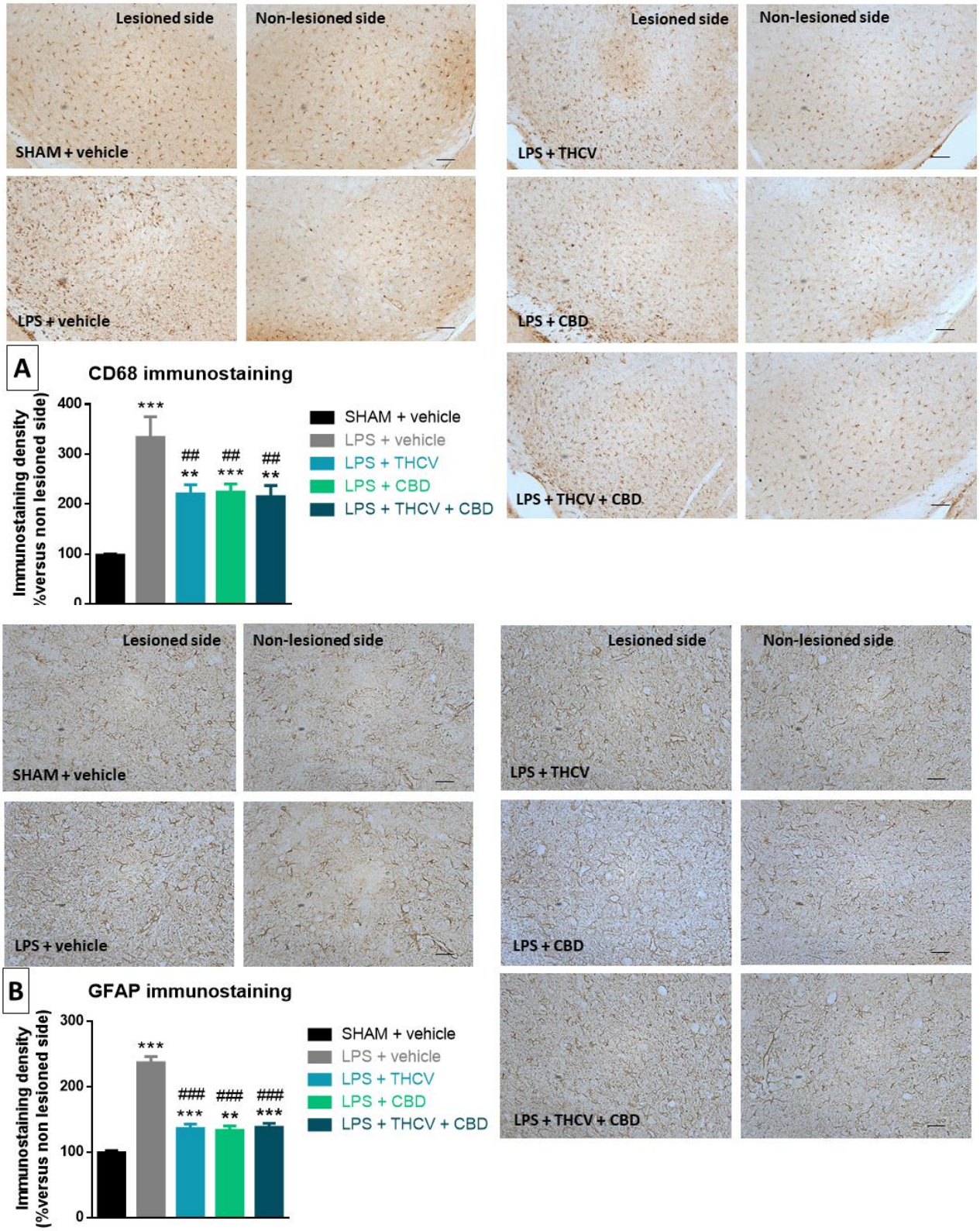

Figure 3. Quantification of CD68 (**A**) and GFAP immunoreactivity (**B**), including representative images (scale bar = 100 µm and 50 µm, respectively), measured in the SN of male mice subjected to unilateral LPS lesions or sham-operated and daily treated with THCV (2 mg/kg, i.p.), CBD (3 mg/kg, i.p.) or both treatments for three weeks. Values correspond to % of the ipsilateral lesioned side vs contralateral non-lesioned side and were expressed as means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Bonferroni test. (**p<0.01, ***p<0.001 vs sham-operated group; ##p<0.01, ###p<0.001 vs vehicle-treated LPS lesioned mice).

Finally, we wanted to evaluate if the treatments were also effective against some associated inflammatory events elicited by LPS lesion, such as the elevated gene expression detected in the striatum in proinflammatory cytokines TNF-α ($F(4,29)=29.09$, $p<0.0001$; **Figure 4A**) and IL-1β ($F(4,28)=8.908$, $p<0.0001$; **Figure 4B**), as well as in proinflammatory enzymes iNOS ($F(4,34)=3.651$, $p<0.05$; **Figure 4C**) and COX-2 ($F(4,34)=8.215$, $p<0.0001$; **Figure 4D**). Our data indicated no effect after CBD treatment, but certain attenuation in those animals treated with THCV, in particular in TNF-α. The combination of both treatments did not improve these effects.

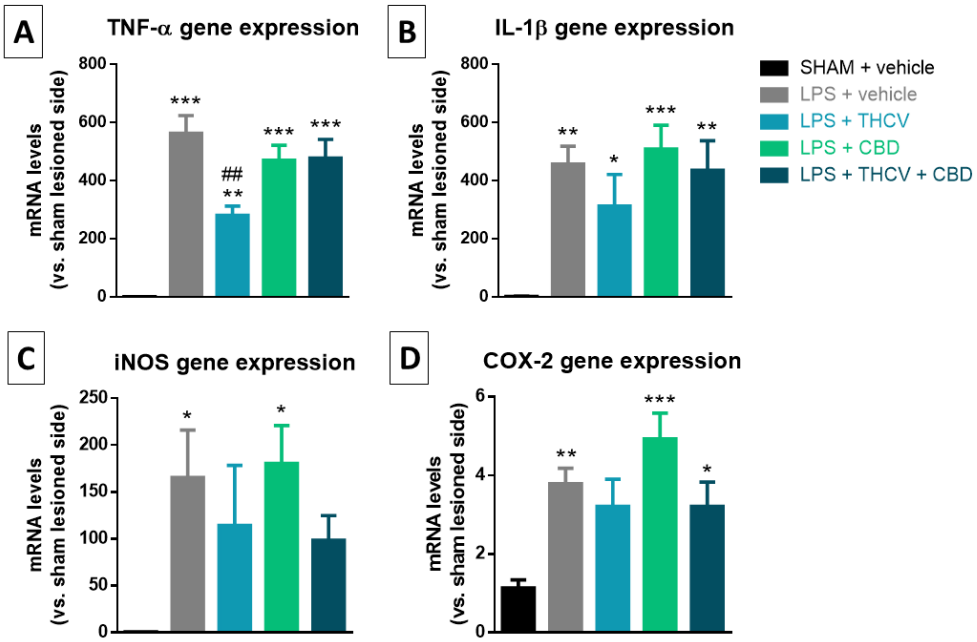

Figure 4. mRNA levels for TNF-α (**A**), IL-1β (**B**), Inos (**C**) and COX-2 (**D**) in the striatum of sham and LPS-lesioned mice daily treated with THCV (2 mg/kg, i.p.), CBD (3 mg/kg, i.p.) or both treatments for three weeks. Values were normalized vs sham mice and correspond to means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Bonferroni test. (*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs sham-operated group; ##$p<0.01$ vs vehicle-treated LPS lesioned mice).

In a second experiment, we studied the effects of the administration of THCV (2 mg/kg) over the motor impairments caused by 6-OHDA lesion, using a computer-aided actimeter, frequently used in our laboratory to detect hypokinetic signs in experimental parkinsonism. The treatment with THCV was able to partially reduce the alterations observed in the ambulatory activity ($F(4,31)=5.323$, $p<0.005$; **Figure 5A**), mean velocity ($F(4,31)=5.229$, $p<0.005$; **Figure 5B**) and resting time ($F(4,31)=4.419$, $p<0.01$; **Figure 5C**), whereas the greatest effects were always found after the treatment with CBD alone. However, the combination of THCV with CBD did not improve these results. Likewise, in the cylinder rearing test, our data demonstrated that 6-OHDA lesion caused again an increased preference for the ipsilateral paw, which was likely reduced by the treatment with THCV, as a loss of statistical significance ($F(4,30)=4.261$, $p<0.01$; **Figure 5D**). Surprisingly, these effects were not only not improved by the combination with CBD, but also partially worsened in this group, while the group treated only with CBD showed the best recovery of the forepaw hemiparesis.

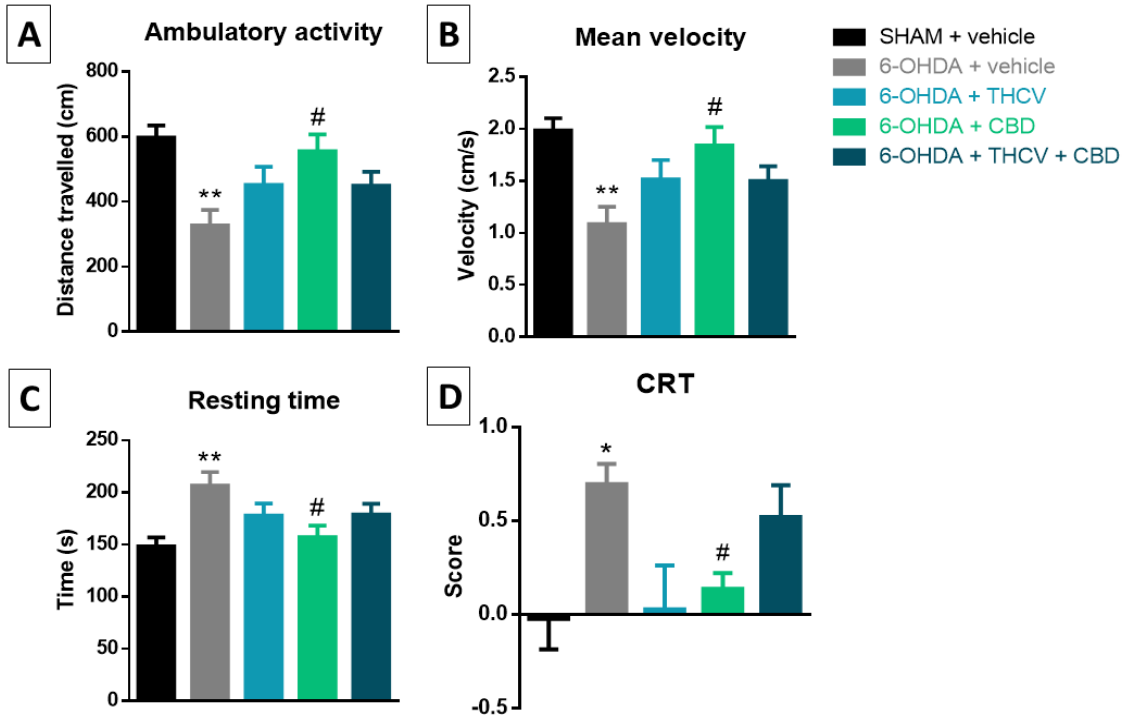

Figure 5. Ambulatory activity (**A**), mean velocity (**B**) and resting time (**C**) measured in a computer-aided actimeter and response in the cylinder rearing test (**D**) of male mice subjected to unilateral LPS lesions or sham-operated and daily treated with THCV (2 mg/kg, i.p.), CBD (3 mg/kg, i.p.) or both treatments for two weeks. Values are means ± SEM of more than 6 animals *per* group. Data were assessed by one-way ANOVA followed by the Bonferroni test (***p<0.005 vs sham-operated group; ##p<0.01 vs vehicle-treated LPS lesioned mice).

The histopathological analysis of the SNpc of these animals reflected a significant loss of TH-containing nigrostriatal neurons caused by the 6-OHDA lesion ($F(4,30)=13.14$, $p<0.0001$; **Figure 6A**), which was importantly prevented by the treatment with both THCV and CBD when administered alone. However, and similarly to the results observed in the CRT, the combination of both treatments had much less effects over the neurodegeneration than any of the treatments on their own. As in the first *in vivo* experiment, we also analyzed the autophagy status by LAMP-1 labelling. Our data showed a significant elevation in LAMP-1 expression in the SN of 6-OHDA-lesioned mice (smaller than in LPS-lesioned mice) and again an important recovery after the treatment with THCV alone ($F(4,30)=4.720$, $p<0.01$; **Figure 6B**). This time, the effect was maintained, although not improved, when administered in combination with CBD, which was also effective on its own. Next, we also wanted to analyse the glial reactivity in the SN, which in the 6-OHDA model is not so intense as in LPS model, as the inflammatory events are not primary but secondary to the neuronal death in this area. The microglial reactivity was measured by CD68 immunostaining, and the increase observed in the lesioned group was reduced by the chronic treatment with THCV ($F(4,30)=25.96$, $p<0.0001$; **Figure 7A**). Again, these effects were not improved by the combination of the treatment with CBD, which had similar effects on its own. In addition, we observed an important reduction, triggered by THCV, in the intense astroglial reactivity present in the SN, measured by GFAP immunostaining ($F(4,29)=6.723$, $p<0.001$; **Figure 7B**). The groups treated with CBD, alone or combined with THCV, also showed a reduction in the astrogliosis, to a similar extent than when only treated with THCV.

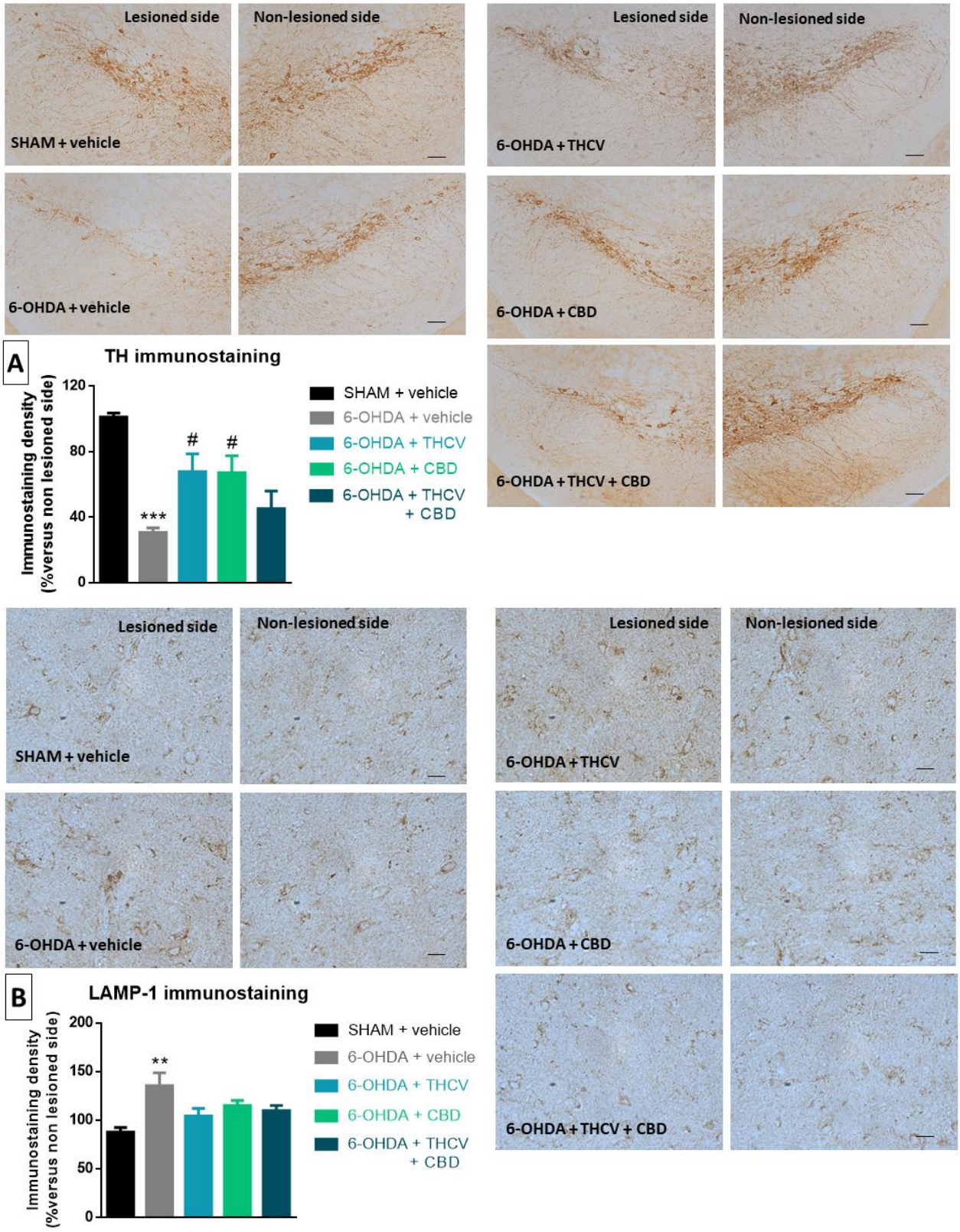

Figure 6. Quantification of TH (**A**) and LAMP-1 immunoreactivity (**B**), including representative images (scale bar = 100 µm and 25 µm, respectively), measured in the SN of male mice subjected to unilateral 6-OHDA lesions or sham-operated and daily treated with THCV (2 mg/kg, i.p.), CBD (3 mg/kg, i.p.) or both treatments for two weeks. Values correspond to % of the ipsilateral lesioned side vs contralateral non-lesioned side and were expressed as means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Bonferroni test (**p<0.01, ***p<0.001 vs sham-operated group; #p<0.05, ###p<0.001 vs vehicle-treated 6-OHDA lesioned mice).

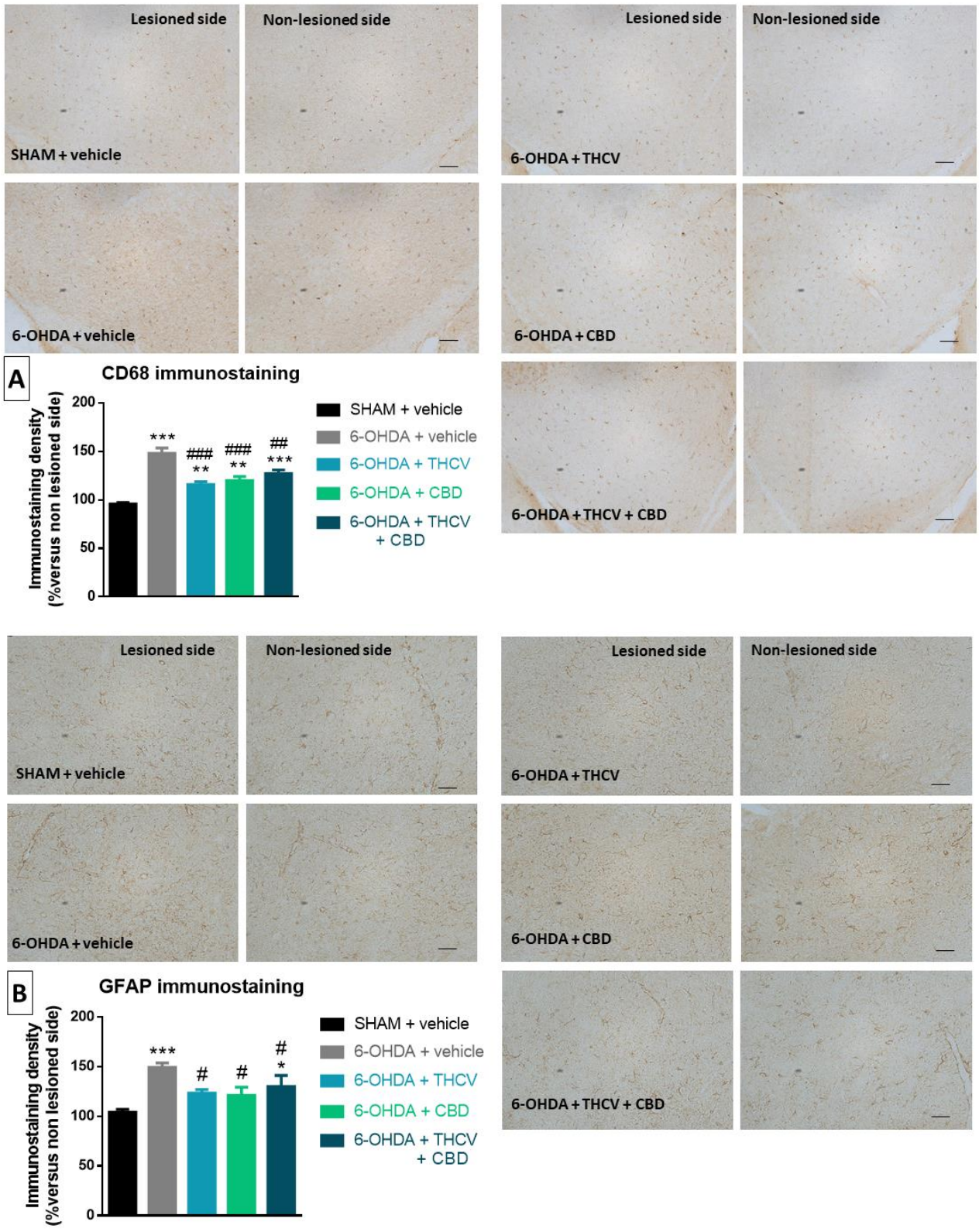

Figure 7. Quantification of CD68 (**A**) and GFAP immunoreactivity (**B**), including representative images (scale bar = 100 µm and 50 µm, respectively), measured in the SN of mice subjected to unilateral 6-OHDA lesions or sham-operated and daily treated with THCV (2 mg/kg, i.p.), CBD (3 mg/kg, i.p.) or both treatments for two weeks. Values correspond to % of the ipsilateral lesioned side vs contralateral non-lesioned side and were expressed as means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Bonferroni test. (*p<0.05, (**p<0.01, ***p<0.001 vs sham-operated group; #p<0.05, ##p<0.01 vs vehicle-treated 6-OHDA lesioned mice).

Lastly, we conducted a series of experiments in a cell-based assay, which consisted in cultured SH-SY5Y cells exposed to 6-OHDA, commonly used as an *in vitro* PD model. Our aim was to further explore the potential targets (PPAR-γ and/or CB2 receptors) involved in beneficial effects found with THCV and CBD, but also to try to find the mechanism behind the unexpected effects of the combination of these compounds, which in some cases seem to be deleterious. First, we carried out a concentration-response experiment to determine the best THCV and CBD concentration able to increase cell survival against the 6-OHDA (200 µM) insult. THCV showed cytoprotection in a concentration-dependent manner ($F(7,32)=17.88$, $p<0.0001$; **Figure 8A**) in the range 50 nM to 500 nM, losing protective effects at higher concentrations. As for CBD, it showed a similar profile, with cytoprotective effects from 50 nM to 5 µM ($F(7,32)=23.52$, $p<0.0001$; **Figure 8B**).

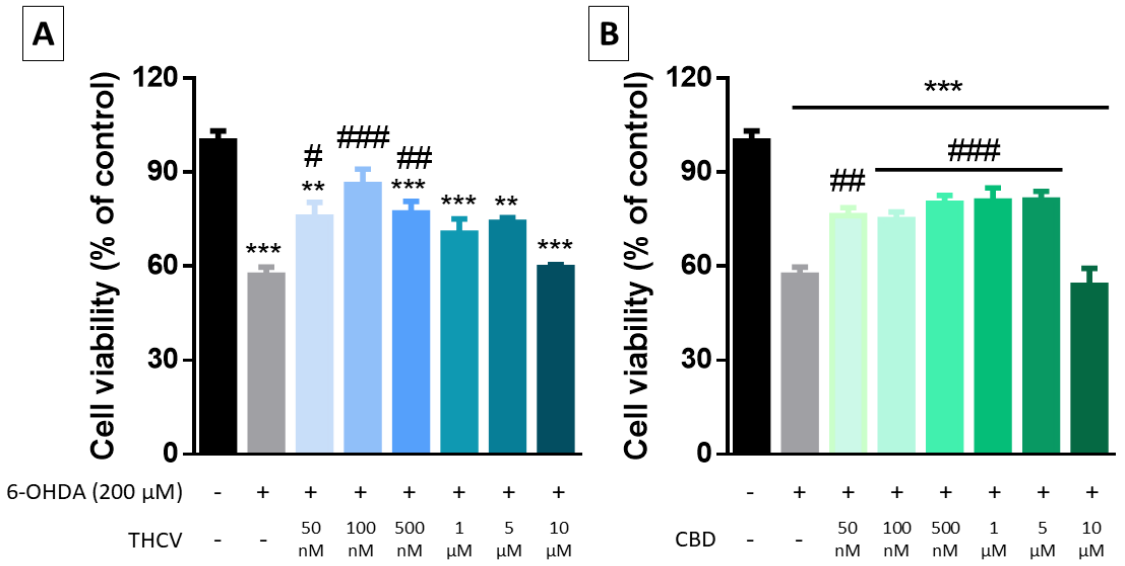

Figure 8. Cell viability measured with the MTT assay in cultured SH-SY5Y cells at 24 h to be treated with (**A**) different concentrations of THCV (from 50 nM to 10 µM) against 6-OHDA (200 µM); (**B**) different concentrations of CBD (from 50 nM to 10 µM) against 6-OHDA (200 µM). In all cases, a group with cells exposed to vehicle was also included to determine the 100% of cell viability. Values represent means ± SEM of at least 4 independent experiments each performed in triplicate. Data were assessed by one-way ANOVA followed by the Bonferroni test (**$p<0.01$, ***$p<0.001$ vs control cells; #$p<0.05$, ##$p<0.01$, ###$p<0.001$ vs cells exposed to 6-OHDA + vehicle).

Once selected the most adequate concentration of THCV (100 nM) and CBD (500 nM), we investigated the effect of their combination against 200 µM 6-OHDA. Again, both THCV and CBD used at their optimal concentrations preserved cell viability when added individually. However, the combination of THCV and CBD at these optimal concentrations was surprisingly not cytoprotective for these cells ($F(4,27)=17.24$, $p<0.0001$; **Figure 9A**), just as we observed in the *in vivo* model of 6-OHDA lesion. In order to confirm if this effect persisted when the compounds are used at subeffective doses, we did a new combination with THCV at 50 nM and CBD at 100 nM, and we observed the same effect with the combination that with both compounds alone, they were cytoprotective but without additive nor synergistic effect ($F(4,19)=20.03$, $p<0.0001$; **Figure 9B**). Also, we tried a different battery of proportions for both compounds, to check if the effects were the same or may be improved (data not shown). The best result was found when using the combination of THCV at 50 nM and CBD at 500 nM, (this is, 1:10 of THCV:CBD instead

of 1:2), but once more it did not reach higher levels of neuroprotection than when used individually (F(4,19)=20.06, p<0.0001; **Figure 9C**).

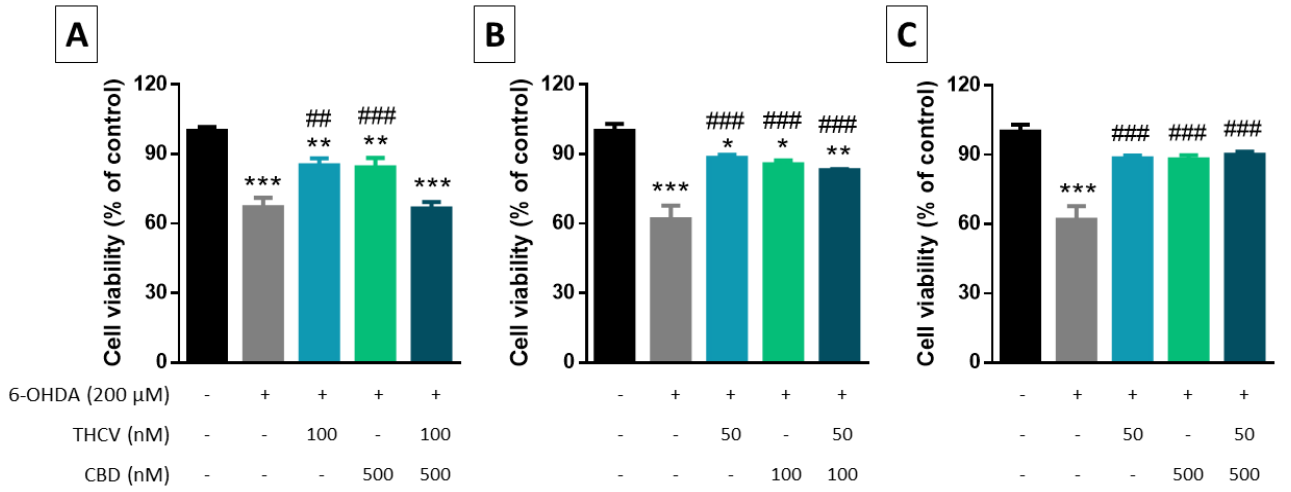

Figure 9. Cell viability measured with the MTT assay in cultured SH-SY5Y cells at 24 h to be treated with (**A**) THCV (100 nM) and/or CBD (500 nM) against 6-OHDA (200 µM); (**B**) THCV (50 nM) and/or CBD (100 nM) against 6-OHDA (200 µM); (**C**) THCV (50 nM) and/or CBD (500 nM) against 6-OHDA (200 µM). In all cases, a group with cells exposed to vehicle was also included to determine the 100% of cell viability. Values are means ± SEM of at least 4 independent experiments each performed in triplicate. Data were assessed by one-way ANOVA followed by the Bonferroni test (*p<0.05, **p<0.01, ***p<0.001 vs control cells; ##p<0.01, ###p<0.001 vs cells exposed to 6-OHDA + vehicle).

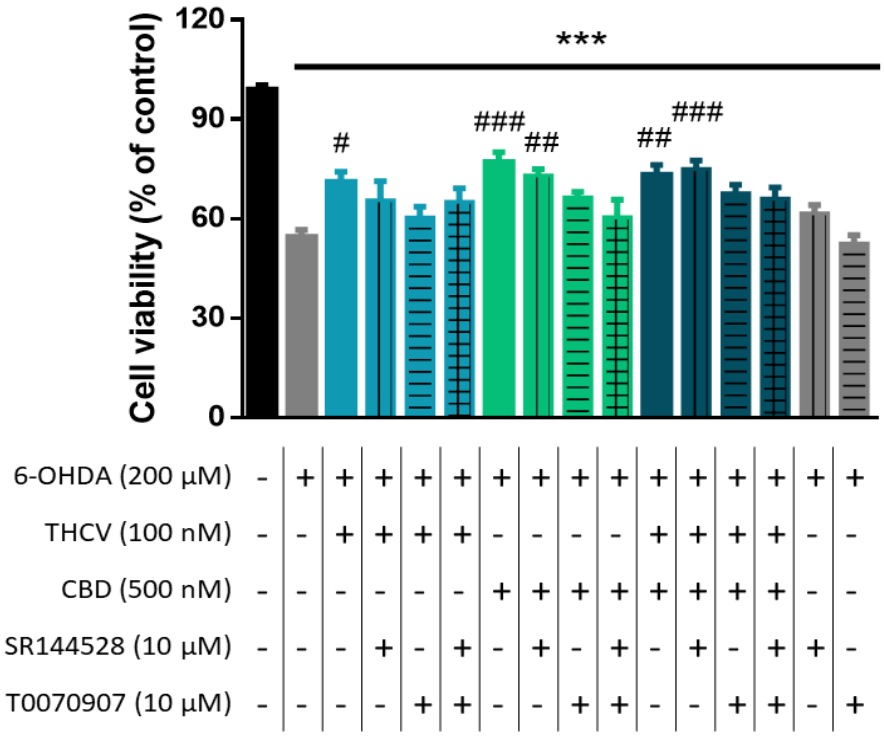

Figure 10. Cell viability measured with the MTT assay in cultured SH-SY5Y cells at 24 h to be treated with THCV (100 nM) and/or CBD (500 nM) against 6-OHDA (200 µM), in the presence or the absence of SR144528 (10 µM), T0070907 (10 µM), or both. In all cases, a group with cells exposed to vehicle was also included to determine the 100% of cell viability. Values are means ± SEM of at least 4 independent experiments each performed in triplicate. Data were assessed by one-way ANOVA followed by the Bonferroni test (***p<0.001 vs control cells; #p<0.05, ##p<0.01, ###p<0.001 vs cells exposed to 6-OHDA + vehicle).

Finally, using the original combination (THCV:CBD as 1:2), which we consider the most similar concentrations to the ones we used *in vivo* (2:3), we conducted the same experiment but, in the presence or absence of the selective antagonist SR144528 (10 μM) to block CB2 receptors, the PPAR-γ receptor inhibitor T0070907 (10 μM) to inactivate PPAR-γ receptors, or the combination of both compounds. Our data confirmed that 200 μM 6-OHDA reduced cell viability up to 50%, which was significantly elevated (up to 70%) with THCV ($F(15,103)=14.63$, $p<0.0001$; **Figure 10**). This cytoprotective effect was slightly reduced with SR144528, T0070907 and the combination of both together, confirming that both PPAR-γ and CB2 receptors contribute to the effect of THCV but not on their own. Regarding CBD, alone or in the combination with THCV, whereas the treatment with T0070907 alone caused a partial reversal, the treatment with SR144528 alone had no effect, then indicating a major contribution of PPAR-γ receptors in the cytoprotective effect of CBD at 500 nM.

4. Discussion

There is large evidence supporting the great potential of a cannabinoid-based therapy for PD, but many of the candidate compounds investigated to date have only been effective for some of the pathophysiological features of the disease. Traditionally, scientific efforts have been focused in finding symptom-relieving effects of cannabinoids, using CB1 antagonists for treating akinesia (Fernández-Espejo et al., 2005; González et al., 2006) or levodopa-induced dyskinesia (García-Arencibia et al., 2009); and CB1 agonists for reducing the tremor (Sañudo-Peña et al., 1998). Given the importance of oxidative stress and inflammation in the neuropathology of PD, cannabinoids have been also evaluated as neuroprotective agents, by studying their effects on the progression of nigral damage based on their antioxidant activity (Lastres-Becker et al., 2005; García-Arencibia et al., 2007; Jiménez-Del-Río et al., 2008) or their anti-inflammatory profile as CB2 agonists (Price et al., 2009; Gómez-Gálvez et al., 2016). However, none of these approaches has been effective enough, which confirmed the need of evaluating more pleiotropic compounds. In this line, THCV has been shown to have a dual effect acting as a symptom-relieving agent (due to its CB1 antagonist activity at low doses, (Pertwee, 2008)), and as an anti-inflammatory compound (due to its CB2 agonist activity) with antioxidant properties through receptor-independent mechanisms (García et al., 2011). Nevertheless, the effect reducing reactive microgliosis and nigral damage both in 6-OHDA and LPS-lesioned rodents shown in these previous studies is limited. Thus, given the promising but limited results shown by THCV, we propose a combination with CBD in order to enhance the neuroprotective properties of both compounds. To accomplish this aim, we have used two PD chronic animal models based on (i) oxidative stress (by 6-OHDA injection) and (ii) neuroinflammation (by LPS injection).

In the LPS-lesioned mice, the bradykinesia-like phenotype is not so marked (possibly because the lesion was unilateral (García et al., 2018)), but we could observe a trend for its reduction in the THCV treated group more than with the combined treatment with CBD. We also conducted the CRT, that may be a more relevant functional endpoint when the lesion is unilateral, and we demonstrated that the hemiparesis caused by LPS-lesion was significantly reversed by THCV treatment. Even though the combination with CBD did not change the effects

of THCV on this parameter, the score reached by the THCV group is already close to the sham-operated group, thus leaving little scope for action for a synergistic or an additive effect. A similar reason might explain most of the histological results. The neuronal death in LPS model is frequently modest (Burgaz et al., 2019), but we did find the first evidence of the potential of THCV targeting autophagy dysfunction, which is one of the main pathogenic mechanisms in PD (Hou et al., 2020). The treatment with THCV was able to markedly reduce the levels of the lysosomal marker LAMP-1, which is abnormally increased in LPS-lesioned mice. The THCV treatment combined with CBD gave the same LAMP-1 levels, although again in all cases very close to the control levels. Likewise, in the study of both microglial and astroglial reactivity in the SN, we observed the same significant effect of THCV reducing neuroinflammation, but not to a greater extent when co-administered with CBD. However, the levels of inflammatory modulators in the striatum were differentially modified by the treatments, as THCV alone was the only one that reached a significant reduction, in particular in TNF-α. The differences between the treatments could be related to the importance of CB2 receptor in LPS-model (Gómez-Gálvez et al., 2016), where its activation by THCV, unlike CBD, achieves a higher anti-inflammatory response in the striatum, the starting point of the neuroinflammation.

In our second experiment, the decrease in the general motor function in the 6-OHDA lesioned mice, was only slightly ameliorated by THCV treatment, whereas it was significantly recovered by CBD treatment. Considering that 6-OHDA lesion provokes intense mitochondrial dysfunction and oxidative stress (Alvarez-Fischer et al., 2008), it seems normal that CBD, with a higher antioxidant activity (Lastres-Becker et al. 2005), could be more effective than THCV (García et al., 2011). However, when co-administered with THCV, we observe a loss of the CBD effect over the general motor function, which seems to indicate that there could be an interference between the mechanisms of action of both compounds. A similar effect was obtained in the CRT, where the better score reduction was obtained with the CBD alone group, followed by the THCV alone group, and last the lesioned mice that received the combined treatment. Unlike LPS-model, 6-OHDA-lesioned mice present an important neuronal loss in the SNpc, which was significantly recovered by THCV or CBD on their own, but not when administered together, reinforcing the idea of a crosstalk between their mechanisms, which causes a loss of the beneficial effects that are observed for each compound individually. Nevertheless, the reduction of autophagy dysfunction in these mice was not affected by this potential interaction, as both THCV, CBD and the combination group loss the statistical significance of LAMP-1 levels with respect to the vehicle-treated 6-OHDA-lesioned mice. The same result was obtained for CD68 and GFAP markers, as all the treated groups showed reduced neuroinflammation levels, so close to the sham group that no additive or synergistic effect between THCV and CBD would be possible. Taken these results together, we can conclude that the effects observed for the combined treatment in both models did not imply significant improvements, neither additive nor synergistic effects.

In order to deepen in the molecular mechanisms around the loss of protective effects shown by the combination of THCV and CBD we conducted additional experiment with the SH-SY5Y cell line. Once we established the optimal cytoprotective concentrations of THCV and CBD (100 and 500 nM, respectively) in the 6-OHDA *in vitro* model, we explored the combination of

both treatments. Similar to the *in vivo* model, the combination group lost the beneficial effects observed with each compound individually, using a variety of combination concentrations. We found that at lower concentrations, especially for THCV, we managed to avoid the loss of cytoprotective effect of THCV and CBD alone, but we could not reach a synergistic effect even at subeffective doses, as it has been described for other combination of phytocannabinoids (Stern et al., 2015). Next, given the involvement of CB2 and/or PPAR-γ in anti-inflammatory and neuroprotective effects of both THCV and CBD *in vivo* (Pertwee, 2008), we investigated whether the selective blockade of these receptors using SR144528 and/or T0070907, respectively, altered the effect of THCV, CBD or the combined treatment. We observed that both CB2 and PPAR-γ receptors have an important role in the effects of THCV, either combined or alone, as there was a partial reversal of its cytoprotective effects with the antagonists. By contrast, the use of SR144528 alone had no effect in reversing the CBD-induced cytoprotection. Therefore, our data support a higher relevance for PPAR-γ, as the effect of its blockade is maintained in the combined treatment group. These effects could be the consequence of a crosstalk between CB2 and PPAR-γ signalling which has been described for some cannabinoid compounds (Youssef et al., 2019). Thus, when we CB2 receptors are blocked, there can still be PPAR-γ signalling, but when we PPAR-γ receptors are blocked part of the signalling activated by CB2 receptors is lost (Picciolo et al., 2020). Moreover, CBD has been shown to indirectly modulate the ECS, leading to an increase in endocannabinoid levels, through inhibitory effects on the inactivation of endocannabinoids, which could facilitate cannabinoid signalling and indirectly activate CB2 receptors (De Petrocellis et al., 2011). Also, CBD has been proved to act as an allosteric modulator of both CB1 (Laprairie et al., 2015) and CB2 (Martinez-Pinilla et al., 2017), the main targets of THCV. Even outside the ECS, there could be other targets involved in the basal ganglia regulation, such as the 5-HT1A receptors, activated by both THCV (Morales et al., 2017) and CBD (Espejo-Porras et al., 2013). Therefore, there seems to be a complex interaction between the pharmacology of THCV and CBD, probably derived from their multi-target activities, and which may make them inadequate for obtaining an additive or synergistic effect. In our hand, it appears that these multitarget activities make more difficult any type of additive effects.

To conclude, we have confirmed the beneficial effects proposed for THCV (García et al., 2011; Espadas et al., 2020), and also added some others (e.g., effect on the hemiparesis of contralateral forepaw and autophagy dysfunction), as a pharmacological strategy for PD, although we could not confirm that such effects may be enhanced by the co-administration with CBD.

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CHAPTER 3

Preclinical evaluation of compounds targeting GPR55: VCE-006.1

In this last chapter, we investigated the potential of a synthetic cannabinoid, VCE-006.1, a GPR55 activator, in two animal models of PD, as well as in some cell-based models. In a complementary study in our research group, this compound was also tested in two transgenic models of amyotrophic lateral sclerosis (ALS).

In the 6-OHDA-lesioned mice, the treatment with VCE-006.1 reversed the motor impairments, and this was apparently caused by an effect of this compound reducing the neuronal death. VCE-006.1 also attenuated microglial and astroglial reactivity, but only partially restored autophagy dysfunction. Similar cytoprotective effects were found *in vitro* in SH-SY5Y cells exposed to 6-OHDA. The treatment with the GPR55 activator in LPS-lesioned mice showed some beneficial effects, but not for glial reactivity and inflammatory events. This lack of anti-inflammatory potential was confirmed in BV2 cells exposed to LPS and treated with VCE-006.1. GPR55 gene expression was analysed and remained unaltered in both models, although GPR55 was downregulated in BV2 cells treated with LPS, which may explain the lack of efficacy of VCE-006.1 in such an assay. The results obtained in ALS confirmed these results, as neither the neurological impairments not he microgliosis and astrogliosis were improved by the treatment with VCE-006.1, albeit modest spinal motor neuron preservation was achieved in both models. Thus, our findings support the view that targeting the GPR55 may afford neuroprotection in experimental PD, but without anti-inflammatory effects.

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Preclinical Investigation in Neuroprotective Effects of the GPR55 Ligand VCE-006.1 in Experimental Models of Parkinson's Disease and Amyotrophic Lateral Sclerosis

Sonia Burgaz [1,2,3], Concepción García [1,2,3], Claudia Gonzalo-Consuegra [1,2,3], Marta Gómez-Almería [1,2,3], Francisco Ruiz-Pino [4], Juan D. Unciti [4], María Gómez-Cañas [1,2,3], Juan Alcalde [1], Paula Morales [5], Nadine Jagerovic [5], Carmen Rodríguez-Cueto [1,2,3], Eva de Lago [1,2,3], Eduardo Muñoz [4,6,7,8] and Javier Fernández-Ruiz [1,2,3,*]

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[1] Instituto Universitario de Investigación en Neuroquímica, Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense, 28040 Madrid, Spain; soniabur@ucm.es (S.B.); conchig@med.ucm.es (C.G.); clagon11@ucm.es (C.G.-C.); margom27@ucm.es (M.G.-A.); mgc@med.ucm.es (M.G.-C.); jualcald@ucm.es (J.A.); carc@med.ucm.es (C.R.-C.); elagofem@med.ucm.es (E.d.L.)
[2] Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), 28040 Madrid, Spain
[3] Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), 28040 Madrid, Spain
[4] Emerald Health Biotechnology España, 14014 Córdoba, Spain; b62rupif@uco.es (F.R.-P.); jdunciti@gmail.com (J.D.U.); fi1muble@uco.es (E.M.)
[5] Instituto de Química Médica, CSIC, 28006 Madrid, Spain; paula.morales@iqm.csic.es (P.M.); nadine@iqm.csic.es (N.J.)
[6] Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), 14004 Córdoba, Spain
[7] Department of Cellular Biology, Physiology and Immunology, University of Córdoba, 14071 Córdoba, Spain
[8] Hospital Universitario Reina Sofía, 14004 Córdoba, Spain
* Correspondence: jjfr@med.ucm.es; Tel.: +34–913941450

Abstract: Cannabinoids act as pleiotropic compounds exerting, among others, a broad-spectrum of neuroprotective effects. These effects have been investigated in the last years in different preclinical models of neurodegeneration, with the cannabinoid type-1 (CB_1) and type-2 (CB_2) receptors concentrating an important part of this research. However, the issue has also been extended to additional targets that are also active for cannabinoids, such as the orphan G-protein receptor 55 (GPR55). In the present study, we investigated the neuroprotective potential of VCE-006.1, a chromenopyrazole derivative with biased orthosteric and positive allosteric modulator activity at GPR55, in murine models of two neurodegenerative diseases. First, we proved that VCE-006.1 alone could induce ERK1/2 activation and calcium mobilization, as well as increase cAMP response but only in the presence of lysophosphatidyl inositol. Next, we investigated this compound administered chronically in two neurotoxin-based models of Parkinson's disease (PD), as well as in some cell-based models. VCE-006.1 was active in reversing the motor defects caused by 6-hydroxydopamine (6-OHDA) in the pole and the cylinder rearing tests, as well as the losses in tyrosine hydroxylase-containing neurons and the elevated glial reactivity detected in the substantia nigra. Similar cytoprotective effects were found in vitro in SH-SY5Y cells exposed to 6-OHDA. We also investigated VCE-006.1 in LPS-lesioned mice with similar beneficial effects except against glial reactivity and associated inflammatory events, which remained unaltered, a fact confirmed in BV2 cells treated with LPS and VCE-006.1. We also

analyzed GPR55 in these in vivo models with no changes in its gene expression, although GPR55 was down-regulated in BV2 cells treated with LPS, which may explain the lack of efficacy of VCE-006.1 in such an assay. Furthermore, we investigated VCE-006.1 in two genetic models of amyotrophic lateral sclerosis (ALS), mutant SOD1, or TDP-43 transgenic mice. Neither the neurological decline nor the deteriorated rotarod performance were prevented with this compound, and the same happened with the elevated microglial and astroglial reactivities, albeit modest spinal motor neuron preservation was achieved in both models. We also analyzed GPR55 in these in vivo models and found no changes in both TDP-43 transgenic and mSOD1 mice. Therefore, our findings support the view that targeting the GPR55 may afford neuroprotection in experimental PD, but not in ALS, thus stressing the specificities for the development of cannabinoid-based therapies in the different neurodegenerative disorders.

Keywords: cannabinoids; GPR55 receptors; VCE-006.1; chromenopyrazole; Parkinson's disease; 6-hydroxydopamine; lipopolysaccharide; amyotrophic lateral sclerosis; mSOD1 mice; TDP-43 transgenic mice

1. Introduction

Phytocannabinoids, the active constituents of the *Cannabis* plant, as well as endocannabinoids and synthetic cannabinoids, have been proposed as promising neuroprotective agents in accidental brain damage (e.g., stroke, brain trauma, spinal injury) and in chronic progressive disorders (e.g., Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Huntington's disease, and others) [1-3]. This potential derives from their pleiotropism and ability to activate numerous cytoprotective targets within the endocannabinoid system, but also outside this signaling system [3]. An important part of these neuroprotective properties described for cannabinoids have been related to the activation of the type-1 cannabinoid (CB_1) receptor [1,2]. This receptor is predominantly located in neurons in the CNS, which facilitates its role in the control of excitotoxic damage in glutamatergic synapses [4], as well as a possible contribution in the autophagy-mediated elimination of protein aggregates [5]. Data supporting CB_1 receptor-mediated neuroprotective effects have been collected in experimental models of Alzheimer's disease [6-8], PD [9,10], ALS [11-13], Huntington's disease [4,14-16], and multiple sclerosis [17,18].

Important neuroprotective effects have also been described for the activation of the type-2 cannabinoid (CB_2) receptor [1-3,19]. This receptor is predominantly located in activated astrocytes and reactive microglial cells in the CNS of neuroinflammatory/neurodegenerative conditions, in which it becomes significantly up-regulated with the purpose to control glial toxicity for neurons as well as other beneficial effects [1,19]. Data supporting CB_2 receptor-mediated neuroprotective effects have been collected in experimental models of Alzheimer's disease and related dementias [7,20-23], PD [7,24-27], ALS [28-32], Huntington's disease [33-35], and multiple sclerosis [36-38].

These broadly-demonstrated neuroprotective effects of cannabinoids have also been extended to additional targets, within or outside the endocannabinoid system, which are also active for cannabinoids [3]. This includes, for example, the nuclear receptors of the peroxisome proliferator-activating receptor (PPAR) family, which have been investigated for their role in the control of inflammatory/neurodegenerative events [39,40] in experimental PD [41-44], and, to a lower extent, in experimental ALS [45] and Alzheimer's disease [46,47]. More recent data have indicated the

orphan G-protein receptor 55 (GPR55) as an additional neuroprotective and anti-inflammatory target [48-50]. This has been investigated mainly in PD given the abundant presence of GPR55 receptors in the basal ganglia [51,52] and the important motor impairment found in mice lacking GPR55 [53].

GPR55 receptor was considered for years as an orphan receptor, but some recent evidence has positioned this receptor as a possible new cannabinoid receptor type [54]. However, such an assumption has been controversial due to the important differences in homology, conformational structure, pharmacology, signaling, and functional relevance shown by GPR55 compared to classic CB_1 and CB_2 receptors [55-57]. The human GPR55 protein has 319 amino acids and is also a member of the rhodopsin-like 7TM/GPCR family [55,57]. It was isolated and cloned in 1999, when it was found to be located in chromosome 2 (2q37) in humans [58]. Its naturally-occurring ligand is lysophosphatidyl inositol (LPI) [59]. Its pharmacology is complex and still remains to be clarified, including some non-cannabinoid compounds that do not bind CB_1/CB_2 receptors (e.g., GSK-494,581, CID-16020046 [60]), but also certain phytocannabinoids (e.g., cannabidiol), endocannabinoids (e.g., anandamide, 2-arachidonoylglycerol) and synthetic cannabinoids (e.g., WIN 55,212-2, HU-210, SR141716, AM251, methanandamide), which may also be active at other cannabinoid receptors [61,62]. GPR55 is widely distributed in the CNS, in particular in the basal ganglia, hippocampus, thalamus, and cerebellum [63], and is also present in the periphery (e.g., vasculature, gastrointestinal tract, bones, lung, spleen, liver, kidney, uterus) [64]. This distribution has prompted research on this receptor in relation to pathogenesis and/or development of novel therapies against different central and peripheral pathologies, including, as mentioned above, neurodegenerative disorders for which targeting GPR55 has been proposed as a promising anti-inflammatory and neuroprotective strategy [48-51].

In the present study, we have further investigated the neuroprotective potential of this new target for cannabinoids, using VCE-006.1, a chromenopyrazole derivative designed, synthesized, and investigated as GPR55 ligand in a previous study of our group [65]. VCE-006.1 is the compound 2-[2-(4-cyclohexylcarbonylpiperazinyl)ethyl]-2,4-dihydro-7-methoxy-4,4-dimethylchromeno[4,3-c]pyrazole (compound 23 in [65]), which showed affinity at the GPR55 receptor analyzed in a label-free cell-impedance-based assay in hGPR55-HEK293 cells, whereas having negligible or poor affinity for the CB_1 and CB_2 receptor (as measured in competitive radioligand assays), respectively [65]. The patent generated with this and other similar compounds [66] was acquired by the company Emerald Health Biotechnology-Spain in 2018, and the compound was renamed as VCE-006.1. In this study, we have extended the analysis of its activity at the GPR55 receptor, using several cell-based assays, which has situated this compound as a potential biased positive allosteric modulator (PAM) for the GPR55 receptor. Next, we have investigated its neuroprotective profile in vitro (cell-based assays) and in vivo (neurotoxin-based models or genetically-modified mice) models of two neurodegenerative diseases, PD and ALS, in which the potential of GPR55 as a neuroprotective target has been claimed [32,51].

2. Results

2.1. Studies on PAM Activity of VCE-006.1

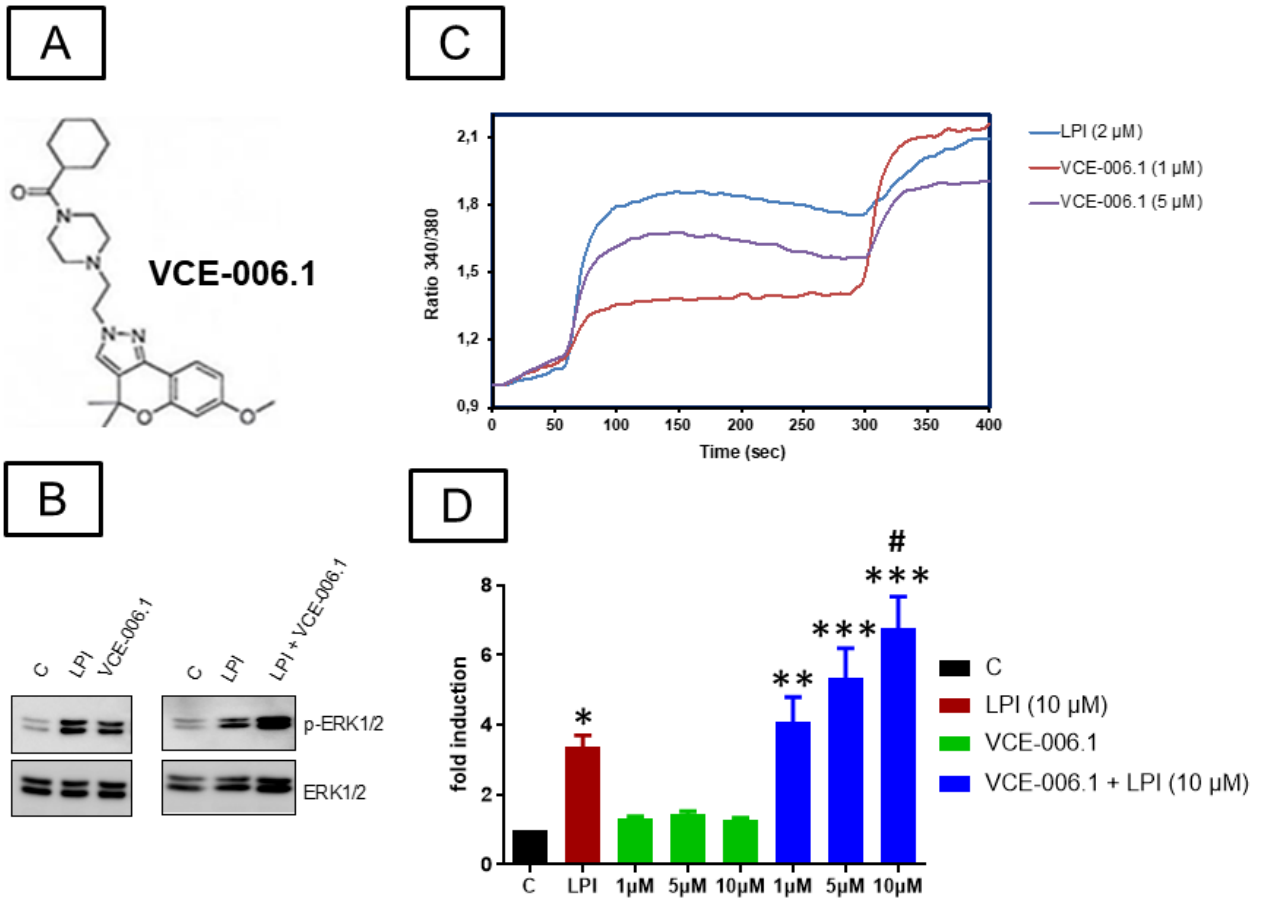

Figure 1. (**A**) Chemical structure of VCE-006.1. (**B**) VCE-006.1 and LPI induces ERK1/2 activation in DU145 cells. The cells were stimulated as indicated and the expression of phospho-ERK1/2 and total ERK1/2 determined by immunoblots. (**C**) VCE-006.1 and LPI induces [Ca^{2+}] immobilization in U937 cells. U937 cells were loaded with Indo1-AM, treated with the compounds, and the calcium mobilization was measured by ratiometric fluorescence as indicated under Materials and Methods. (**D**) GPR55 activity of VCE-006.1 at different concentrations (1, 5, and 10 μM) in the absence or the presence of 10 μM LPI on HEK293T-GPR55-CRE-luc cells. Results are expressed as the fold induction of GPR55 activity and represent means ± SEM of data generated in 6 independent experiments, each conducted in triplicates. Statistical significance was determined by one-way ANOVA followed by the Tukey test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ vs. control (basal) and VCE-006.1 alone; $^{\#}p < 0.05$ vs. LPI and VCE-006.1 (1μM) + LPI).

Our first objective was to further explore the activity of VCE-006.1 (see chemical structure in Figure 1A) at the GPR55. Previous studies [65] have indicated VCE-006.1 to be a selective ligand of this receptor with activity as a partial agonist and having no relevant affinity at the classic CB_1 and CB_2 receptors tested in competitive radioligand binding assays. Here, we have explored canonical GPR55 signalling pathways in cells expressing the native receptor (DU145 and U937 cells) and in cells overexpressing the receptor (HEK-293-GPR55 cells). We found that both LPI and VCE-006.1 induced ERK1/2 phosphorylation in DU145 cells and that a combination of both further increased this phosphorylation (Figure 1B). Ca^{2+} mobilization in response to VCE-006.1 and LPI was studied in U937 cells, and as depicted in Figure 1C, both compounds were able to induce Ca^{2+} mobilization, with LPI being more potent than VCE-006.1, suggesting a different mode of action for each

compound. Next, we stimulated HEK293-GPR55-CRE-Luc cells with either VCE-006.1 or LPI, separately or in combination, and the luciferase activity was measured as indicative of cAMP induction. VCE-006.1 did not induce CRE-Luc activity but significantly enhanced the effect of LPI as a potential orthosteric ligand ($F(7,40) = 17.36$, $p < 0.0001$; Figure 1D). Altogether, our results showed that VCE-006.1 activated GPR55 in a biased manner compared to LPI, showing characteristics of both partial orthosteric agonist and PAM depending on the specific cell assay used.

2.2. Studies in Experimental PD

Our second objective was to investigate this compound when administered chronically in two neurotoxin-based models of PD, as well as in some cell-based models of this disease. We first used a classic PD model of mitochondrial damage, 6-OHDA-lesioned mice, which proved the expected hemiparesis in the cylinder rearing test (Figure 2A) and an elevated latency to descend in the pole test (Figure 2B). VCE-006.1 was active in reversing these motor defects caused by 6-OHDA in the cylinder rearing test ($F(3,29) = 17.49$, $p < 0.0001$; Figure 2A) and in the pole test ($F(3,27) = 8.803$, $p < 0.0005$; Figure 2B), effects evident in 6-OHDA-lesioned mice, but absent in sham-operated mice.

These benefits with VCE-006.1 were associated with a reduction in the loss of TH-containing neurons caused by a 6-OHDA lesion in the substantia nigra ($F(3,27) = 25.57$, $p < 0.0001$; Figure 3A,B). The 6-OHDA lesion also caused a modest elevation of LAMP-1 immunostaining, a marker of autophagy, which was attenuated by the treatment with VCE-006.1 ($F(3,29) = 4.77$, $p < 0.01$; Figure 3C,D). Our histological analysis of the substantia nigra also proved an elevated glial reactivity detected in this structure when lesioned with 6-OHDA, visible for Cd68 immunolabelling (reflecting reactive microgliosis) and with GFAP immunostaining. Both responses were notably attenuated by the treatment with VCE-006.1 (Cd68: $F(3,29) = 15.43$, $p < 0.0001$; Figure 4A,B; GFAP: $F(3,29) = 22.72$, $p < 0.0001$; Figure 4C,D). VCE-006.1 had no effect on these markers in sham-operated mice.

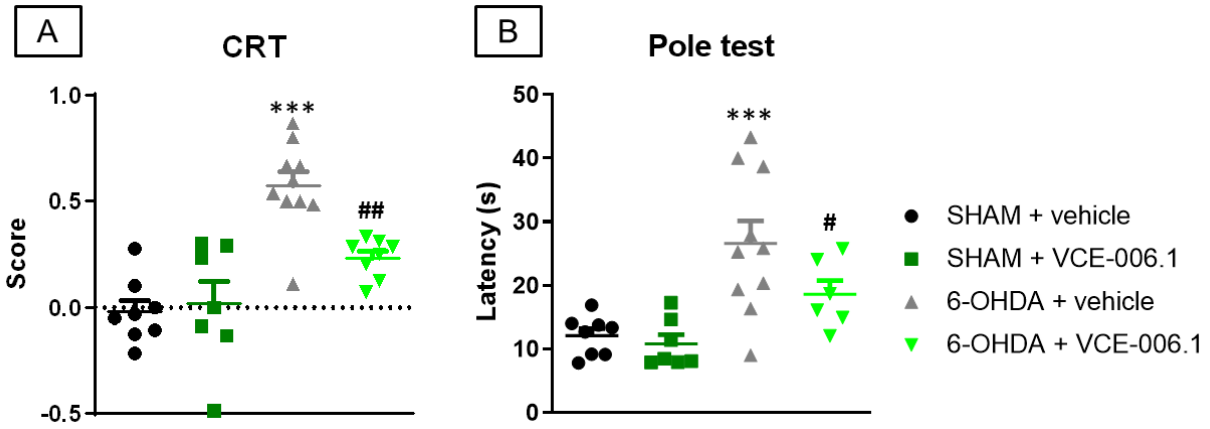

Figure 2. Response in the cylinder rearing test (**A**) and in the pole test (**B**) of male mice subjected to unilateral 6-OHDA lesions or sham-operated and daily treated with VCE-006.1 (20 mg/kg, i.p.) for 2 weeks. Values are means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Tukey test (***$p < 0.005$ vs. the two sham-operated groups; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the vehicle-treated 6-OHDA lesioned mice).

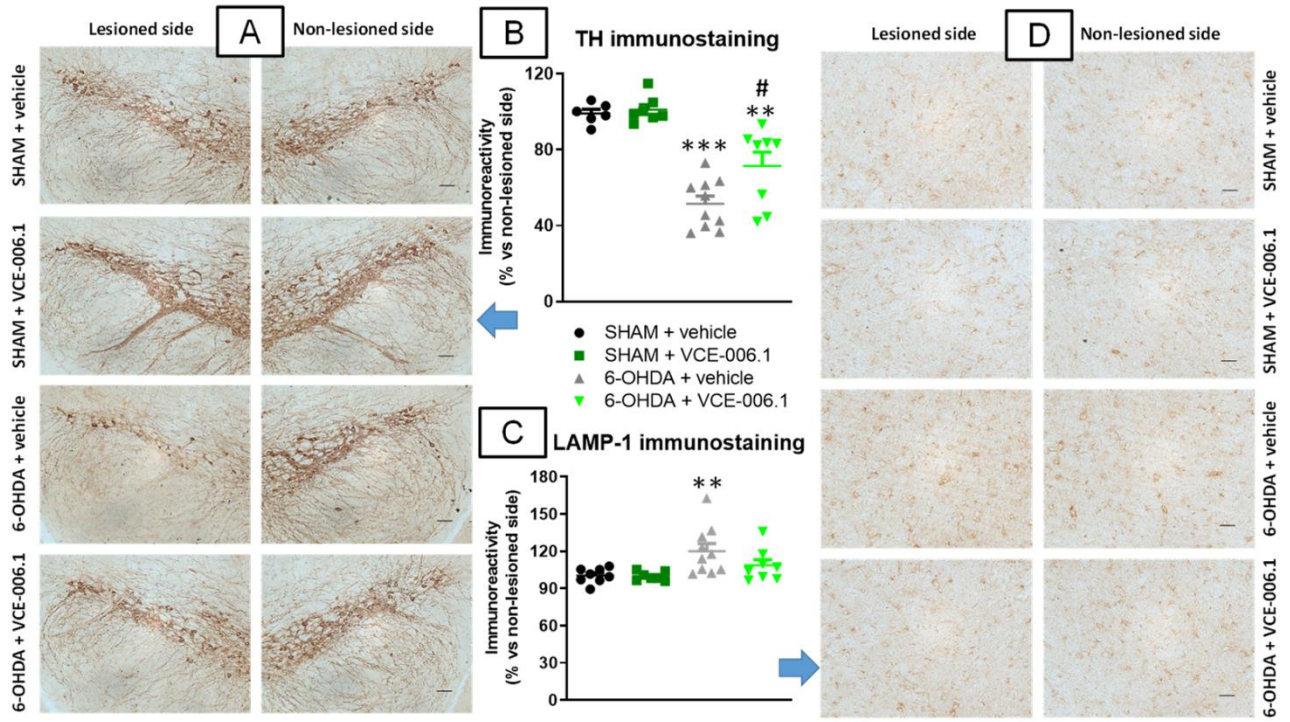

Figure 3. Quantification of TH (**B**) and LAMP-1 (**C**) immunoreactivities, including representative images (**A**) (TH; scale bar = 100 μm) and (**D**) (LAMP-1; scale bar = 50 μm)), measured in a selected area of the substantia nigra pars compacta of male mice subjected to unilateral 6-OHDA lesions or sham-operated and daily treated with VCE-006.1 (20 mg/kg, i.p.) for 2 weeks. Values correspond to % of the ipsilateral lesioned side vs. contralateral non-lesioned side and are expressed as means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Tukey test (**$p < 0.01$, ***$p < 0.005$ vs. the two sham-operated groups; $^{\#}p < 0.05$ vs. the vehicle-treated 6-OHDA lesioned mice).

In a second experiment, we investigated whether VCE-006.1 also exerts similar cytoprotective effects in vitro in SH-SY5Y cells, which express GPR55 [67], exposed to 6-OHDA. Our data revealed that 6-OHDA reduced cell viability up to close to 50% in these cells, which was attenuated by VCE-006.1 in a concentration-related manner with a maximum at 1 μM ($F(6,40) = 40.80$, $p < 0.0001$), lower effects at higher concentrations (5 and 10 μM), and no effect at 20 μM (Figure 5).

Next, we also investigated VCE-006.1 in an inflammatory model of PD, LPS-lesioned mice, having relatively similar beneficial effects. Again, LPS-lesioned mice exhibited motor defects in the cylinder rearing test (hemiparesis) and in the pole test (elevated latency to descend the pole), which were attenuated by the treatment with VCE-006.1 (CRT: $F(2,17) = 9.34$, $p < 0.005$; Figure 6A; pole test: $F(2,19) = 11.75$, $p < 0.0005$; Figure 6B). These benefits of VCE-006.1 on the neurological state of LPS-lesioned mice were again accompanied by higher survival or TH-positive neurons in the substantia nigra ($F(2,19) = 3.45$, $p < 0.05$; Figure 7A,B), an effect that was modest and reflected in the loss of statistically significant differences vs. sham-operated animals.

However, this effect, surprisingly, was not accompanied by a reduction in the LPS-induced elevation of the autophagy marker LAMP-1 ($F(2,19) = 42.56$, $p < 0.0001$; Figure 7C). The same happened with the reactive microgliosis (elevated Cd68 immunoreactivity; $F(2,19) = 45.80$, $p < 0.0001$; Figure 7D) and astroglial reactivity (elevated GFAP immunolabelling; $F(2,19) = 69.94$, $p < 0.0001$; Figure 7E), which remained elevated in LPS-lesioned mice irrespective of VCE-006.1 treatment.

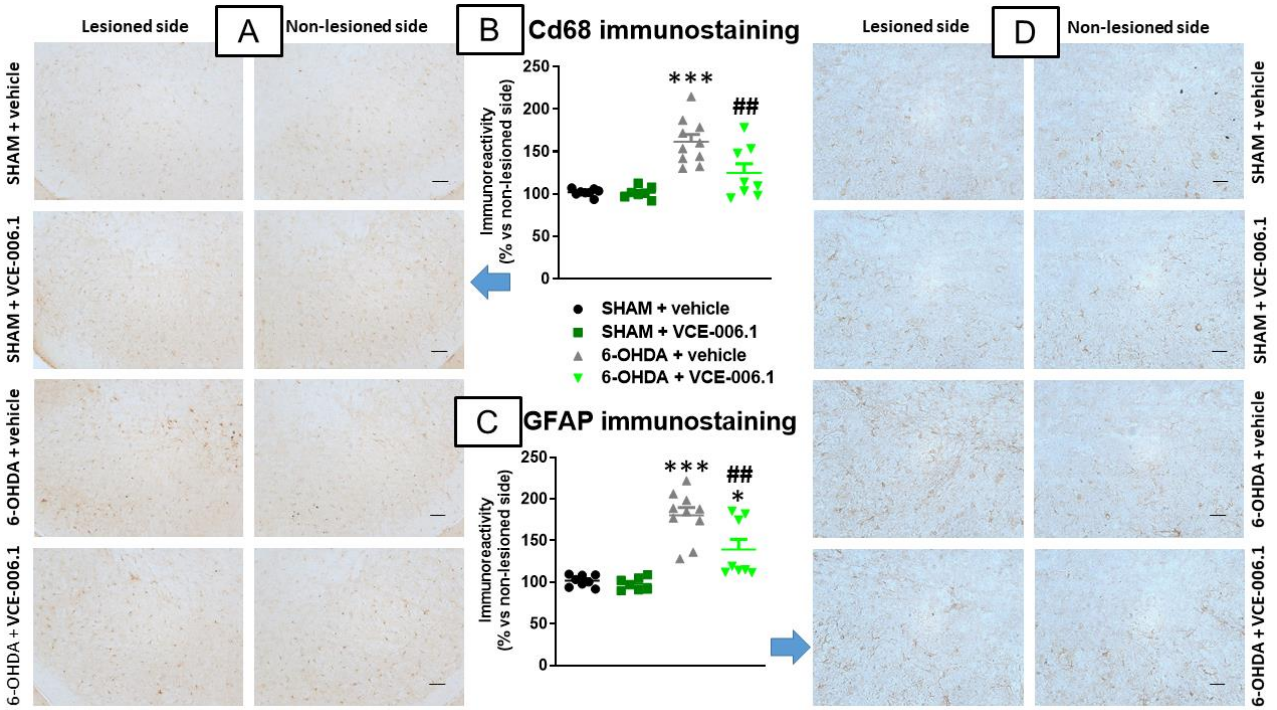

Figure 4. Quantification of Cd68 (**B**) and GFAP (**C**) immunoreactivities, including representative images (**A**)(Cd68; scale bar = 100 μm) and (**D**) (GFAP; scale bar = 50 μm)), measured in a selected area of the substantia nigra pars compacta of male mice subjected to unilateral 6-OHDA lesions or sham-operated and daily treated with VCE-006.1 (20 mg/kg, i.p.) for 2 weeks. Values correspond to % of the ipsilateral lesioned side vs. contralateral non-lesioned side and are expressed as means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Tukey test (*$p < 0.05$, ***$p < 0.005$ vs. the two sham-operated groups; $^{\#\#}p < 0.01$ vs. the vehicle-treated 6-OHDA lesioned mice).

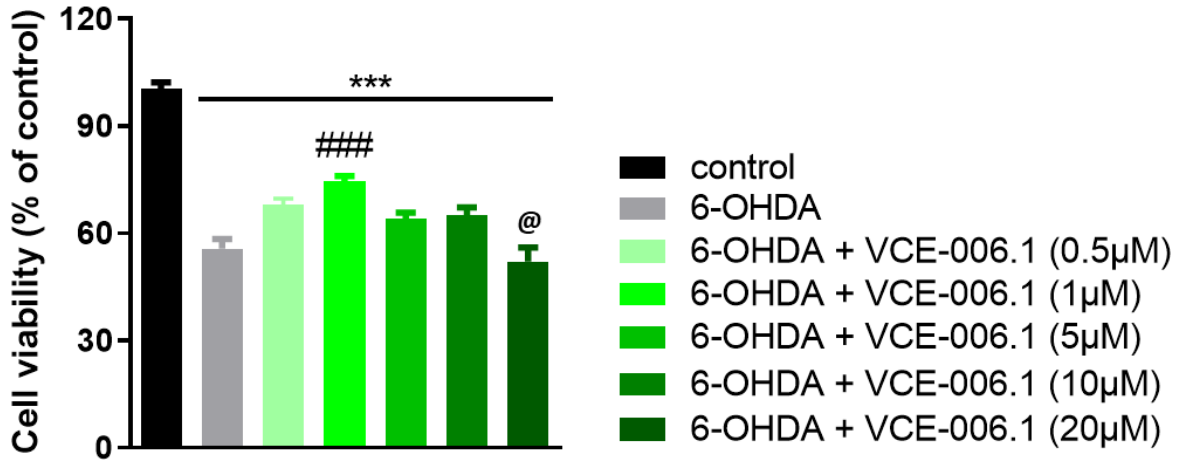

Figure 5. Cell viability measured with the MTT assay in cultured SH-SY5Y cells at 24 h to be treated with different concentrations of VCE-006.1 (0.5, 1, 2, 5, 10, and 20 μM) against 6-OHDA (200 μM). In all cases, a group with cells exposed to vehicle was also included to determine the 100% of cell viability. Values are means ± SEM of at least 4 independent experiments, each performed in triplicate. Data were assessed by the one-way ANOVA followed by the Tukey (***$p < 0.005$ vs. control cells; $^{\#\#\#}p < 0.005$ vs. cells exposed to 6-OHDA + vehicle; $^{@}p < 0.05$ vs. cells treated with the other VCE-006.1 concentrations).

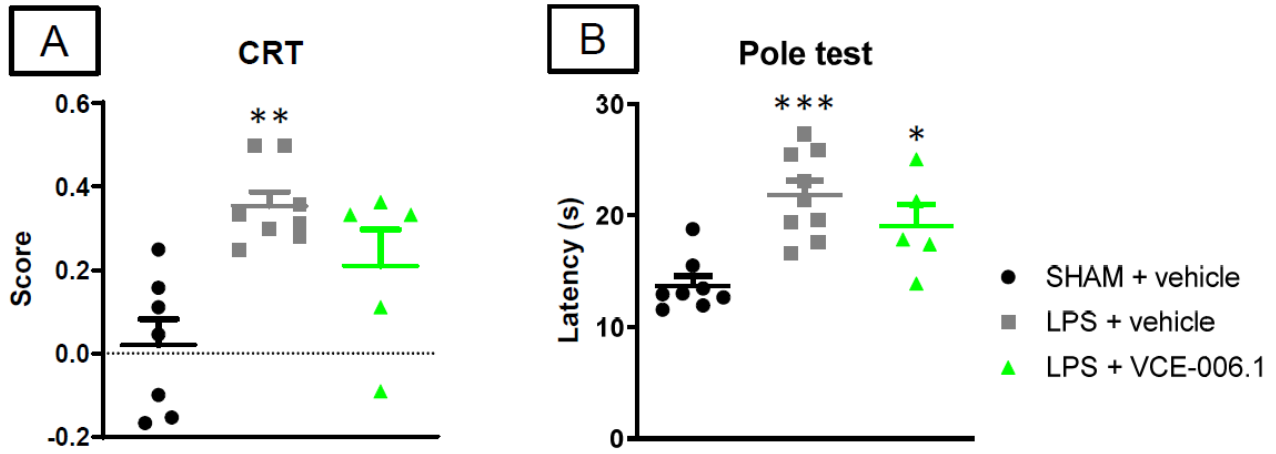

Figure 6. Response in the cylinder rearing test (**A**) and in the pole test (**B**) of male mice subjected to unilateral LPS lesions or sham-operated and daily treated with VCE-006.1 (20 mg/kg, i.p.) for 2 weeks. Values are means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Tukey test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ vs. the two sham-operated groups).

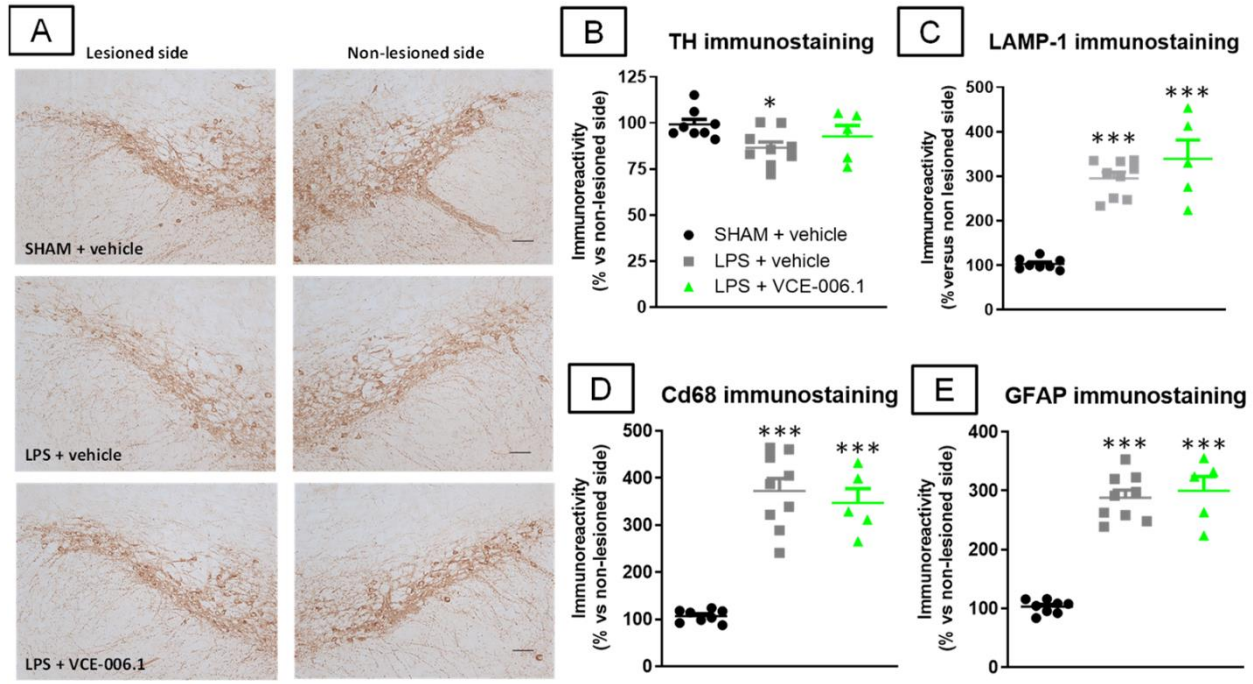

Figure 7. Quantification of TH (**B**), LAMP-1 (**C**), Cd68 (**D**), and GFAP (**E**) immunoreactivities, including representative images for TH immunostaining ((**A**); scale bar = 100 μm), measured in a selected area of the substantia nigra pars compacta of male mice subjected to unilateral LPS lesions or sham-operated and daily treated with VCE-006.1 (20 mg/kg, i.p.) for 2 weeks. Values correspond to % of the ipsilateral lesioned side vs. contralateral non-lesioned side and were expressed as means ± SEM of more than 5 animals per group. Data were assessed by one-way ANOVA followed by the Tukey test (*$p < 0.05$, ***$p < 0.005$ vs. the two sham-operated groups).

Such absence of VCE-006.1 effects against glial reactivity was also evident against some associated inflammatory events elicited by LPS lesion, for example the elevated gene expression detected in the striatum in proinflammatory cytokines TNF-α ($F(2,18) = 33.34$, $p < 0.0001$; Figure 8A) and IL-1β ($F(2,18) = 9.41$, $p < 0.005$; Figure 8B), as well as in proinflammatory enzymes iNOS ($F(2,16) = 4.24$, $p < 0.05$; Figure 8C) and COX-2 ($F(2,17) = 9.13$, $p < 0.005$; Figure 8D), which remained unaltered

after VCE-006.1 treatment. This was also evident for the LPS-induced reduction in the CB_1 receptor ($F(2,18) = 28.63$, $p < 0.0001$; Figure 8E), elevation of the CB_2 receptor ($F(2,18) = 31.31$, $p < 0.0001$; Figure 8F), and no effect in PPAR-γ ($F(2,18) = 1.14$, ns; Figure 8G).

Lastly, the absence of VCE-006.1 effects against glial reactivity and associated inflammatory events detected in LPS-lesioned mice was also confirmed in BV2 cells (which also express GPR55 [68]) treated with LPS and VCE-006.1, as the elevated levels of gene expression detected for TNF-α ($F(2,15) = 15.14$, $p < 0.0005$; Figure 9A) and IL-1β ($F(2,15) = 12.21$, $p < 0.001$; Figure 9B) after LPS again remained unaltered by the treatment with VCE-006.1. This may be related to the strong reduction in GPR55 mRNA levels found in BV2 cells treated with LPS in the absence or presence of VCE-006.1 in comparison with control cells ($F(2,15) = 10.53$, $p < 0.005$; Figure 9C). However, the analysis of gene expression for GPR55 in the striatum of LPS-lesioned mice proved no changes in this receptor ($F(2,18) = 0.57$, ns; Figure 9D), and the same happened in 6-OHDA-lesioned mice ($F(3,22) = 0.65$, ns; Figure 9E).

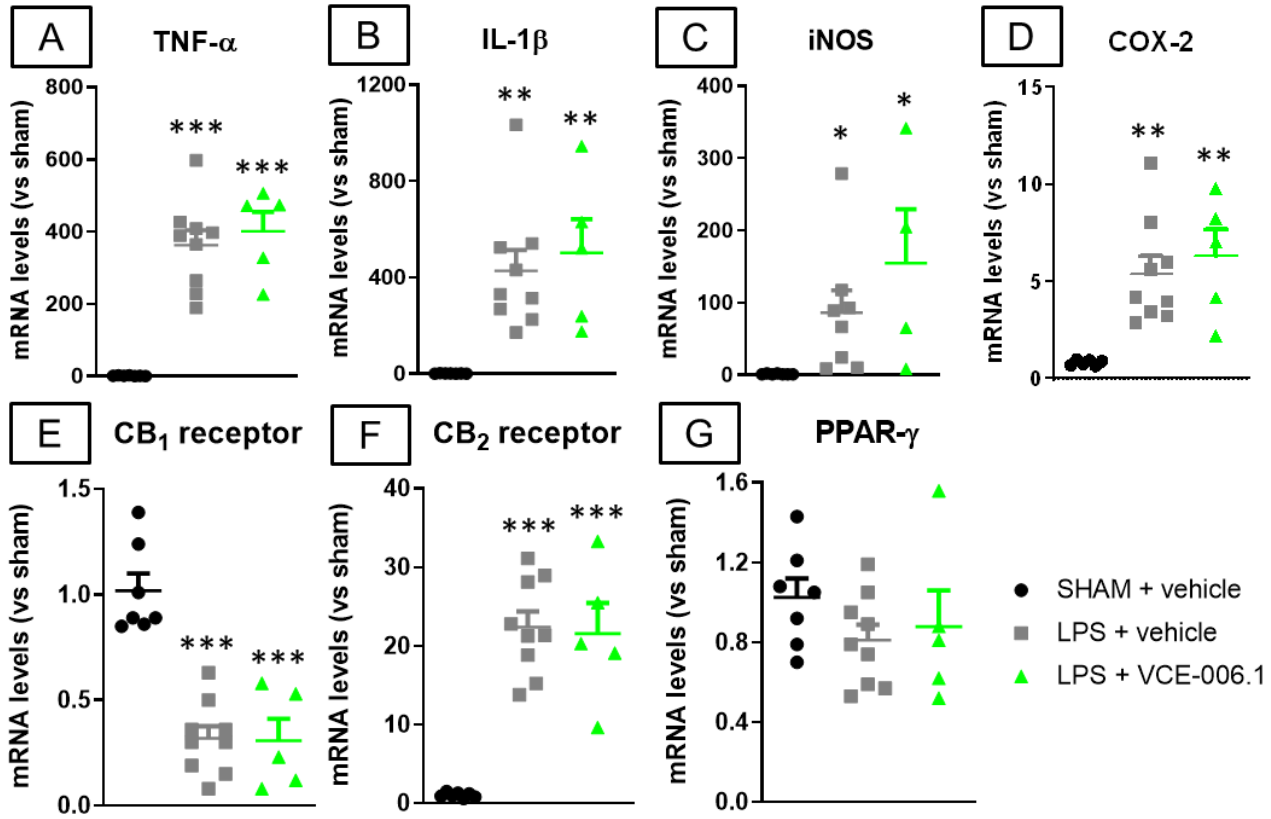

Figure 8. mRNA levels for TNF-α (**A**), IL-1β (**B**), iNOS (**C**), COX-2 (**D**), CB_1 receptor (**E**), CB_2 receptor (**F**), and PPAR-γ (**G**) measured by qPCR in the striatum of male mice subjected to unilateral LPS lesions or sham-operated and daily treated with VCE-006.1 (20 mg/kg, i.p.) for 2 weeks. GAPDH was used as an endogenous reference gene for data normalization. Values correspond to fold of change vs. sham-operated controls and are expressed as means ± SEM of more than 5 animals per group. Data were assessed by one-way ANOVA followed by the Tukey test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ vs. the two sham-operated groups).

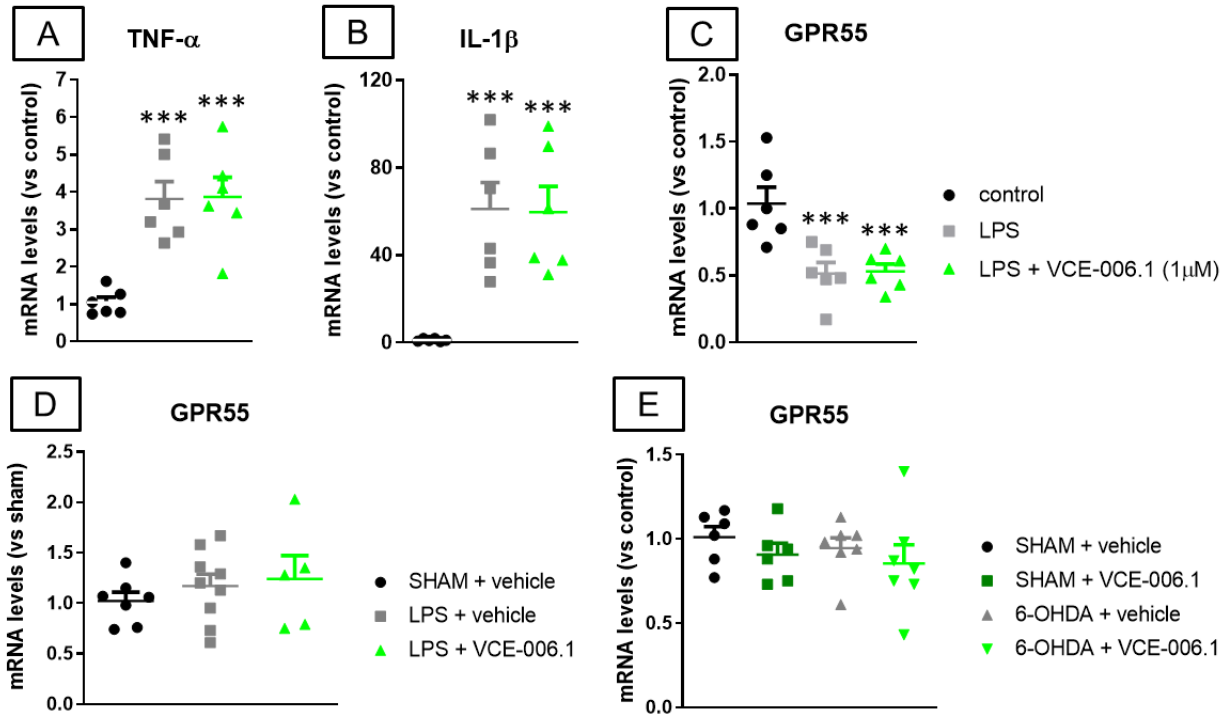

Figure 9. mRNA levels for TNF-α (**A**), IL-1β (**B**), and GPR55 (**C**) measured by qPCR in BV2 cells exposed to LPS and/or VCE-006.1 (1 μM) for 20 hours, and mRNA levels for GPR55 measured by qPCR in the striatum of male mice subjected to unilateral 6-OHDA (**D**) or LPS (**E**) lesions or sham-operated and daily treated with VCE-006.1 (20 mg/kg, i.p.) for 2 weeks. In all cases, GAPDH was used as an endogenous reference gene for data normalization, and values correspond to fold change vs. controls and are expressed as means ± SEM of more than 5 animals per group. Data were assessed by one-way ANOVA followed by the Tukey test (***p < 0.005 vs. the control group).

2.3. Studies in Experimental ALS

Our third objective was to investigate VCE-006.1 when administered chronically in two genetic murine models of ALS. We first used the classic mSOD-1 model which showed several motor abnormalities such as: (i) a progressive reduction in the time on wire (2-way interaction: F(10,155) = 13.25, p < 0.0001; Figure 10A) visible in the hanging wire test; (ii) a progressively marked deterioration in the rotarod performance (2-way interaction: F(18,270) = 15.43, p < 0.0001; Figure 10B) detected in the rotarod test; and (iii) a rapid elevation in a specific neurological score for ALS signs recapitulated in mice (2-way interaction: F(18,288) = 10.23, p < 0.0001; Figure 10C). VCE-006.1 was not active against any of these neurological decline signs, then indicating no effects at the functional level. However, the strong loss of Nissl-stained motor neurons visible in the ventral horn of the spinal cord (lumbar levels) in mSOD-1 mice was partially attenuated by the chronic treatment with VCE-006.1 (F(2,31) = 98.79, p < 0.0001; Figure 10D,E), although this does not have any influence on possible neurological recoveries as seen in the above behavioural data. This may be in part related to the persistence of higher levels of glial reactivity in the ventral horn of the spinal cord (lumbar levels) in mSOD-1 mice after the treatment with VCE-006.1 (GFAP immunolabelling: F(2,30) = 53.34, p < 0.0001; Figure 11A,B); Iba-1 immunolabelling: F(2,31) = 62.56, p < 0.0001; Figure 11C,D), which were similar to mSOD-1 mice treated with vehicle.

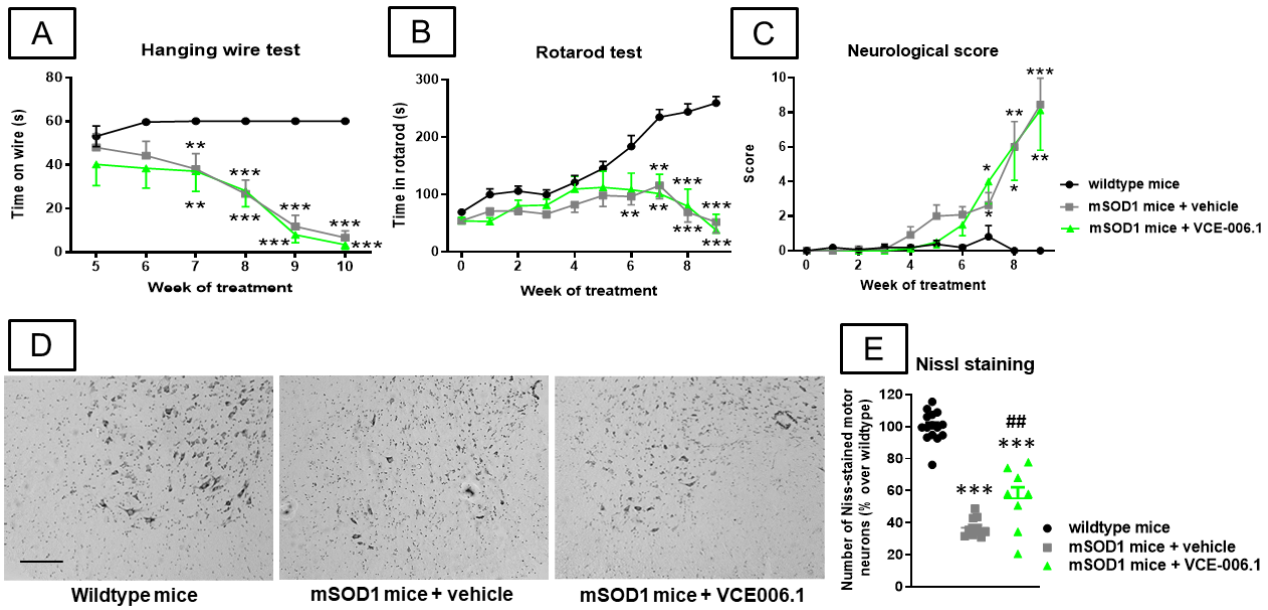

Figure 10. Hanging wire response (**A**), rotarod performance (**B**), and neurological score (**C**), analyzed mSOD1 transgenic and wild-type male mice at specific weeks during a chronic treatment from 63 day-old to 125 day-old with VCE-006.1 (20 mg/kg, daily and i.p.) or vehicle, and quantification of the number of Nissl-stained motor neurons (**E**), including representative images ((**D**); scale bar = 100 μm), in the lumbar ventral horn (marked with a dotted line) of the spinal cord in all experimental groups after the chronic treatment. Values are means ± SEM of more than 6 animals per group. Behavioral data were assessed by two-way ANOVA (with repeated measures), whereas Nissl staining data were assessed by one-way ANOVA, in both cases followed by the Tukey test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ vs. wild-type mice; $^{\#\#}p < 0.01$ vs. mSOD1 mice treated with vehicle).

Next, we investigated the same issue in an alternative and more recent ALS model based on the RNA-binding protein TDP-43. Again, TDP-43 transgenic mice showed several motor abnormalities such as: (i) a progressively higher clasping response (2-way interaction: $F(8,88) = 4.50$, $p < 0.0001$; Figure 12A); and (ii) a progressively marked deterioration in the rotarod performance (2-way interaction: $F(8,88) = 4.46$, $p < 0.0001$; Figure 12B) detected in the rotarod test. Again, VCE-006.1 was not active against any of these motor signs, then indicating no effects at the functional level, despite the strong loss of Nissl-stained motor neurons visible in the ventral horn of the spinal cord (lumbar levels) in TDP-43 transgenic mice was partially attenuated by the chronic treatment with VCE-006.1 ($F(2,21) = 82.28$, $p < 0.0001$; Figure 12C,D).

Again, we may attribute this effect in part to the persistence of higher levels of glial reactivity in the ventral horn of the spinal cord (lumbar levels) in TDP-43 transgenic mice after the treatment with VCE-006.1 (GFAP immunolabelling: $F(2,21) = 21.08$, $p < 0.0001$; Figure 13A,B); Iba-1 immunolabelling: $F(2,20) = 8.82$, $p < 0.005$; Figure 13C,D), which were similar to TDP-43 transgenic mice.

Lastly, as in the experimental models of PD, we also analyzed GPR55 gene expression in these in vivo ALS models. Our data indicated that GPR55-mRNA levels did not experience any changes in the case of mSOD1 mice compared to wild-type animals when analyzed at a late symptomatic phase (123 days; Figure 14A), and the same happened with TDP-43 transgenic mice at two specific

ages: 65 (early symptomatic stage; Figure 14B) and 105 days (advanced symptomatic phase; Figure 14C).

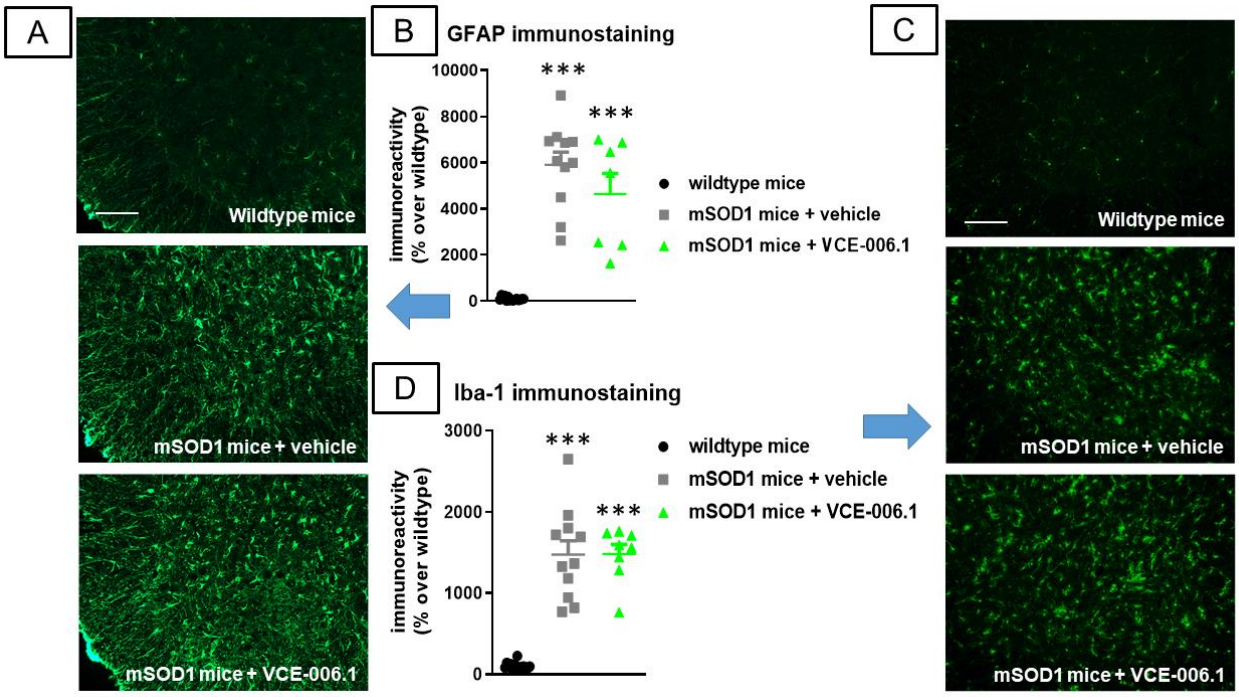

Figure 11. Quantification of GFAP (**B**) and Iba-1 (**D**) immunoreactivities, including representative images ((**A**) and (**C**), respectively; scale bar = 100 µm), in the lumbar ventral horn (marked with a dotted line) of the spinal cord in wild-type and mSOD1 transgenic mice after a chronic treatment from 63 day-old to 125 day-old with VCE-006.1 (20 mg/kg, daily and i.p.) or vehicle. Values are means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Tukey test (***$p < 0.005$ vs. wild-type mice).

3. Discussion

The orphan receptor GPR55 has emerged in the last years as a potential new component of the endocannabinoid signaling system [54], despite its differences with the classic CB_1 and CB_2 receptors [55-57], as well as a promising neuroprotective target for the development of novel therapies for neurodegenerative conditions [48-52]. One of the key areas, involving GPR55 activity in the CNS, is the control of movement and motor coordination, which is supported by the fact that motor-related areas (e.g., basal ganglia, cerebellum) are within the CNS structures with higher GPR55 expression [63]. In addition, GPR55-deficient mice develop, among others, important impairments in motor control and coordination [53]. This possibly explains that neurodegenerative disorders such as Alzheimer's disease and related dementias have been explored for determining the neuroprotective potential of GPR55-targeting compounds only recently [69,70], whereas movement-related disorders, in particular PD, are within those neurodegenerative pathologies investigated earlier and more extensively in relation with the GPR55 ligands [51,52,71,72]. Our present study has been designed to pursue the objective of developing a GPR55-based neuroprotective therapy for PD and also by other motor-related pathologies, for example, ALS. To do that, we used a chromenopyrazole derivative, VCE-006.1, which a priori showed selective properties as a partial agonist at the GPR55 receptors [65]. Our first objective was to extend the characterization of this compound to its activity

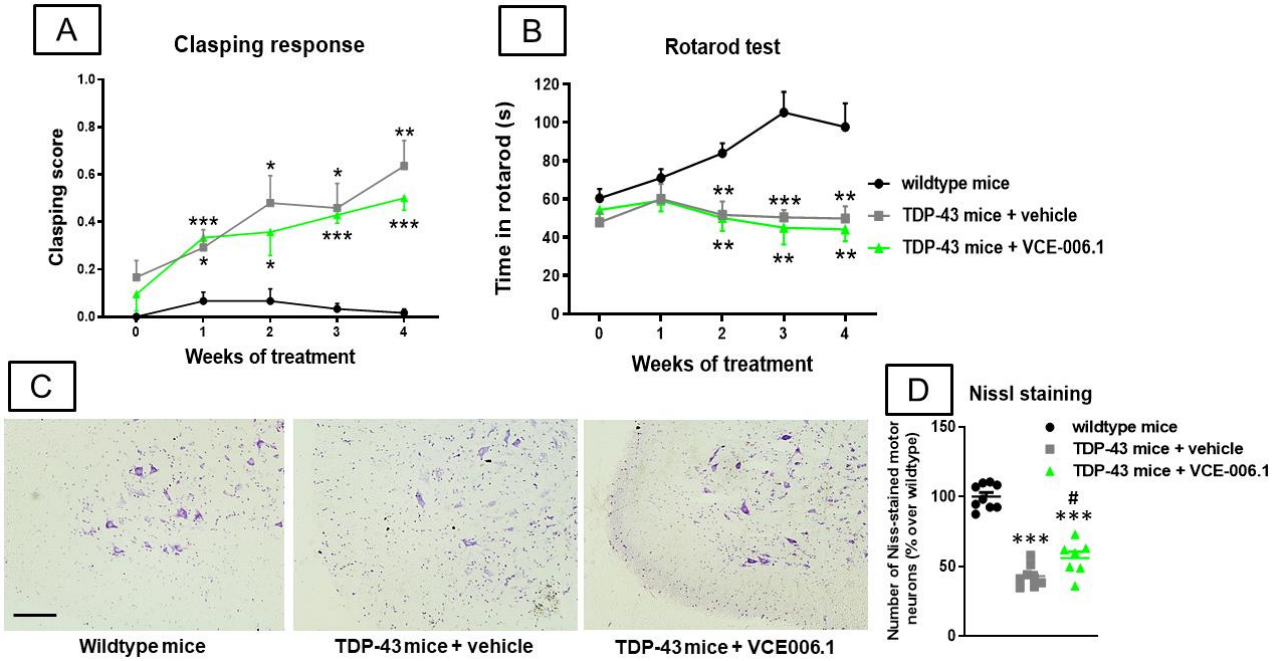

Figure 12. Clasping response (**A**) and rotarod performance (**B**) analyzed TDP-43 transgenic and wild-type male mice at specific weeks during a chronic treatment of 30 days with VCE-006.1 (20 mg/kg, daily and i.p.) or vehicle, and quantification of the number of Nissl-stained motor neurons (**D**), including representative images ((**C**); scale bar = 100 μm), in the lumbar ventral horn (marked with a dotted line) of the spinal cord in all experimental groups after the chronic treatment. Values are means ± SEM of more than 6 animals per group. Behavioral data were assessed by two-way ANOVA (with repeated measures), whereas Nissl staining data were assessed by one-way ANOVA, in both cases followed by the Tukey test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ vs. wild-type mice; $^{\#}p < 0.05$ vs. TDP-43 mice treated with vehicle).

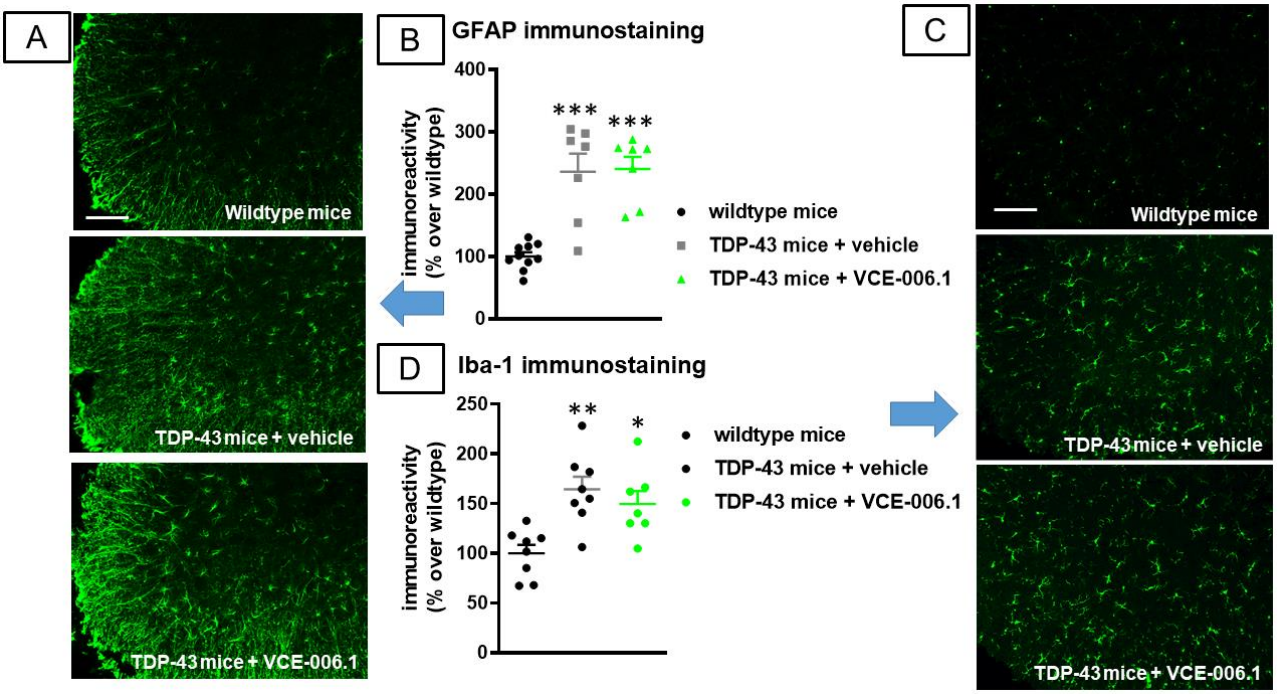

Figure 13. Quantification of GFAP (**B**) and Iba-1 (**D**) immunoreactivity, including representative images ((**A**) and (**C**), respectively; scale bar = 100 μm), in the lumbar ventral horn (marked with a dotted line) of the spinal cord in wild-type and TDP-43 transgenic mice after chronic treatment of 30 days with VCE-006.1 (20 mg/kg, daily and i.p.) or vehicle. Values are means ± SEM of more than 6 animals per group. Data were assessed by one-way ANOVA followed by the Tukey test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ vs. wildtype mice).

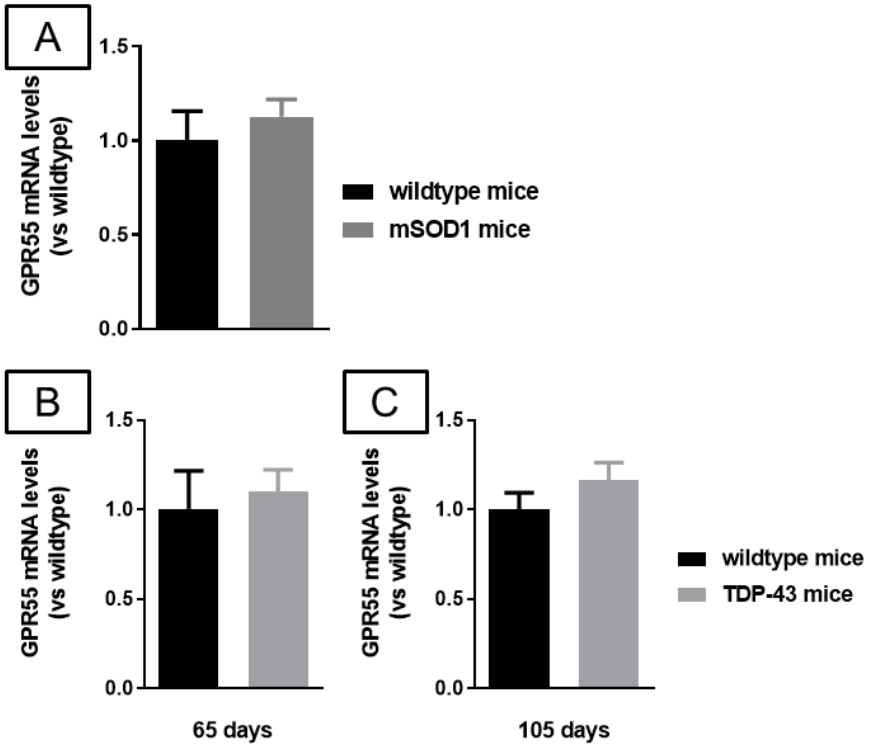

Figure 14. mRNA levels for GPR55 measured by qPCR in the spinal cord of male mSOD1 (at 123 days of age; (**A**)) or TDP-43 (at 65 (**B**) and 105 (**C**) days of age) transgenic mice, and their corresponding wild-type mice. GAPDH was used as an endogenous reference gene for data normalization. Values correspond to fold change vs. controls and are expressed as means ± SEM of more than 5 animals per group. Data were assessed by the unpaired Student's *t*-test.

at the GPR55 receptor, using specific cell assays that revealed a biased activity of VCE-006.1 on this receptor as a partial orthosteric agonist or PAM, depending on the specific cell assay used.

Once we confirmed this activity of VCE-006.1 at the GPR55 receptor, we wanted to explore whether this enables the compound to afford neuroprotection in cells and murine models of the two neurodegenerative diseases indicated before, i.e., PD and ALS. Our experiments in PD demonstrated that VCE-006.1 was highly active in the preservation of TH-containing nigral neurons damaged in this disease, and that this has an important reflect in the improvement of motor defects associated with this damage. In our study, this neuroprotective effect was evident in two in vivo models of PD generated by 6-OHDA or, to a lower extent, LPS lesions in mice, and was also confirmed in an in vitro cell-based model (SH-SY5Y cells exposed to 6-OHDA). Similar benefits have been observed with other GPR55-acting compounds using additional experimental models, such as MPTP-lesioned mice and a murine model of haloperidol-induced catalepsy [51], and the same happens with more recent studies conducted by Martínez-Pinilla and coworkers [52,71]. However, whereas the neuroprotection seen in 6-OHDA-lesioned mice with VCE-006.1 in our study was accompanied by an attenuation of the reactive gliosis elicited by the neurotoxin, this did not occur in the LPS-lesioned mice, in which the inflammatory response caused by LPS has been proposed to be the primary cause of further neuropathological events (e.g., loss of TH-positive neurons, motor defects). These paradoxical effects remain to be investigated, but, in support of this in vivo effect, the lack of VCE-006.1 effect against glial reactivity and associated inflammatory events (elevated generation of

proinflammatory cytokines) was also evident in BV2 cells treated with LPS and VCE-006.1. This could be related to an LPS-induced down-regulation of GPR55 receptors in the BV2 cells, although such down-regulation was not found in LPS-lesioned mice, and the same was seen in 6-OHDA-lesioned mice. In addition, in preliminary studies carried out with post mortem tissues from PD patients and control subjects, we detected apparently similar levels of GPR55 and an equivalent cell distribution, although this will require further confirmation (García, Burgaz and Fernández-Ruiz, unpublished results). To make the issue more complicated and justify the need for additional studies, a previous experiment also conducted in BV2 cells, and in part in rat microglial cell primary cultures, showed activity of LPI against LPS-induced nitric oxide production and iNOS expression [50]. By contrast, a similar study was carried out with anandamide, which also binds GPR55; instead, LPI resulted in inactivity [73].

As indicated before, we also investigated VCE-006.1 in another motor-related neurodegenerative disorder, ALS, using two genetic models of this pathology, the classic mSOD-1 model and the more recent TDP-43 transgenic mice. In both cases, our results confirmed that VCE-006.1 was poorly active, exerting only partial preservation of spinal motor neurons, which was not sufficient to reverse the intense neurological decline and muscle strength deterioration seen in these animals during the progression of the pathological phenotype. This may be related to the lack of effect of VCE-006.1 on the elevated microglial and astroglial reactivities seen in both models, a fact that, in this case, was not associated with a reduction in the levels of GPR55 receptors, which resulted in being similar to those found in the corresponding wild-type mice for both TDP-43 transgenic and mSOD-1 mice. Combining neuroprotection (preservation of motor neurons) and anti-inflammatory (attenuation of glial reactivity) effects appear to be an important determinant for disease-modifying effects of cannabinoids in experimental ALS. For example, cannabinoids targeting the CB_2 (e.g., HU-308) or the PPAR-γ receptors (e.g., VCE-003.2) afforded important levels of neuroprotection, being able to preserve motor neurons and to attenuate glial reactivity, which results in an improvement against the neurological (motor) deterioration [30,32,45]. However, such neurological improvement was not observed in studies that used cannabinoids that were not active at the same time against both the loss of motor neurons and the elevated glial reactivity [74]. Therefore, we assume that the potential of VCE-006.1 for ALS would require its combination with other cannabinoids also active at other endocannabinoid-related targets (e.g., CB_2 receptors, PPAR-γ receptors). We also have evidence that VCE-006.1 does not activate PPAR-γ receptors (Muñoz et al., unpublished results).

4. Materials and Methods

4.1. Synthesis and Characterization as PAM of VCE-006.1 in Cell-Based Assays

VCE-006.1 (2-[2-(4-cyclohexylcarbonylpiperazinyl)ethyl]-2,4-dihydro-7-methoxy-4,4-dimethylchromeno[4,3-c]pyrazole) was designed, synthesized, and characterized for the first time as a partial agonist at the GPR55 receptor by Morales and coworkers (compound 23 in [65]). In this new study, we have further characterized its biological activity profile both in HEK-293 cells overexpressing GPR55 and in cell lines expressing the native receptor.

4.1.1. Determination of ERK 1/2 activation.

DU145 cells expressing endogenous GPR55 were stimulated with either VCE-006.1 (5 μM), LPI (2 μM), or a combination of both for 30 min. Then, cells were washed with phosphate-buffered saline (PBS) and proteins extracted in lysis buffer (50 mM Tris–HCl pH 7,5, 150 mM NaCl, 10% glycerol, and 1% NP-40) supplemented with 10 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 1 μg/ml pepstatin and aprotinin, and 1 μl/ml saturated PMSF. Thirty μg of proteins were boiled at 95 °C in Laemmli buffer and electrophoresed in 10% SDS/PAGE gels. Total ERK was used as a loading control. Separated proteins were transferred to PVDF membranes, and after blocking with non-fat milk in TBST buffer, primary antibodies were added. The washed membranes were incubated with appropriate secondary antibodies coupled to horseradish peroxidase that were detected by an enhanced chemiluminescence system (USB). Antibodies against total and phospho-ERK1/2 were purchased from Sigma-Aldrich (Madrid, Spain).

4.1.2. Ca^{2+} Mobilization Assay.

U937 cells expressing endogenous GPR55 receptor were incubated for 1 h at 37°C in Tyrode's salt solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 12.0 mM $NaHCO_3$, and 5.6 mM D-glucose) containing 5 μM Indo1-AM (Invitrogen, Waltham, MA, USA) for 30 min at 37°C in the dark. Cells were then harvested, washed three times with buffer to remove extracellular Indo1 dye, readjusted to 10^6 cells/ml in the appropriate buffer, and analyzed in a spectrofluorometer operated in the ratio mode (model F-2500; Hitachi Ltd., Tokyo, Japan) under continuous stirring and at a constant temperature of 37°C using a water-jacketed device. After a 5-min accommodation to equilibrate temperatures, samples were excited at 338 nm, and emission was collected at 405 and 485 nm, corresponding to the fluorescence emitted by Ca^{2+} bound and -free Indo1, respectively. The cells were stimulated with either LPI or VCE-006.1, and maximal ratio values for calculations were determined by the addition at the end of the measurements of 10 μM ionomycin. $[Ca^{2+}]i$ changes are presented as changes in the ratio of bound to free calcium (340 nm/380 nm).

4.1.3. cAMP Signaling Induced by GPR55 Activation.

The determination of GPR55 activity was carried out using the HEK293T-GPR55 cells stably transfected with the human GPR55 cDNA. Briefly, HEK293T-GPR55 cells were transiently transfected with 0.2 μg of the reporter plasmid CRE-Luc that contains six consensus cAMP-responsive elements (CRE) linked to the firefly luciferase reporter gene using Roti©-Fect (Carl Roth, Karlsruhe, Germany). Transfected cells were treated with either VCE-006.1, LPI, or a combination of both. After 6 h of stimulation, cells were washed twice with PBS 1x and lysed in 100 μl lysis buffer containing 25 mM Tris-phosphate (pH 7.8), 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol for 15 min at room temperature in a horizontal shaker. Luciferase activity was measured using a TriStar2 Berthold/LB942 multimode reader (Berthold Technologies, Bad Wildbad, Germany) following the instructions of the luciferase assay kit (Promega, Madison, WI, USA). The RLUs (relative light units) were calculated, and the results were expressed as fold induction over unstimulated cells. The experiment was performed 5–6 times.

4.2. Animals and Cell Experiments

4.2.1. PD Experiments

Male C57BL/6 mice were housed in a room with a controlled photoperiod (08:00–20:00 light) and temperature (22 ± 1°C). They had free access to standard food and water and were used at adult age (3–4 month-old; 25–30 g weight). All experiments were conducted according to national and European guidelines (directive 2010/63/EU), as well as conformed to ARRIVE guidelines and approved by the "Comité de Experimentación Animal" of our university (PROEX: 056/19).

In a first experiment, male C57BL/6 mice were subjected to stereotaxic unilateral application of 6-hydroxydopamine (6-OHDA) or saline [24,75]. To do that, mice were anesthetized (ketamine 40 mg/kg + xylazine 4 mg/kg, i.p.) 30 min after pretreatment with desipramine (25 mg/kg, i.p.), and then 6-OHDA free base (2 μl at a concentration of 2 μg/μl saline in 0.2% ascorbate to avoid oxidation) or saline (for control mice) were injected stereotaxically into the right striatum at a rate of 0.5 μl/min, using the following coordinates: + 0.4 mm AP, −1.8 mm ML and −3.5 mm DV, as described in [75]. Once injected, the needle was left in place for 5 min before being slowly withdrawn, thus avoiding reflux and a rapid increase in intracranial pressure. Control animals were sham-operated and injected with 2 μl of saline using the same coordinates. The lesions were generated using unilateral injection, the advantage of which is that contralateral structures serve as controls for the different analyses. After the application of 6-OHDA or saline, animals were subjected to a daily treatment with VCE-006.1 (20 mg/kg, i.p.) or vehicle (cremophor-saline, 1:18) for two weeks, at the end of which (24 hours after the last injection), they were analyzed in the pole test and the cylinder rearing test just before being killed by rapid and careful decapitation and their brains rapidly removed. Brains were divided coronally into two parts, following the procedure described by Palkovits and Brownstein [76]. The anterior halves were used to dissect the striatum (both ipsilateral and contralateral sides separately), and tissues were rapidly frozen by immersion in cold 2-methylbutane and stored at −80 °C for qPCR analysis. The posterior halves containing the midbrains were fixed for one day at 4°C in fresh 4% paraformaldehyde prepared in 0.1 M PBS, pH 7.4. Samples were cryoprotected by immersion in a 30% sucrose solution for a further day, and finally stored at −80 °C for immunohistochemical analysis in the substantia nigra.

In a second experiment, mice were anesthetized (ketamine 40 mg/kg + xylazine 4 mg/kg, i.p.) and subjected to unilateral injections of *S. Minnesota* LPS (Sigma-Aldrich, Madrid, Spain) into two points of the right striatum following the procedure developed by Hunter et al. [77]. We used the following stereotaxic coordinates from bregma: + 1.1 mm AP, −1.8 mm ML, and −3.5 mm DV, as well as −0.3 mm AP, −2.5 mm ML, and −3.2 mm DV (see details in [77]). At each intrastriatal coordinate, 5 μg of LPS in a volume of 1 μl of saline was injected slowly (0.5 μl/30 s), and the needle was again left in place for 5 min before being slowly withdrawn. This avoids generating reflux and a rapid increase in intracranial pressure. Control animals were sham-operated and injected with 1 μl of saline using the same coordinates. Again, the lesions were generated using unilateral administration, the advantage of which is that contralateral structures serve as controls for the different analyses. After the application of LPS or saline, animals were subjected to a daily treatment with VCE-006.1 (20 mg/kg, i.p.) or vehicle (cremophor-saline, 1:18) for two weeks, at the end of

which (24 hours after the last injection), they were analyzed in the pole test and the cylinder rearing test just before being killed by rapid and careful decapitation and their brains rapidly removed and processed as described before 6-OHDA-lesioned mice.

In a third experiment, cultures of SH-SY5Y neuronal cell line (kindly provided by Dr. Ana Martínez, CIB-CSIC, Madrid, Spain) were used to induce cell death with 6-OHDA and to investigate in vitro the possible cytoprotective effects of VCE-006.1, following a procedure described previously [78]. To this end, SH-SY5Y cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS), 2 mM Ultraglutamine, and 1% antibiotics (Lonza, Verviers, Belgium) under a humidified 5% CO_2 atmosphere at 37°C. For cytotoxicity experiments, cells were seeded at 60,000 cells/well in 96-well plates and maintained under a humidified atmosphere (5% CO_2) at 37°C overnight. For experiments, 24 hours after seeding, cells were treated with the vehicle (DMEM + 0.1% DMSO) or with five different concentrations of VCE-006.1 (0.5, 1, 2, 5, 10, and 20 μM; selected according to [65]), 60 minutes before being exposed to 200 μM 6-OHDA (or saline) following our previously published studies with different concentrations of 6-OHDA in these cells [43,44]. Cells were incubated 24 hours before the neuronal death was analyzed with the MTT assay (Panreac AppliChem., Barcelona, Spain). Data of cell viability were normalized in relation to the corresponding control group (cells exposed to vehicles for 6-OHDA and VCE-006.1).

In a fourth experiment, cultured BV-2 cells were maintained in DMEM (Lonza, Verviers, Belgium) supplemented with 10% FBS (Sigma-Aldrich, Madrid, Spain), 2 mM Ultraglutamine, and antibiotics (Lonza, Verviers, Belgium) in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were plated at a density of 45×10^4 cells per well in 12-well culture plates and incubated in DMEM with a reduction of FBS to 1%. Three hours later, cells were treated with 0.5 μg/ml LPS (from *Escherichia coli* 055:B5, Sigma-Aldrich, Madrid, Spain), alone or in combination with VCE-006.1, used at a concentration of 1 μM, and added 1 hour before LPS. Twenty hours after the addition of LPS, media were removed, and cell pellets were collected for analyzing mRNA levels of GPR55, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) using qPCR analysis.

4.2.2. ALS Experiments

Experiments were conducted with two mouse colonies: (i) B6SJL-Tg(SOD1*G93A)1Gur/J transgenic (mSOD1 mice) and non-transgenic littermate sibling mice bred in our animal facilities from initial breeders provided by Dr. Rosario Osta (LagenBio-Ingen, University of Zaragoza, Spain), and (ii) Prp-hTDP-43(A315T) transgenic and non-transgenic littermate sibling mice bred in our animal facilities from initial breeders purchased from Jackson Laboratories (Bar Harbor, ME, USA). In both cases, animals were subjected to genotyping for identifying the presence or absence of the transgene containing the SOD-1 or the TDP-43 mutation (see details in [30,45], respectively). As in PD experiments, all animals were housed in a room with controlled photoperiod (08:00–20:00 light) and temperature (22 ± 1 °C) with free access to standard food or, in the case of TDP-43 transgenic mice, to a high-fat jelly diet (DietGel Boost, ClearH20, Portland, ME, USA) [79], and water. All experiments were conducted according to local and European rules (directive 2010/63/EU), as well

as conformed to ARRIVE guidelines. They were approved by the ethical committees of our university and the regulatory institution (PROEX: 056/19).

In a first experiment, wild-type and mSOD-1 transgenic mice were identified by numbered ear marks, and prior to the start of the different experiments, they were randomly allocated to the different treatment groups. We treated B6SJL-Tg(SOD-1*G93A)1Gur/J transgenic male mice with VCE-006.1, synthesized as previously described [65], and administered i.p. to mice at the dose of 20 mg/kg. Additional transgenic mice, as well as wild-type animals, were treated with vehicle (cremophor-saline, 1:18). The treatment was initiated when animals were 63 days old and prolonged daily up to the age of 18 weeks (125 days of age). During this period, animals were weighed every day and subjected to several neurological analyses and behavioral tests at specific time points. Twenty-four hours after the last injection, animals were euthanized by rapid decapitation, and their spinal cords were dissected and removed. The spinal cords (lumbar level) to be used for histology were fixed for one day at 4 °C in 4% formaldehyde solution in PBS. Samples were then cryoprotected by immersion in a 30% sucrose solution for a further day, and finally stored at −80 °C for Nissl staining and immunohistochemical analysis. The spinal samples (also lumbar area) to be used for qPCR analyses were collected and rapidly frozen by immersion in cold 2-methylbutane and stored at −80 °C for qPCR analysis.

In a second experiment, we treated non-transgenic and Prp-hTDP-43(A315T) transgenic male mice with VCE-006.1, again synthesized as previously described [65] and administered i.p. to mice at the dose of 20 mg/kg. Additional transgenic mice, as well as wild-type animals, were treated with vehicle (cremophor-saline, 1:18). The treatment was initiated when animals were 65 days old and prolonged daily up to the age of 95 days, the same treatment window used in our previous study [30], which extends from early symptomatic phases (around the 9th week of age) up to an advanced stage (around the 13th week of age). Animal weight was logged daily. Weight loss of 20% was established as the human end-point. Rotarod performance and clasping reflex to detect dystonia were recorded weekly during the 4 weeks of the treatment period (including a recording just before the first injection). All animals were euthanized by rapid decapitation at the age of 95 days, at least 24 hours after the last administration. Their spinal cords were rapidly removed and processed as described for mSOD-1 mice.

4.3. Behavioral Recording

4.3.1. Pole Test

Mice were placed head-upward on the top of a vertical rough-surfaced pole (diameter 8 mm; height 55 cm), and the time until animals descended to the floor was recorded with a maximum duration of 120 s. When the mouse was not able to turn downward and instead dropped from the pole, the time was taken as 120 s (default value) (see details in [44]).

4.3.2. Cylinder Rearing Test.

Given that the lesion was unilateral in the experiment with 6-OHDA or LPS, this test attempted to quantify the degree of forepaw (ipsilateral, contralateral, or both) preference for wall contacts

after placing the mouse in a methacrylate transparent cylinder (diameter: 15.5 cm; height: 12.7 cm [80]). Each score was made out of a 3 min trial with a minimum of 4 wall contacts.

4.3.3. Neurological Score.

Mice were evaluated for neurological decline using a numerical scale published previously [45]. The scale ranged from 0 to 15 distributed in three sub-scales (0–5) concentrated on ambulation, strength analysis, and hind-foot reflex test. A final score of 0 corresponds to animals that are not symptomatic, whereas a score of 15 reflects a state of total functional loss in hindlimbs and postural control. The assessment of ambulation was carried out by placing the animal inside a corridor (10 × 10 × 80 cm) while evaluating postural control and the way in which hindlimbs were leaned during motion. The strength test evaluated the animal's ability to drag and offer resistance when the tail was pulled softly to the opposite direction in which the animal moves. Lastly, the hind-foot reflex test evaluated the stiffness of the limbs and their coordination when the mouse was suspended by the tail 10 cm over the surface. The final score was calculated from the sum of values reached in each sub-scale.

4.3.4. Rotarod Test.

Mice were evaluated for possible motor weakness using the rotarod test, using an LE8200 device (Panlab, Barcelona, Spain). Mice were exposed to a period of acclimation and training (first session: 0 r.p.m. for 30 s; second and third sessions: 4 r.p.m. for 60 s, with periods of 10 min between sessions), followed 30 min later by the assay. Mice were placed into the apparatus, and the rotational speed was increased from 4 to 40 r.p.m. over a period of 300 s to measure the time to fall off. Mice were tested for 3 consecutive trials with a rest period of approximately 15 min between trials, and the mean of the 3 trials was calculated.

4.3.5. Clasping Response.

Dystonia was evaluated by picking up the mouse by the base of the tail for 30 seconds so that the mouse was facing downwards away from any object. The position of the hindlimbs was observed and scored following the scale reported by Guyenet et al. [81]. Animals were scored as follows: 0 if the hindlimbs were consistently extended away from the abdomen; 1 if one hindlimb was retracted toward the abdomen; 2 if both hindlimbs were partially retracted toward the abdomen; 3 if both hindlimbs were entirely retracted and touching the abdomen. Mice were tested for three consecutive trials, and the mean clasping score of the three trials was calculated.

4.3.6. Hanging Wire Test.

The latency of mice to fall from a wire cage top, which was slowly inverted and suspended at approximately 30 cm to the floor, was also used as an index of motor weakness. The test was repeated three times to obtain the mean value of the three trials.

4.4. Histological Procedures

4.4.1. Tissue Slicing

In the PD experiment, brains were sliced in coronal sections (containing the substantia nigra) in a cryostat (30 μm thick) and collected on antifreeze solution (glycerol/ethylene glycol/PBS; 2:3:5) and stored at −20 °C until used for immunostaining. In the ALS experiment, fixed spinal cords were sliced with a cryostat at the lumbar level (L4-L6) to obtain coronal sections (20 μm thick) that were collected on gelatin-coated slides. Sections were used for procedures of Nissl-staining and immunostaining.

4.4.2. Immunohistochemistry Analysis in the PD Experiment.

Brain sections containing the substantia nigra were mounted on gelatin-coated slides and, once adhered, washed in 0.1 M potassium PBS (KPBS) at pH 7.4. Endogenous peroxidase was blocked by 30 min incubation at room temperature in peroxidase blocking solution (Dako Cytomation, Glostrup, Denmark). After several washes with KPBS, sections were incubated overnight at room temperature with the following polyclonal antibodies: (i) rabbit anti-tyrosine hydroxylase (TH) (Chemicon-Millipore, Temecula, CA, USA) used at 1/200; (ii) rat anti-mouse Cd68 antibody (AbD Serotec, Oxford, UK) used at 1/200; or (iii) rabbit anti-mouse GFAP antibody (Dako Cytomation, Glostrup, Denmark) used at 1/200. In the case of LAMP-1 immunostaining, we used the hybridoma monoclonal rat anti-mouse LAMP-1 antibody 1D4B, which was deposited by Dr. J. Thomas in the Developmental Studies Hybridoma Bank (DSHB; Hybridoma Product 1D4B), created by the NICHD (NIH, Bethesda, MD, USA) and maintained at The University of Iowa, Department of Biology, Iowa City, IA, USA. Dilutions were carried out in KPBS containing 2% bovine serum albumin and 0.1% Triton X-100 (Sigma Chem., Madrid, Spain). After incubation, sections were washed in KPBS, followed by incubation with the corresponding biotinylated secondary antibody (1/200) (Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature. Avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine substrate–chromogen system (Dako Cytomation, Glostrup, Denmark) were used to obtain a visible reaction product. Negative control sections were obtained using the same protocol with omission of the primary antibody. A Leica DMRB microscope and a DFC300FX camera (Leica, Wetzlar, Germany) were used for the observation and photography of the slides, respectively. For quantification of TH, LAMP-1, GFAP, or Cd68 immunostaining in the substantia nigra, we used the NIH Image Processing and Analysis software (ImageJ; NIH, Bethesda, MD, USA) using 4–5 sections, separated approximately by 200 μm, and observed with 5x-20x objectives depending on the method and the brain area under quantification. In all sections, the same area of the substantia nigra pars compacta was analyzed. Analyses were always conducted by experimenters who were blinded to all animal characteristics. Data were expressed as a percentage of immunostaining intensity in the ipsilateral (lesioned) side over the contralateral (non-lesioned) side.

4.4.3. Nissl Staining

Slices were used for Nissl staining using cresyl violet, as previously described [82], which permitted us to determine the effects of particular treatments on cell numbers. A Leica DMRB

microscope (Leica, Wetzlar, Germany) and a DFC300Fx camera (Leica) were used to study and photograph the tissue, respectively. To count the number of Nissl-stained motor neurons (> 400 μm^2) in the ventral horn, high-resolution photomicrographs were taken with a 10x objective under the same conditions of light, brightness, and contrast. Counting was carried out with ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2012). At least 6 images per animal were analyzed to establish the mean of all animals studied in each group. Analyses were always conducted by experimenters who were blinded to all animal characteristics. In all analyses, data were transformed to the percentage over the mean obtained in the wild-type group for each parameter.

4.4.4. Immunofluorescence Analysis in the ALS Experiment.

Spinal slices were used for the detection and quantification of GFAP or Iba-1 immunofluorescence. After preincubation for 1 hour with Tris-buffered saline with 0.1% Triton X-100 (pH 7.5), sections were sequentially incubated overnight at 4° C with the following polyclonal antibodies: (i) anti-Iba-1 (Wako Chemicals, Richmond, VI, USA) used at 1:500; or (ii) anti-GFAP (Dako Cytomation, Glostrup, Denmark) used at 1:200, followed by washing in Tris-buffered saline and a new incubation (at 37° C for 2 hours) with an anti-rabbit secondary antibody conjugated with Alexa 488 (Invitrogen, Carlsbad, CA, USA). A DMRB microscope and a DFC300Fx camera (Leica, Wetzlar, Germany) were used for slide observation and photography. The mean density of immunolabelling was measured in the selected areas. Again, all data were transformed to the percentage over the mean obtained in the wild-type group for each parameter.

4.5. Real Time qRT-PCR Analysis

Tissues (striatum and spinal cord) from in vivo experiments and cell pellets from the in vitro experiments were also used for qRT-PCR analysis. Total RNA was isolated from the different samples using Trizol reagent (Sigma-Aldrich, Madrid, Spain). The total amount of RNA extracted was quantitated by spectrometry at 260 nm and its purity from the ratio between the absorbance values at 260 and 280 nm. After genomic DNA was removed (to eliminate DNA contamination), single-stranded complementary DNA was synthesized from up to 1 μg of total RNA using the commercial kits Rneasy Mini Quantitect Reverse Transcription (Qiagen, Hilgen, Germany) and iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The reaction mixture was kept frozen at −20 °C until enzymatic amplification. Quantitative RT-PCR assays were performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) to quantify mRNA levels for TNF-α (ref. Mm99999068_m1), IL-1β (ref. Mm00434228_m1), iNOS (ref. Mm01309902_m1), COX-2 (ref. Mm00478372_m1), CB_1 receptor (ref. Mm00432621_s1), CB_2 receptor (ref. Mm00438286_m1), GPR55 (ref. Mm03978245_m1), and PPAR© (ref. Mm01184322_m1), using GAPDH expression (ref. Mm99999915_g1) as an endogenous control gene for normalization. The PCR assay was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and the threshold cycle (Ct) was calculated by the instrument's software (7300 Fast System, Applied Biosystems, Foster City, CA, USA). Expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

4.6. Statistics

Data were assessed using one-way or two-way (repeated measures) ANOVA, as required, followed by the Tukey test, or using the Student's *t*-test, as required, using GraphPad Prism, version 8.00 for Windows (GraphPad Software, San Diego, CA, USA). A *p*-value lower than 0.05 was used as the limit for statistical significance. The sample sizes in the different experimental groups were always ≥ 5.

5. Conclusions

Therefore, our findings support the view that targeting the GPR55 with cannabinoids able to activate this receptor may afford neuroprotection in experimental PD, in particular, in models associated with mitochondrial dysfunction as in 6-OHDA-lesioned mice. Some beneficial effects were also found in LPS-lesioned mice, but with no effect against the intense glial activation occurring in this model. Future studies are projected to explore whether VCE-006.1 could also be active in mutant α-synuclein-based models of PD. Such a question is important to determine whether VCE-00.1 activity occurs exclusively in toxin-based models of PD or may also be found in models based on gene modifications. The need for this confirmation derives in part from the fact that VCE-006.1 was poorly active in experimental genetic models of ALS, although it is also possible that its development in this disease would require its combination with other cannabinoids active at additional endocannabinoid-related targets, in particular, anti-inflammatory targets. Collectively, these results demonstrate the specificities for the development of cannabinoid-based therapies for the different neurodegenerative disorders.

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Sample Availability: Samples of the compounds are available from the authors.

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DISCUSSION

Neurodegenerative diseases can be considered as one of the main health and economic challenges of this century, due to the progressive increase in human life span during the last decades, which gives these diseases more opportunities to be visible. Being aging the main risk factor for the development of these disorders, and assuming that life span will keep increasing in coming years, the expectation is that disorders based on the degeneration of certain brain areas will continue growing and resulting in worsening cognitive and physical impairments (Pringsheim et al., 2014). Most of these disorders remain not only with an unknown origin, which hinders the research, but also without an effective disease-modifying treatment, as in many cases they are just symptom-alleviating agents, and they are not very potent.

PD is the second most prevalent degenerative disorder after AD, and the first movement disease in the CNS. Except in the familiar cases caused by a mutation in a specific gene, the cause of PD seems to be multifactorial, including genetic and environmental risk factors which interact with each other, together with aging and the neuromelanin accumulation it entails. Clinically, the neurodegeneration begins in the basal ganglia, where the dopaminergic neurons of the SNpc die, causing the denervation of the striatum. Consequently, there is a misbalance between the direct and indirect pathway which manifests as hypokinetic symptoms, such as bradykinesia, rigidity, immobility, as well as resting tremor (Kalia and Lang, 2015). Currently, these symptoms may be mainly treated with L-DOPA, used to restore dopamine tone in the basal ganglia. Nevertheless, when used for a long time this therapy causes the appearance of dyskinesias due to the pulsatile stimulation of the striatum, which requires the reduction of L-DOPA doses to avoid the worsening of these side effects, which finally may become irreversible (Heumann et al., 2014). Considering the multiple neuropathological events implied in the neurodegenerative process, together with the aggregation of α-synuclein, the therapeutical strategy for PD may need to be multitarget, or to target signalling system with a broad spectrum of action. In this sense, the pharmacological manipulation of the ECS may have an impact in the motor symptoms and in particular against the disease progression of PD.

As presented in the Introduction, there is large evidence supporting the relevance of the ECS in the pathophysiology of neurodegenerative diseases, and specifically in PD, as well as wide research on the variety of potentially useful cannabinoid targets and cannabinoid compounds (Fernández-Ruiz, 2019). This assortment of targets and compounds is implied in a high number of biological processes, which allows us to design different pharmacological strategies or combinations of them, in order to achieve a disease modifying treatment for PD. Thus, the aim of this Doctoral Thesis has been to progress in the development of a cannabinoid-based pharmacological therapy in PD by means of preclinical evaluation of such therapies in different models of PD, concentrating our research mainly in three major targets: PPAR-γ, CB2 and GPR55.

The activation of PPAR-γ by thiazolidinediones, which are non-cannabinoid ligands, has shown to be effective in different PD experimental models and has entered in clinical investigation (Carta et al., 2015). However, these compounds present many clinical side effects such as weight gain, fluid retention, and osteoporosis (Rosen, 2010), whereas small molecules as cannabinoids, also active at this nuclear receptor, may provide a more secure anti-inflammatory and neuroprotective activity and this has become one of the objectives of this

work. The benefits of the activation of PPAR-γ by cannabinoids, such as CBG, have already been proved in neurodegenerative diseases (Granja et at al., 2012). However, EHP company developed a series of CBG derivatives with a better pharmacokinetic profile and potency, that were also effective in experimental autoimmune encephalomyelitis (Carrillo-Salinas et al., 2014) and HD (Díaz-Alonso et al., 2016), disorders that share several pathological events with PD. One of those compounds, VCE-003.2, showed an anti-inflammatory and neuroprotective effect in the classic inflammatory model of PD generated by intrastriatal application of LPS in mice (García et al., 2018). Interestingly, this study also demonstrated that the effects of VCE-003.2 were exerted through its binding to a regulatory site at the PPAR-γ receptor, which differs from the canonical binding site (Hughes et al., 2014), which means that this compound could fine tune PPAR-γ activity without necessarily competing with endogenous ligands.

Given the therapeutic interest of VCE-003.2 for PD, in the first chapter we have developed the validation of its oral formulation, more useful for future clinical development, in different *in vitro* and *in vivo* models of PD, including the study of the potential molecular mechanisms involved in its anti-inflammatory and neuroprotective effects. The administration of oral VCE-003.2 reproduced, in general, the benefits found when this compound was given i.p. (García et al., 2018), so it also showed a positive effect on LPS-induced astrogliosis and the generation of some proinflammatory markers, as well as on autophagy impairment. These effects resulted in a partial recovery of the dopaminergic neuronal loss of the SN and a functional recovery of the hemiparesis in the LPS-lesioned mice measured by specific behavioural tests. The effective dose of VCE-003.2 was two-fold higher than in the previous work (García et al., 2018), but this may respond to a lower oral absorption, of 10-12% biodisponibility, when formulated in lipid solvents (Aguareles et al., 2019). Given the characteristics of the LPS-lesioned mouse model of PD, we assume that the activation of PPAR-γ by VCE-003.2 should be strongly related to anti-inflammatory effects. However, such activation may also play a key role in regulating the cellular antioxidative defence response, for example activating Nrf2 signalling (Lee, 2017). Likewise, many phytocannabinoids, such as CBG, have antioxidant properties (Hill et al., 2012), which may persist in its derivative VCE-003.2. Thus, we continued the evaluation of this compound using another classic model of PD, based on 6-OHDA intrastriatal injection, in which the main pathological feature is oxidative stress instead of neuroinflammatory events. The chronic oral administration of VCE-003.2, at the same dose than in the previous study (Burgaz et al., 2019), strongly reduced neurodegeneration in the SN, and even restored the levels of dopamine and of its metabolite DOPAC in the striatum, indicating a functional recovery. This improvement associated with VCE-003.2 treatment also reached the motor impairments present on the 6-OHDA lesioned mice, as well as the modest and secondary inflammatory response in the damaged area of the SN, reducing both microgliosis and astrogliosis. These results are also supported by the activity of VCE-003.2 in other neurodegenerative diseases with common pathological events, in which it has been found to be neuroprotective and to induce neurogenesis, for example in a preclinical model of HD (Aguareles et al., 2019), and of amyotrophic lateral sclerosis (Rodriguez-Cueto et al., 2018).

Despite the strong potential of VCE-003.2 in PD models demonstrated in these studies, we wondered whether its effects could be maintained with additional derivatives that may offer

some advantages compared to VCE-003.2 (eg. easier synthesis or better solubility/ bioavailability). Thus, this aminoquinone needs a two-step synthesis and shows a limited oral absorption because of its highly lipophilic profile. Since the conversion of the resorcinol to paraquinone is essential for the increased PPAR-γ activity of cannabinoids (Granja et al., 2012), we evaluated two new derivatives: CBGA-Q, a one-step synthesis starting from cannabigerol acid; and CBGA-Q-Salt, a water-soluble derivative with *a priori* a better bioavailability. Both of them were active when given orally, but in all cases with less efficacy than VCE-003.2, specially regarding the functionality reflected in the DA/DOPAC ratio in the striatum. These results suggest that there may be a differential mechanism among these compounds, in addition to PPAR-γ activation, that involves VCE-003.2 in neuroprotection and perhaps in neurogenesis in PD, through regulation of neuronal progenitor proliferation and neuronal differentiation (Quintanilla et al., 2014). Indeed, when we studied their profile *in vitro*, we could observe that, unlike VCE-003.2, these new analogues exerted their neuroprotective properties through their binding to the canonical binding site of PPAR-γ, reinforcing the above-mentioned hypothesis of an alternative (though not exclusive) mechanism of action for VCE-003.2. For example, it has been seen that VCE-003.2 is capable to induce a transient activation of ERK and mTORC1 signalling pathways in Hib5 hippocampal progenitor cell line (Díaz-Alonso et al., 2016) and to promote neuronal-like differentiation as revealed by CTIP2 reporter activation in neutralized mouse embryonic teratocarcinoma P19 cells (Aguareles et al., 2019). However, these effects were reversed by PPAR-γ antagonists, indicating, in this case, that the effects were due to its binding to the canonical binding site. Thus, at this point it appears that for VCE-003.2, the quinone modification at positions 1,4 is essential for binding to the canonical binding site and the modification at position 2 (ethylamine) is required for the alternative one and probably for other targets or signalling pathways activated by this cannabinoid which still need to be elucidated.

Once demonstrated that VCE-003.2 given orally is active as neuroprotective against inflammatory events and mitochondrial dysfunction, two key pathogenic mechanisms in PD, we wanted to explore whether it may be also active against α-synuclein dysregulation and aggregation, which represents a third pathogenic event in the triad of insults that destroy dopaminergic neurons (Han et al., 2020). As indicated in the corresponding chapter, managing α-synuclein aggregation in PD models is a difficult objective, but in our study, we used a new model of PD based on AAV-mediated overexpression of mutant A53T α-synuclein (α-SYN) which, following previous studies of some of our collaborators has confirmed its usefulness for investigating PD therapeutics (Rodríguez-Pérez et al., 2018; Castro-Sánchez et al., 2018). In addition to our classical behavioural and histological analysis, for which we found in this case an aggressive but PD-like phenotype *a priori* difficult to reverse, we analysed the transcriptomic signature of the striatum of the α-SYN-exposed mice. These analyses proved several important changes in specific genes presumably located in striatal cells under the control of dopaminergic innervation. We found DEGs related to different pathological events, including inflammatory processes, both of DAM and astrogliosis, phagocytosis and lysosomal degradation, dysregulation of ECS elements, alteration of UPR, apoptotic pathways, etc., all events highly related to PD and other similar neurodegenerative disorders.

As for VCE-003.2 treatment in this model, we found a motor recovery which did not correlate with parallel neuroprotection or anti-inflammatory actions in the SN as expected. However, in the striatum, although there were no significant changes in the DEGs, there was an enrichment in pre-ranked hallmark pathways, with an opposite directionality from the previous analysis of the damaged non-treated group, suggesting a potential of VCE-003.2 on the reversion of the transcriptomic changes caused by the α-synuclein overexpression. More precisely, there was an increased expression of *Trem2*, which may be a protective response of VCE-003.2 against the damage, as it has been described how TREM2 deficiency implies proinflammatory microglial activation that aggravates neurodegeneration in PD models (Guo et al., 2019). There was also an increase in the interferon regulons after the treatment with VCE-003.2, probably associated with the role of PPAR-γ against viral infections in the CNS (Layrolle et al., 2021), and of the regulon of Spi1, which could mean a specialized microglial contribution (Satoh et al., 2014). For the rest of the above mentioned DEGs we could find trends to reversion with the VCE-003.2, which should be further explored in future studies. The increased expression of CB1 may be useful for modulating the proteasome system (Salgado-Mendialdúa et al., 2018); the decrease in the regulon of NFκB could be a potential mechanism against neuroinflammation related to PPAR-γ activation (Cai et al., 2018); the increase in the regulon of Atf6 is probably a beneficial response, contributing to the recovery of the UPR signalling in PD models (Gupta et al., 2021); and the coordinated downregulation of genes related with the cell-cycle, such as Sox10, Prdm14, and the E2F transcription factors. An increased expression of E2F1 and its role in DNA damage has been related with the modulation of neuronal apoptosis (Verdaguer et al., 2007), and specifically in neurons of the SN of PD patients (Höglinger et al., 2007). In the MPTP model, the blockade of E2F1 avoids DNA duplication and achieves neuroprotection and the deficiency of E2F1 protects against neuronal death in the SN (Höglinger et al., 2007), suggesting the downregulation of E2F1 observed in the group that received VCE-003.2 is protective against the motor impairment caused by α-synuclein overexpression. We are unaware of the mechanisms behind this effect, but they may probably be related with PD pathophysiology, as the blockade of E2F1 causes the disinhibition of complex I of mitochondria, which ends up in a neuroprotective effect (Alvira et al., 2007), and recent experimental analysis have newly revealed E2F1 as a target of PPAR-γ signalling (Vattulainen-Collanus et al., 2016). A similar effect was found for the E2F4 regulon, which is affected by mutations in the *GBA* gene, and related with cognitive impairment in PD patients (Jiang et al., 2020), and for the regulon of p53, which increased can contribute to the exacerbated apoptotic cell death observed in PD-affected brains (Checler and Alves da Costa, 2014), and thus its downregulation would aid to the effect of VCE-003.2 in the α-synuclein model. Interestingly p53 shares apoptotic route with E2F1, and their inhibition has been suggested as a potential neuroprotective strategy (Camins et al., 2007).

To unravel which of these proposed mechanisms are implied in the VCE-003.2 protective response, we would need to develop an experiment with a new cohort of animals, lesioned with a lower dose of virus. This would allow to reduce the nigral damage, thus facilitating to observe recoveries in neuronal death and/or glial reactivity by VCE-003.2. It would also allow to increase the sample sizes in which we could also extend the RNAseq analyses to confirm the preclinical data obtained in the striatum. On the other hand, we also plan to develop an *in vitro*

model of α-synuclein-transfected cells (Nascimento et al., 2020) where we can explore more precisely the effects of VCE-003.2 over α-synuclein aggregation, which may reinforce the proposal of this compound for its clinical development.

However, even these promising preliminary results have a chance to be improved, the present data support what as we proposed in the Introduction, that a multi-target strategy may be the best option for a multi-factorial pathology as PD. In this respect, recent research has proposed a crosstalk between CB2 and PPAR-γ signalling for some cannabinoid compounds (Youssef et al., 2019), which could be an interesting pharmacological approach aiming for a greater anti-inflammatory effect than when targeting each of them on their own. The activation of CB2 receptors using cannabinoid compounds has demonstrated to be useful against local inflammation and gliosis in LPS-lesioned mice (García et al., 2011; Gomez-Gálvez et al., 2016), reducing neurotoxic and neuroinflammatory events and preserving dopaminergic neurons in MPTP-lesioned mice (Chung et al., 2016; Shi et al., 2017), and partially reducing dopamine depletion in the striatum in 6-OHDA model (García-Arencibia et al., 2007). Consequently, we considered the evaluation of the combination of these CB2 activating effects with the previous PPAR-γ activation we described for VCE-003.2.

In this context, EHP developed a series of quinone derivatives of CBD (VCE-004 series), which act as dual CB2/PPAR-γ agonists. The 3-hydroxiquinone derivative VCE-004.8 has shown to be active at both receptors (del Río et al., 2016), to have great potential as a therapeutic agent in systemic sclerosis (García-Martín et al., 2019), as well as to be an anti-inflammatory and remyelination agent in multiple sclerosis (Navarrete et al., 2020). Using the 6-OHDA model, we found that the oral formulation of VCE-004.8 (EHP-101) was also active as a disease-modifying agent in this PD model. Its main effect was anti-inflammatory, as it managed a total reversion of microgliosis and astrogliosis, which were accompanied by modest recoveries in the survival of TH+ neurons survival and in the functional activity at the behavioural tests. This anti-inflammatory effect is consistent with the previous findings of VCE-004.8 on multiple sclerosis (Navarrete et al., 2020), as well as in other inflammatory pathologies which affect peripheral systems (García-Martin et al., 2021). However, VCE-004.8 did not show additional potency compared to VCE-003.2 or the other CBGA derivatives (Burgaz et al., 2021a). This could be related to the fact that, whereas CB2 receptors remain unaltered in the 6-OHDA model (García et al., 2011), PPAR-γ receptor expression is increased in the striatum of these mice (Burgaz et al., 2021a). An alternative explanation may be that the combination of these targets exclusively potentiates the anti-inflammatory activity, which in the 6-OHDA model is secondary to neuronal deterioration. Thus, VCE-004.8 may work better in models of PD based on inflammation-driven neurodegeneration, such as LPS or rotenone-lesioned mice. In addition, CB2 receptor becomes upregulated both in LPS model (García et al., 2011) and rotenone model (Concannon et al., 2016), which has been found to facilitate the anti-inflammatory and neuroprotective effect of compounds selectively activating this receptor in these models (Gómez-Gálvez et al., 2016; Javed et al., 2016).

Next, we managed to confirm the contribution of each receptor, at least for 6-OHDA insult, using the *in vitro* model of SH-SY5Y cells. We found that both receptors were necessary to reach the maximal reversal of the cytoprotective effect, but with a major role of PPAR-γ in the effects

of VCE-004.8. These results seem consistent with the variety of signalling pathways activated by PPAR-γ receptors as a transcription factor (Cai et al., 2018), including the possibility of their activation by the CB2 receptor itself (Picciolo et al., 2020), and the greater relevance of CB2 receptor in inflammatory models mentioned above. Finally, the reversal of these effects by a PPAR-γ antagonist also confirmed the binding of VCE-004.8 to the canonical site of the receptor (Hughes et al., 2014), unlike VCE-003.2, which mainly exerted its effects in our PD models through the regulatory binding site of PPAR-γ (García et al., 2018; Burgaz et al., 2021a).

To continue with the investigation of a multi-target strategy to fight against the pathological events that cooperate to kill nigral neurons in PD, we considered another CB2 agonist, again looking for the anti-inflammatory properties of targeting this receptor (Price et al., 2009; Gómez-Galvez et al., 2016), but with a pleiotropic profile. The phytocannabinoid THCV is a CB2 agonist, which has shown anti-inflammatory and antioxidant effects in two animal models of PD (García et al., 2011), as well as a symptom-relieving capacity, due to its CB1 antagonist activity at low doses (Pertwee, 2008). However, the effects of THCV treatment over reactive microgliosis and neuronal damage in both LPS and 6-OHDA models were limited, especially if compared with the treatment with CBD, which has a potent antioxidant activity as well as many available targets in and outside the ECS, such as PPAR-γ (Esposito et al., 2011). So, we resolved to evaluate if the combination of both phytocannabinoids could imply an additive or synergistic effect compared to their individual effects (García-Fuente et al., 2018; Anand et al., 2021), which may serve to improve their efficacy. However, in LPS-lesioned mice, the efficacy of the combined treatment was the same than for THCV and CBD separately, both in the behavioural tests and in the histological analysis of neurodegeneration, autophagy dysfunction and neuroinflammation. We only found changes comparing the levels of inflammatory modulators in the striatum, as THCV alone was the only one that reached a significant reduction, in particular in TNF-α. These differences between the treatments could explain the relevance of CB2 receptor in LPS-model (Gómez-Gálvez et al., 2016), in which its activation by THCV, unlike CBD, achieves a higher anti-inflammatory response in the striatum, the starting point of the neuroinflammation. Regarding the 6-OHDA model, we obtained similar results, with no differences in neuroinflammation and autophagy markers for any of the treatments. However, the motor impairments caused by the lesion were only partially ameliorated by THCV treatment (alone or combined), whereas they were significantly recovered by CBD treatment. Considering that 6-OHDA lesion provokes intense oxidative stress (Alvarez-Fischer et al., 2008), it seems likely that CBD, with a higher antioxidant activity (Lastres-Becker et al. 2005), could be more effective than THCV (García et al., 2011), although it would not explain why the combined treatment was at least as effective as CBD on its own. Moreover, the analysis of neuronal survival in the SN also revealed a loss of those beneficial effects that are observed for each compound individually, reinforcing the idea of a negative crosstalk between their mechanisms of action.

Despite the ineffectiveness (or lack of additional effectiveness) of the combination of THCV and CBD, we wanted to investigate the mechanisms behind such potential interaction, in this case as an attempt to elucidate if it is possible, the reasons for the absence of additive (or at least similar) effects. Our *in vitro* data, obtained with SH-SY5Y neurons challenged with 6-

OHDA, showed that the effect of each compound was lost with the combination of both, just as in the *in vivo* model. However, we found that using subeffective concentrations, this undesired interaction was avoided or at least reduced, maintaining the individual effect of each cannabinoid. This result suggests that the concentrations we selected for the combined treatment were too high, causing an excessive response, and that it would have been better the use of subeffective concentrations, as described for other combinations of phytocannabinoids (Stern et al., 2015). Next, we aimed to check if the crosstalk between THCV and CBD mechanisms of action was due to CB2 and PPAR-γ receptors. We observed that both targets have an important role in the effects of THCV alone or in combination, and, as expected, only PPAR-γ is involved in the effects of CBD. The participation of both receptors in the effects of THCV could be due to the crosstalk between CB2 and PPAR-γ signalling described for some cannabinoids (Youssef et al., 2019), which could cause the loss of the signalling activated by CB2 receptors when PPAR-γ receptors are inhibited (Picciolo et al., 2020), just as we observed in the first part of Chapter 2 for VCE-004.8. Moreover, CBD is known to indirectly modulate the ECS, leading to an increase in endocannabinoid levels, which could facilitate cannabinoid signalling and indirectly activate CB2 receptors (De Petrocellis et al., 2011). Also, CBD has been proved to act as an allosteric modulator of both CB1 (Laprairie et al., 2015) and CB2 (Martinez-Pinilla et al., 2017), which again may interfere with the effects exerted by THCV over these receptors. Even outside the ECS, there are other targets which could be involved in the regulation of the basal ganglia functions, such as the 5-HT1A receptors, activated by both THCV (Morales et al., 2017) and CBD (Espejo-Porras et al., 2013). Collectively, we can say there is a complex interaction between the pharmacology of THCV and CBD, probably derived from their multi-target activities, that will require further research, and that for the moment does not seem suitable for obtaining an additive or synergistic effect. However, we do not discard further studies on THCV as a pharmacological strategy for PD, as we have reinforced the beneficial effects already proposed for THCV as neuroprotective and anti-inflammatory agent (García et al., 2011); as anti-hypokinetic (García et al., 2011) and anti-dyskinetic (Espadas et al., 2020), and also added some others, described in this Doctoral Thesis for the first time, such as its capability to impact on the autophagy dysfunction, another important pathological feature of PD (Hou et al., 2020).

Although our data have importantly contributed to the preclinical evidence around the ECS as a therapeutical target for PD, there are still other possibilities which have not been so deeply studied, such as targeting the GPR55 receptor. Some of the areas implied in control of movement, such as basal ganglia and cerebellum are also the ones within the CNS with higher GPR55 expression (Marichal-Cancino et al., 2017). This fact, together with the impairment in motor control and coordination observed in GPR55-deficient mice (Wu et al., 2013), situates this receptor a potential target for PD. However, the pharmacology of this receptor has provided some controversial results, which make difficult to choose between an activating or inactivating strategy. On one hand, GPR55 agonists have been described as anti-inflammatory (Minimahata et al., 2020), neuroprotective (Hill et al., 2019) and active against motor impairments (Celorrio et al., 2017). On the other hand, GPR55 antagonists have also shown anti-inflammatory properties (Saliba et al., 2018), anti-nociceptive effects (Okine et al., 2020) and are able to reduce motor impairments (Fatemi et al., 2021). Given the ambiguity found in

the literature, we started our evaluation by studying the effects of a GPR55 activator, VCE-006.1 (Morales et al., 2016), which was not active at CB1/CB2 receptors and we found to act in a biased manner at GPR55, both as partial orthosteric agonist and positive allosteric modulator.

Our experiments in PD models demonstrated that VCE-006.1 was active in the preservation of dopaminergic neurons damaged in the SN, which had an important reflect in the improvement of motor defects associated with such damage. This neuroprotective effect was higher in the 6-OHDA model (and also confirmed in the *in vitro* cell-based model of SH-SY5Y cells exposed to 6-OHDA) than in LPS-lesioned mice, in which there was just a slight recovery of these parameters. In the 6-OHDA-lesioned mice, the treatment with VCE-006.1 also caused an attenuation of the reactive gliosis elicited by the neurotoxin as a secondary event to neuronal death, whereas this did not occur in the LPS-lesioned mice, in which the inflammatory response is the primary cause of further neuropathological events, then being much more exacerbated. The lack of VCE-006.1 effect against glial reactivity and the increase of proinflammatory cytokines and enzymes was also evident in BV2 cells treated with LPS. This could have been related to an LPS-induced downregulation of GPR55 receptors in the BV2 cells (Pietr et al., 2009), but such effect was not found in LPS-lesioned mice, and the same was seen in 6-OHDA-lesioned mice. As mentioned above, what is clear about the pharmacology of GPR55 is its complexity, even more if we consider the presence in CNS of heteromers of GPR55 with CB1 or CB2 (Martínez-Pinilla et al., 2020). According to the general aim of this Thesis, it may be interesting, as part of the multi-target strategy, to study the pharmacological potential of these heteromers in PD. However, there are already some studies in this field, which do not propose the CB1-GPR55 heteromers as targets to afford PD-related neuroprotection (Martínez-Pinilla et al., 2019). In relation with CB2-GPR55 heteromers, their study has been more related to immune dysregulation in the CNS (Zhou et al., 2016), being especially interesting the proposed functional crosstalk between these receptors in microglial cells (Malek et al., 2015).

In a complementary study conducted in our research lab, the treatment with the GPR55 activator VCE-006.1 was evaluated in two genetic models of ALS, mSOD1 and TDP-43. In both cases, VCE-006.1 was poorly active, exerting only a partial preservation of the motor neurons in the spinal cord, which did not reverse the neurological decline and muscle weakness that progressively affect these animals. This situation is not rare in ALS, as the survival of motor neurons does not imply the preservation of the neuromuscular junction, which could remain dysfunctional in these mice (Verma et al., 2022). As for neuroinflammation, we observed the same results than in LPS-lesioned mice, as VCE-006.1 showed no anti-inflammatory activity. The levels of microgliosis and astrogliosis in these ALS models are not only very intense, but also a crucial feature in the neuropathology of this disease (Rodríguez-Cueto et al., 2021). Combining neuroprotection and anti-inflammatory effects seems to be determinant for disease-modifying effects of cannabinoids in experimental ALS. Previous studies described how cannabinoids targeting CB2 (e.g., HU-308; Espejo-Porras et al., 2019) or PPAR-γ receptors (e.g., VCE-003.2; Rodríguez-Cueto et al., 2018) preserved motor neurons and attenuated glial reactivity, which resulted in an improvement against the neurological deterioration and muscle atrophy of these ALS models. Therefore, these results indicate that the potential of VCE-006.1 does not rely on anti-inflammatory activity, reinforcing our results obtained in PD models, and

that GPR55 activation as a therapeutical strategy for PD will require its combination with other endocannabinoid-based targets, such as CB2 and PPAR-γ, as demonstrated in the previous chapters of this Thesis.

Lastly, although the pharmacological manipulation of the ECS has provided promising results in preclinical models of PD as a novel disease-modifying therapy, there still are some issues that require our attention. Although we have always evaluated our compounds in more than one PD model, they are still partial models, which limits their translationality. Two of the original aims of this Thesis were to implement other models in our laboratory and to develop new models (double-hit models), but we have only been able to fulfil the first one with the AAV-α-synuclein model used in the last part of Chapter 1. However, we are fully aware that the development of an ideal experimental model to reproduce all the phenotypic and pathological events of PD remains to be the key point, as such a model would be determinant for a full understanding of PD pathogenesis. To further progress in this aim, we are interested in introducing new models in our research, such as the PD model based on neuromelanin aggregation provoked by overexpression of tyrosinase activity (Carballo-Carbajal et al., 2019). This model is characterised by the formation of α-synuclein aggregates, hypokinetic symptoms, and dopaminergic degeneration of the nigrostriatal neurons, so authors claim a pathological correlation between these events, and neuromelanin aggregation as the underlying pathological mechanism of aging. Likewise, other type of interesting but not so used models, are the prodromal models (Taguchi et al., 2020). An ideal model of prodromal PD is one that reproduces several PD-specific premotor symptoms followed by the slowly progressive dopaminergic neurodegeneration. These models allow researchers to start a pharmacological strategy before the neuronal death is too severe. In this sense, we cannot forget the need for early biomarkers of PD, which will give us the opportunity to apply this same strategy in PD patients (Lotankar et al., 2017). Moreover, the use of a combination of biomarkers might enhance the specificity and sensitivity over a single measure, just as it happens with the pharmacology, considering the complexity and multifactorial origin of PD (Li and Le, 2020).

However, considering the unknown etiology and the increasing number of risk factors associated, it remains an aim difficult to achieve. Consequently, we have concentrated our efforts in the pharmacological research, which has provided large evidence of the value of the ECS as a therapeutical target for PD. Summarising, we have evaluated different cannabinoid compounds in three different PD models, which mainly represent one or two of the pathological events occurring in PD. For the LPS model, which reproduces inflammation-driven neurodegeneration, we have found that the best pharmacological strategy would be activating PPAR-γ (with VCE-003.2) or activating CB2 receptor (with THCV), and we hypothesise that activating both of them (with VCE-004.8) may potentiate these effects. However, the treatment with VCE-004.8 did not achieve the same motor improvements described for VCE-003.2 or THCV, as in those cases the symptom-relieving effect seems to respond to their activity at other targets, such as E2F1 downregulation or CB1 antagonism, respectively. For the 6-OHDA model, which reproduces mitochondrial dysfunction and oxidative stress we have found that the best pharmacological strategy would be activating PPAR-γ (with VCE-003.2) or activating GPR55 (with VCE-006.1), as well as the off-target and the receptor-independent antioxidant

mechanisms of the treatment with CBD. For the α-synuclein overexpression model, which reproduces the protein aggregation-derived neuronal death, we have found that activating PPAR-γ (with VCE-003.2) has beneficial effects, although the underlying mechanism may not be the same as in the other models, due to the lack of neuroprotective effects at the level of the SNpc. As a result, we consider the treatment with VCE-003.2 as our chosen therapy, given its multitarget effects which allow us to have a broad-spectrum activity without the disadvantages of the polypharmacology.

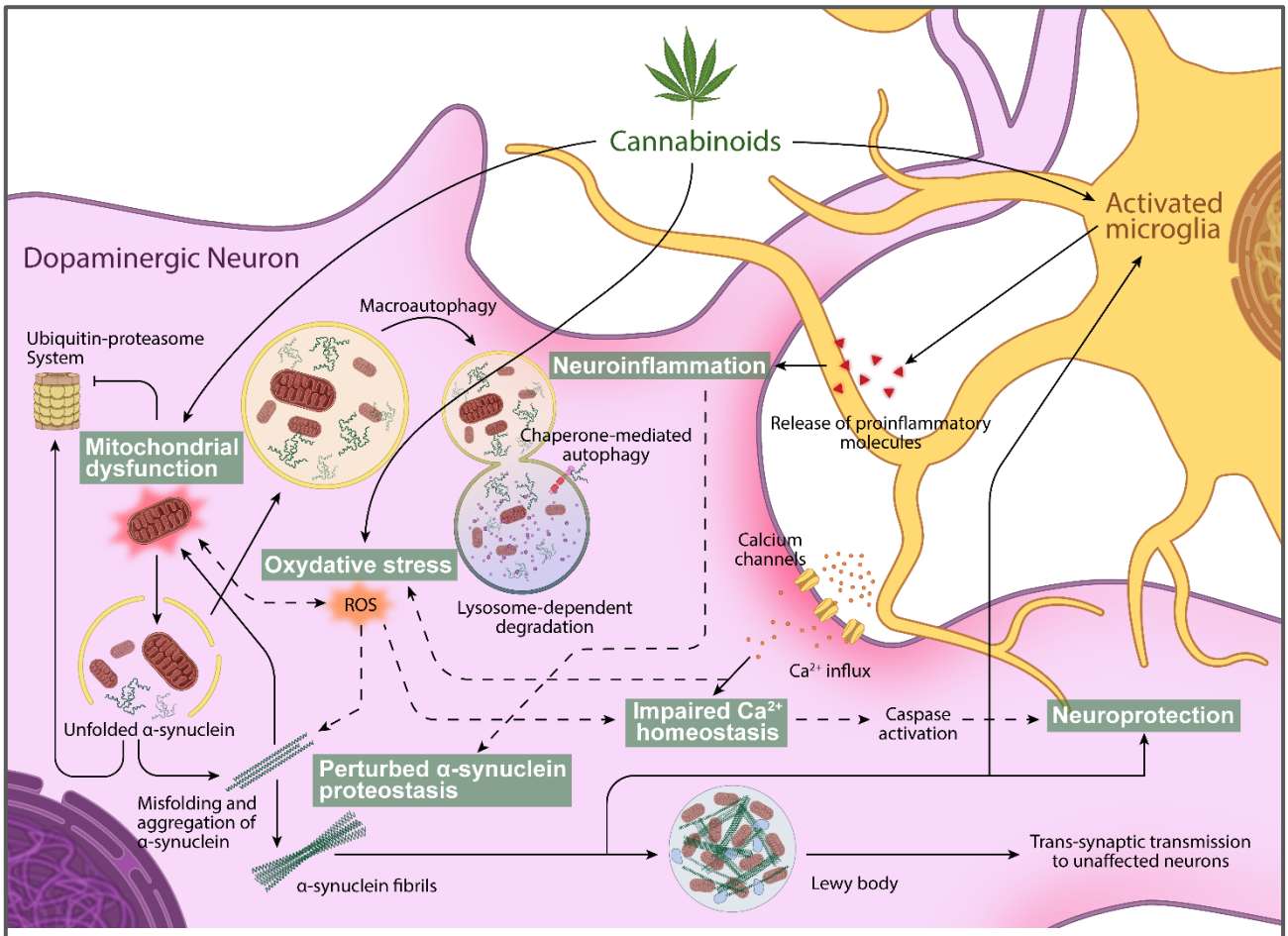

Figure 14. Molecular mechanisms involved in PD targeted by cannabinoids. Schematic diagram depicting the reduction in the interactions between major molecular pathways implicated in the pathogenesis of PD after a cannabinoid-based treatment

As an overall conclusion, the results shown in this Thesis allow us to confirm that the treatment with cannabinoids is antioxidant, anti-inflammatory and neuroprotective in different preclinical models of PD. As we hypothesised, the activation of the cannabinoid targets PPAR-γ, CB2 and GPR55 is effective as a pharmacological strategy for PD, although in some cases targeting just one of them may be not sufficient as a disease-modifying treatment. Thus, we propose the use of multitarget strategies based on cannabinoid compounds (in combination or with broad-spectrum activity) in preclinical models and later in the clinical field. However, we also suggest a cautious approach when combining multitarget compounds, since at certain circumstances they may have deleterious effects (or losses of efficacy) due to undesired off-

target activities or to an excessive dose in total. Hence, further research must be done to unveil the multiple mechanisms involved in the beneficial effects of cannabinoid compounds and its combinations against the multiple neuropathological events occurring in PD.

CONCLUSIONS

1. The activation of PPAR-γ receptor by an oral formulation of the CBG derivative VCE-003.2 is an effective pharmacological strategy in three experimental models of PD, based on neuroinflammation, mitochondrial dysfunction, and α-synuclein dysregulation, showing neuroprotective, anti-inflammatory and symptom-relieving effects.

2. The activation of PPAR-γ receptor by two oral formulations of CBGA derivatives, CBGA-Q and CBGA-Q-Salt, is an effective pharmacological strategy in two preclinical models of PD, but with lower efficacy than VCE-003.2.

3. The effects of VCE-003.2 were exerted through its binding to the alterative/regulatory binding site of PPAR-γ receptor, whereas the effects of the CBGA derivatives were due to their binding to the canonical binding site of PPAR-γ receptor.

4. The activation of CB2 and PPAR-γ receptors by an oral formulation of the CBD derivative VCE-004.8 is an effective pharmacological strategy in a model of PD based on oxidative stress and mitochondrial dysfunction with neuroprotective and anti-inflammatory effects.

5. The activation of CB2 receptor by the phytocannabinoid THCV, together with the blockade of CB1 receptor and the partial activation of other ECS-targets, is an effective pharmacological strategy in two experimental models of PD, especially in the inflammation-driven neurodegeneration model, with neuroprotective, anti-inflammatory and symptom-relieving effects.

6. The activation of CB2 receptor by the phytocannabinoid THCV, in combination with the activation of other ECS-targets by the phytocannabinoid CBD, does not appear to be effective pharmacological strategy in two preclinical models of PD.

7. The activation of GPR55 receptor by the synthetic cannabinoid VCE-006.1 is an effective pharmacological strategy in a model of PD based on oxidative stress and mitochondrial dysfunction, with neuroprotective and symptom-relieving effects, but it was not active in a model of PD based on neuroinflammation, nor in two ALS transgenic models.

As an overall conclusion, the activation of the ECS-targets such as PPAR-γ, CB2 and GPR55, by different cannabinoids is effective as a pharmacological strategy in preclinical models of PD, as they provide beneficial responses against the multiple neuropathological events occurring in this disease.

1. La activación del receptor PPAR-γ mediante una formulación oral del derivado del CBG VCE-003.2 es una estrategia farmacológica eficaz en tres modelos experimentales de EP, basados en la neuroinflamación, la disfunción mitocondrial y la desregulación de α-sinucleína, mostrando efectos neuroprotectores, anti-inflamatorios y de alivio de los síntomas.

2. La activación del receptor PPAR-γ mediante dos formulaciones orales de derivados de CBGA, CBGA-Q y CBGA-Q-Salt, es una estrategia farmacológica eficaz en dos modelos preclínicos de EP, pero menor eficacia que el VCE-003.2.

3. Los efectos del VCE-003.2 fueron ejercidos a través de su unión al sitio alternativo/regulador del receptor PPAR-γ, mientras que los efectos de los derivados del CBGA derivatives se debieron a la activación del sitio de unión canónico del receptor PPAR-γ.

4. La activación de los receptores CB2 y PPAR-γ mediante una formulación oral de un derivado del CBD, VCE-004.8, es una estrategia farmacológica eficaz en un modelo de EP basado en el estrés oxidativo y la disfunción mitocondrial con efectos neuroprotectores y anti-inflamatorios.

4. La activación del receptor CB2 por el fitocannabinoide THCV, junto con el bloqueo del receptor CB1 y la activación parcial de otras dianas del SEC, es una estrategia farmacológica eficaz en dos modelos experimentales de EP, especialmente en el modelo de neurodegeneración inducida por inflamación, con efectos neuroprotectores, anti-inflamatorios y de mejora de los signos motores.

5. La activación del receptor CB2 por el fitocannabinoide THCV, en combinación con la activación de otras dianas del SEC por el fitocannabinoide CBD, no parece ser una estrategia farmacológica eficaz en dos modelos preclínicos de EP.

6. La activación del receptor GPR55 por el cannabinoide sintético VCE-006.1 es una estrategia farmacológica efectiva en un modelo de EP basado en el estrés oxidativo y la disfunción mitocondrial, con efectos neuroprotectores y de alivio de las alteraciones motoras, pero no fue activa en un modelo de EP basado en la neuroinflamación, ni en dos modelos transgénicos de ELA.

Como conclusión general, la activación de dianas del SEC, como PPAR-γ, CB2 y GPR55, por diferentes cannabinoides es eficaz como estrategia farmacológica en modelos preclínicos de EP, ya que proporcionan respuestas beneficiosas contra los múltiples eventos neuropatológicos que ocurren en esta enfermedad.

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ANNEX

Participation in scientific publications

García, C., Gómez-Cañas, M., Burgaz, S., Palomares, B., Gómez-Gálvez, Y., Palomo-Garo, C., Campo, S., Ferrer-Hernández, J., Pavicic, C., Navarrete, C., Luz Bellido, M., García-Arencibia, M., Ruth Pazos, M., Muñoz, E., & Fernández-Ruiz, J. (2018). **Benefits of VCE-003.2, a cannabigerol quinone derivative, against inflammation-driven neuronal deterioration in experimental Parkinson's disease: possible involvement of different binding sites at the PPARγ receptor**. *Journal of neuroinflammation*, *15*(1), 19.

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